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As Extracellular Glutamine Levels Decline, Asparagine Becomes an Essential Amino Acid

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Summary

When mammalian cells are deprived of glutamine, exogenous asparagine rescues cell survival and growth. Here we report that this rescue results from use of asparagine in protein synthesis. All mammalian cell lines tested lacked cytosolic asparaginase activity and could not utilize asparagine to produce other amino acids or biosynthetic intermediates. Instead, most glutamine-deprived cell lines are capable of sufficient glutamine synthesis to maintain essential amino acid uptake and production of the glutamine-dependent biosynthetic precursors – with the exception of asparagine. While experimental introduction of cytosolic asparaginase could enhance the synthesis of glutamine, increase TCA cycle anaplerosis and the synthesis of nucleotide precursors, cytosolic asparaginase suppressed the growth and survival of cells in glutamine-depleted media in vitro and severely compromised the in vivo growth of tumor xenografts. These results suggest that the lack of asparaginase activity represents an evolutionary adaptation to allow mammalian cells to survive pathophysiologic variations in extracellular glutamine.

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Declaration of Interests. C.B.T. is a co-founder of Agios Pharmaceuticals and a member of the board of directors of Merck and Charles River Laboratories.

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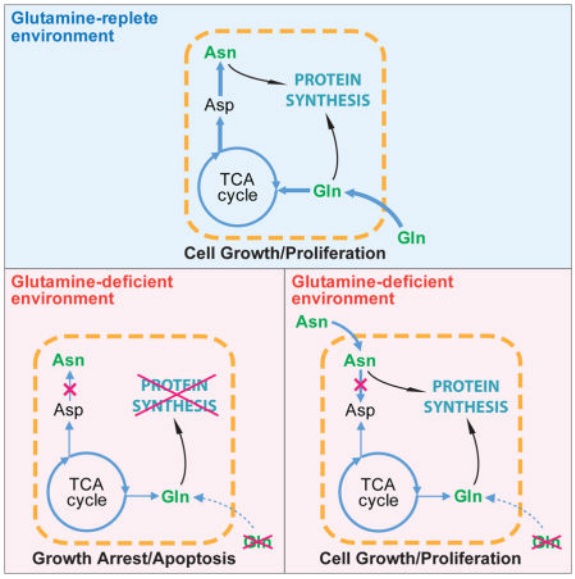
eTOC Blurb

Exogenous asparagine can rescue growth and survival of glutamine-deprived cells. XXX et al show that asparagine is not catabolized to produce other amino acids or biosynthetic intermediates but rather maintains protein synthesis. Introduction of asparaginase suppressed the growth and survival of glutamine-depleted cells and compromised the growth of tumor xenografts.



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glutamine; asparagine; translation; asparaginase

Introduction

Most cultured mammalian cells fail to proliferate or even survive in the absence of exogenous glutamine, despite possessing the enzymatic machinery for its de novo synthesis from the tricarboxylic acid (TCA) cycle carbon and free ammonium. Studies from the past decade convincingly demonstrate that glutamine, a versatile donor of nitrogen and carbon atoms for diverse biosynthetic reactions, is consumed by proliferating cells in excess of other amino acids. Major biosynthetic products of glutamine include nucleotides, non-essential amino acids and the anaplerotic substrates of the TCA cycle (Vander Heiden and DeBerardinis, 2017). Indeed, glutamine-derived α-ketoglutarate (α-KG), a key intermediate of the TCA cycle, has been proposed to be the key determinant of cellular glutamine dependency. Thus, a cell permeable α-KG was shown to be sufficient to rescue glutamine-depletion-induced apoptosis in MYC-transformed cells (Wise et al., 2008; Yuneva et al., 2007). More recently, asparagine, the amino acid most structurally similar to glutamine, was also found to be sufficient to reduce glutamine depletion-induced apoptosis in MYC-transformed cells (Zhang et al., 2014). In most species, either asparagine or glutamine can be used as substrates to maintain TCA cycle anaplerosis following intracellular deamidation initiated by cytosolic asparaginase or glutaminase, respectively. However, in mammals

asparaginase expression is restricted to the liver, kidney and smooth muscle and is not observed in proliferative tissues, or cancer cell lines. This raises the question of how exogenous asparagine is utilized to sustain cell survival and growth when extracellular glutamine becomes depleted.

The cellular consumption of glutamine rises in concordance with the growth rate. Accumulating evidence suggests that glutamine becomes preferentially depleted in the milieu of a variety of tumor types in vivo, or in the tissues where the vascular supply is compromised (Kamphorst et al., 2015; Marquez et al., 1989; Rivera et al., 1988; Roberts et al., 1956). Even within the same tumor tissue, the levels of glutamine were found to be substantially lower in the tumor core as compared to the peripheral regions (Pan et al., 2016). These results suggest that cell growth in vascularly compromised tissues may need to employ adaptive mechanisms in order to maintain glutamine levels necessary to sustain cell survival and proliferation. One potential mechanism to maintain glutamine levels under exogenous glutamine limitation is via its de novo biosynthesis (Bott et al., 2015; Carey et al., 2015; Tardito et al., 2015).

Here we show that asparagine rescued cell proliferation in the setting of exogenous glutamine deficit without being catabolized. The restoration of cell proliferation correlated with a restoration of global protein synthesis, and required glutamine synthetase (GLUL). Exogenous asparagine was used exclusively for protein synthesis and not for either TCA cycle anaplerosis or glutamine synthesis. In contrast to lower metazoan organisms, we found that mammalian cells lack the ability to catabolize asparagine to aspartate and free ammonia, which makes them unable to utilize asparagine's carbon and nitrogen atoms towards the biosynthesis of glutamine when the latter is depleted. We demonstrate that the expression of the yeast (ASP1) or zebrafish (zASPG) asparaginase in mammalian cells restores the capacity of mammalian cells to use asparagine as a biosynthetic substrate. However, the cytosolic activity of these asparaginases becomes a liability in the setting of exogenous glutamine limitation in culture. Under physiological concentrations of asparagine (<0.1 mM), expression of a functional asparaginase rendered cancer cells incapable of engaging in protein synthesis and cell proliferation when exogenous glutamine became limiting. Expression of a cytosolic asparaginase also suppressed tumor cell growth in vivo, indicating altogether that the access to exogenous asparagine is critical for cell survival and growth as vascularly compromised tissues and tumors become depleted of glutamine.

Results

Asparagine rescues cell proliferation in the absence of exogenous glutamine

Glutamine depletion-induced apoptosis has been shown to be suppressed by exogenous asparagine (Zhang et al., 2014). This pro-survival effect of asparagine was evident across a variety of cell lines, regardless of their tissue of origin or method of transformation (Supplemental Fig. S1A). For example, we found that in a number of breast cancer cell lines, supplementation with asparagine allowed cell proliferation in the absence of exogenous glutamine (Fig. 1A). In contrast, a cell-permeable form of α-KG, while restoring intracellular α-KG and citrate levels, did not rescue proliferation (Fig. 1B and S1B, left panel), which suggests that asparagine-mediated rescue of proliferation in the absence of

glutamine is independent of restoring the anaplerotic input into the TCA cycle. To determine whether the effect of asparagine on cell proliferation in the absence of glutamine is dose-dependent, we manipulated the concentration of glutamine or asparagine in the medium. We found that under glutamine depletion, asparagine restored the maximal proliferation at concentrations as low as 10~25 μM, while higher asparagine levels did not further enhance cell proliferation (Fig. 1C, left). In addition, supplementation with additional ammonium and aspartate (alone or in combination) also failed to promote proliferation further (Fig. S1B, middle and right panels). Equimolar concentrations (5~50 μM) of glutamine failed to maintain cell proliferation as well, indicating that asparagine is superior to glutamine in sustaining cell proliferation when present at low concentrations. Indeed, 250 μM glutamine was needed to surpass the level of cell proliferation supported by 25 μM asparagine (Fig. 1C, right).

Asparagine rescues translation in the absence of exogenous glutamine

Similar to glutamine, asparagine is utilized as a preferred nitrogen source to activate TOR-dependent translation and growth in yeast (Cardenas et al., 1999; Godard et al., 2007; Hardwick et al., 1999). In addition, both glutamine and asparagine have been shown to facilitate the uptake of essential amino acids (EAAs) for protein synthesis (Krall et al., 2016; Nicklin et al., 2009). To test the hypothesis that asparagine may rescue cell proliferation in the absence of glutamine via restoring the import of EAAs, we performed gas chromatography-mass spectrometry (GC-MS) analysis to quantify the intracellular levels of EAAs in glutamine-deprived cells. Surprisingly, the levels of the leucine, isoleucine, methionine, phenylalanine, threonine, tyrosine and valine were significantly increased in glutamine-deprived cells and returned to levels observed in cells grown in complete medium when glutamine-deficient medium was supplemented with asparagine (Fig. 1D). A similar pattern (leucine as an example) is seen in every cell line we have tested (Fig. S1C, left), and the comparable effects were also observed with non-essential amino acids that are present in the standard DME medium (such as serine) (Fig. S1C, right). To determine whether the observed increase in amino acid levels was due to autophagy resulting from glutamine depletion (Cheong et al., 2011), which may stimulate protein catabolism and amino acid accumulation, we measured the autophagy flux when glutamine was withdrawn or replaced with asparagine. As measured by LC3 conjugation, autophagy flux was not altered under these conditions (Fig. S1D). We also measured amino acid depletion in the culture medium in the conditions indicated. The depletion of leucine, serine, or methionine from the medium correlated in timing and extent with the accumulation of cellular protein as the cells proliferated (Fig. S1E–S1F).

We hypothesized that the influx of other amino acids could compromise the ability of cells to retain intracellular glutamine to support cell survival and proliferation due to the existing import/antiport exchanges between glutamine and other amino acids via system L (Nicklin et al., 2009). To test this, we supplemented low glutamine-containing medium with varied concentrations of EAAs plus serine and glycine (MEM/S/G). Indeed, elevated extracellular levels of EAAs compromised cell proliferation and survival in T47D and SF188 cells under low glutamine conditions, while no inhibitory effect was observed under glutamine-replete

state (Fig. S1G–S1H). However, this effect was relatively minor at 0.5x MEM, which contains close to physiological levels of system L-imported amino acids (Table S1).

The marked increase of intracellular amino acids in glutamine-depleted cells and its restoration back to glutamine-replete levels by asparagine supplementation prompted us to investigate whether this increase was due to the suppression of protein synthesis in glutamine-starved cells. We also hypothesized that asparagine supplementation may act via facilitating translation under glutamine-depleted conditions. Asparagine supplementation was able to restore translation in the absence of glutamine, as measured by the incorporation of puromycin, a tyrosyl-tRNA mimetic (Fig. 1E). Even in cells in which asparagine primarily rescues survival, asparagine supplementation was able to suppress the translation inhibition induced by glutamine withdrawal (Fig. S1I). In addition, we observed that accumulation of short-lived proteins, such as HIF1α and c-Myc (Fig. 1F), triggered by blocking their targeted proteolytic degradation with a proteasome (in case of both HIF1α and c-Myc) or a lysosome inhibitor (in case of HIF1α) was profoundly compromised in glutamine-depleted cells. Asparagine supplementation resulted in a marked restoration of this accumulation.

Asparagine facilitates the expression of GLUL under glutamine deprivation

Protein synthesis cannot proceed in the absence of glutamine. Proliferating cells have to rely on de novo glutamine biosynthesis when the exogenous glutamine is limited. The rate limiting enzyme for glutamine biosynthesis is glutamine synthetase (GLUL), which has been shown to play a critical role for cell proliferation of murine embryonic stem (mES) cells and glioma cells in the absence of exogenous glutamine (Carey et al., 2015; Tardito et al., 2015). Notably, GLUL protein was previously shown to be upregulated in glutamine-depleted cells in a post-transcriptional manner (Nguyen et al., 2016). Therefore, we asked whether the availability of asparagine, owing to its ability to facilitate translation in the absence of exogenous glutamine, may be critical for enabling adaptive upregulation of GLUL when extracellular glutamine is depleted. Strikingly, we found that GLUL protein levels showed little or no induction following glutamine depletion in the lines tested unless exogenous asparagine was added. Asparagine supplementation significantly increased GLUL levels in all cell lines tested (Fig. 2A, S2A). The accumulation of GLUL protein was not due to increased GLUL mRNA (Fig. S2B), suggesting that increased transcription was not responsible for its accumulation. To further interrogate the critical role of GLUL in the adaptation of cells to glutamine limitation, we treated cells with methionine sulfoximine (MSO), a GLUL inhibitor. This resulted in a complete suppression of asparagine-induced translation (Fig. 2B, S2C), as well as asparagine-dependent cell proliferation (Fig. 2C) under glutamine-limited conditions, while having no effect on glutamine-replete cells.

While asparagine facilitated GLUL protein expression in glutamine-depleted cells, replacing glutamine with other NEAAs (including alanine, aspartate, glutamate or proline) individually had no effect on GLUL protein levels (Fig. 2D), which is consistent with the inability of these amino acids to rescue proliferation of glutamine-depleted cells (Fig. 2E). Furthermore, the observed effect on GLUL protein levels was specific to glutamine depletion, and not a feature of the general amino acid stress response induced by glutamine

withdrawal. Indeed, depletion of valine trigged a comparable upregulation of ATF4, a principal amino acid stress response effector, yet it did not induce GLUL protein regardless of the presence or absence of asparagine (Fig. 2F).

In addition to suppressing the general amino acid response elicited by glutamine depletion, asparagine also markedly restored mTORC1 activity (Fig. 2G), which has previously been shown to become compromised in the setting of glutamine withdrawal. Notably, the observed restoration was specific to the proteinogenic L-isoform of asparagine (Fig. 2H).

To determine whether GLUL is required for cell proliferation in glutamine-deficient medium supplemented with asparagine, we used CRISPR/Cas9 to genetically inactivate GLUL, leading to the elimination of GLUL protein (Fig. S2D). GLUL depletion by three independent sgRNA had no effect on cell proliferation when exogenous glutamine was provided (Fig. 2I, left). In contrast, proliferation was profoundly compromised in GLUL-depleted cells when glutamine-deficient medium was supplemented with asparagine (Fig. 2I, right). Introduction of an inducible mouse GLUL construct into GLUL-depleted cells resulted in a marked restoration of asparagine-driven proliferation (Fig. 2J–2K). Consistently with their effects on proliferation, MSO treatment as well as sgRNAs directed against GLUL also inhibited the short-lived protein accumulation under the same conditions (Fig. S2E–S2G).

Despite a marked restoration of protein synthesis and proliferation, asparagine supplementation did not restore intracellular glutamine levels in glutamine-deprived cells (Fig. S2H, left), and did not cause any glutamine accumulation in conditioned medium (Fig. S2H, right). In contrast, glutamate, which must be synthesized de novo when glutamine is absent, accumulated in the conditioned medium from glutamine-deprived cells. This accumulation was significantly repressed by asparagine supplementation (Fig. S2I), suggesting that glutamate is both undergoing increased conversion to glutamine and being utilized to support on-going protein synthesis in asparagine-supplemented cells. Asparagine-driven repression of glutamate levels in the medium was dependent on GLUL activity, as no such effect was observed in GLUL-depleted cells (Fig. S2J). To further verify that glutamine is being synthesized de novo in glutamine-deprived cells, we performed metabolic tracing experiments. We hypothesized that the majority of carbon for the de novo synthesis of glutamine must be derived from glucose, as glucose-derived pyruvate can serve as an anaplerotic substrate for the TCA cycle in cultured cells, when glutamine is unavailable. Indeed, exposing glutamine-deprived cells to [U-^{13}C] glucose for 6 hours revealed that glucose carbons become readily incorporated into glutamine; moreover, asparagine supplementation facilitated this pattern (Fig. S2K).

Asparagine is not used as a substrate for glutamine biosynthesis due to its inability to be catabolized to aspartate in mammalian cells

Recently, it has been suggested that stromal cells might use asparagine as a carbon source to synthesize glutamine and thus supply neighboring tumor cells with glutamine when tissue levels of glutamine are low (Yang et al., 2016). For asparagine-derived carbon to support glutamine biosynthesis, asparagine needs to be first catabolized to aspartate (Fig. 3A). In bacteria and yeast, this reaction is catalyzed by cytosolic asparaginase (Dunlop and Roon,

1975; Jones and Mortimer, 1970; Peterson and Ciegler, 1969); however, cytosolic asparaginase activity has not been reliably reported in mammalian cells (Fig. 3A). To determine whether asparagine can be catabolized to aspartate and free ammonium by cells in the setting of glutamine depletion, we measured the intracellular levels of aspartate and the free ammonium released into the medium. Glutamine depletion significantly reduced the intracellular aspartate and free ammonium release (Fig. 3B and S3A). This was expected as glutaminase-driven deamidation of glutamine is the main source of ammonium production, and the resulting glutamate is the driver of the transamination of oxaloacetate in proliferating cells. Neither the levels of intracellular aspartate nor extracellular free ammonium were restored by asparagine in T47D cells (Fig. 3B and Fig. S3A). Indeed, none of the TCA cycle intermediates or the NEAA alanine and proline could be restored by asparagine in T47D cells under glutamine deprivation (Fig. S3B–S3C).

To determine whether the inability to catabolize asparagine is a general feature of mammalian cells, we tested a panel of mammalian cell lines from diverse tissues of origin, and found no meaningful restoration of aspartate by asparagine in any of the lines tested (Fig. S3D). To rule out the possibility that the failure to detect asparagine catabolism is due to the artifact of mammalian cell culture, we infused mice with [U-^{13}C]-asparagine for 2 hours. Labeled asparagine readily entered various mouse tissues, with 30~60% of tissue asparagine content becoming labeled after just 2 hours (Fig. S3E). However, asparagine-derived carbons did not contribute meaningfully to the pools of aspartate or TCA cycle intermediates in any tissue (Fig. S3F and data not shown). In contrast to mammalian tissues and cell lines, we found that cell lines derived from fruit fly or from zebrafish readily utilized asparagine to maintain intracellular aspartate even beyond the levels normally achieved via the use of glutamine (Fig. 3C).

Mammalian asparaginase homologues lack either sufficient expression and/or activity for asparagine use as a metabolic substrate

The human genome encodes two genes with homology to the cytosolic asparaginases of lower organisms. The first is hASPG, a human cytosolic asparaginase (Karamitros and Konrad, 2014). Expression of hASPG was found to be less than 1 per 10^6 transcripts in all mammalian cell lines tested (Uhlen et al., 2015) (Fig. S4F) and not detectable in the cell lines tested here. Furthermore, ectopically expressed hASPG did not display significant asparaginase activity in T47D cells under assay conditions where comparably expressed zebrafish (zASPG) asparaginase exhibited robust and specific activity (Fig. S4I). This is consistent with the high $S_{0.5}$ of human asparaginase in comparison to other species; namely, 0.4 mM for bacterial and 11 mM for human isoform (Karamitros and Konrad, 2014). Since the key catalytic residues are conserved between bacterial, yeast and human asparaginases (Karamitros and Konrad, 2014), the reason behind a the reduced enzymatic activity of human asparaginase remains to be elucidated.

The other human gene with homology to asparaginases from lower organisms is asparaginase-like 1 (ASRGL1). Although its reported function is hydrolysis of β–aspartyl residues from the N-termini of proteins (Cantor et al., 2009), ASRGL1 is expressed in a wide variety of mammalian cell lines, including several of those studied here (Fig. S4G and

S4H, left). At the levels of endogenous expression of ASRGL1 we were unable to detect amidase activity in the cytosol of the studied cell lines, including T47D, MCF7 and MDA-MB-468 (Fig. S4H, right). As a positive control, we transduced T47D cells with a retrovirus-encoded ASRGL1, achieving ~100-fold increase in its expression (Fig. S4J). At this level of ASRGL1 expression we were able to detect a modest amidase activity (Fig. S4I). These results are consistent with a relatively high reported K_m (~3.4 mM) of ASRGL1 for asparagine (Cantor et al., 2009). Taken together, at physiologic levels of expression and physiologic levels of asparagine, ASRGL1 is unlikely to be a meaningful source of deamidation of asparagine in the cell lines tested.

Expression of asparaginase from lower organisms restores the capacity of mammalian cells to utilize asparagine as a biosynthetic substrate

To determine whether the restoration of asparaginase activity in human cell lines is sufficient to drive asparagine catabolism and its utilization as a biosynthetic substrate, we transduced SF188 cells with a lentiviral vector that drives the ectopic expression of a FLAG-tagged yeast (ASP1) or zebrafish (zASPG) asparaginase in a doxycycline-inducible manner, and confirmed the cytosolic localization of the expressed protein in the transduced cells (Fig. S4A–S4B). As mentioned above, extracts from ASP1- or zASPG-transduced cells showed a robust and specific asparaginase activity indicated by ammonium release, when asparagine, but not glutamine, was used as the substrate (Fig. S4C). In addition, ectopic expression of ASP1 or zASPG induced the release of ammonium (Fig. S4D) into conditioned medium, as well as replenished pools of aspartate, glutamate and TCA cycle intermediates, when glutamine-deficient medium was supplemented with asparagine (Fig. 3D). By tracing the fate of a supplemented [U-^{13}C]-asparagine, we confirmed that it directly contributed to the intracellular pools of aspartate, malate, citrate and glutamate of ASP1- or zASPG-expressing cells in the absence of exogenous glutamine (Fig. 3E). In fact, in cells expressing zASPG, asparagine was the dominant source for aspartate, malate and citrate even when glutamine was present (Fig. S4E).

As has been recently reported, proliferating mammalian cells require an intact respiratory chain to support the production of aspartate to maintain nucleotide production. This explains the inhibition of proliferation when mammalian cell lines are treated with complex I or III inhibitors and this inhibition was shown to be rescued by exogenous aspartate (Birsoy et al., 2015; Sullivan et al., 2015). In contrast, we found that asparagine was unable to rescue cell proliferation in control cells treated with rotenone or antimycin A. However, expression of ASP1 or zASPG allowed asparagine to rescue cell proliferation in the presence of respiratory chain inhibitors (Fig. 3F). In SF188 cells transduced with ASP1 or zASPG, asparagine also enhanced cell proliferation in glutamine-deficient medium, which was inhibited by the GLUL inhibitor MSO (Fig. 3G). This is consistent with the ability of these cells to increase asparagine-dependent TCA cycle anaplerosis and glutamate/glutamine biosynthesis (Fig. 3D).

Expression of zebrafish asparaginase (zASPG) can compromise in vitro and in vivo cell proliferation

Thus far, our findings suggest that intracellular asparagine might be the most limiting NEAA for maintaining protein synthesis when exogenous glutamine is deficient. Indeed, since proliferation of glutamine-deprived cells is resumed upon addition of asparagine alone, this indicates that other missing non-essential amino acids can be readily synthesized by cells once the condition of asparagine sufficiency has been met. The absence of asparaginase activity in proliferating mammalian cells might represent a mechanism which serves to prioritize asparagine preservation when both extracellular glutamine and asparagine levels are depleted. To test this hypothesis, we transduced T47D cells with a constitutively expressed FLAG-tagged zebrafish asparaginase (zASPG) or a control vector. We reasoned that reducing the extracellular asparagine concentration might compromise the ability of zASPG-transduced cells to proliferate specifically in the absence of exogenous glutamine. As expected, at concentrations of asparagine less than 0.1 mM, which is representative of the asparagine levels in human plasma, zASPG-transduced cells failed to proliferate in the absence of exogenous glutamine (Fig. 4A, red box). In contrast, at high concentrations of exogenous asparagine (> 1 mM), zASPG-transduced cells displayed a marked proliferative advantage when exogenous glutamine was removed (Fig. 4A). Consistent with the effect on proliferation, we found that intracellular asparagine levels fell to almost undetectable levels in the zASPG-transduced cells when glutamine was absent and exogenous asparagine is low (≤0.1 mM) (Fig. 4B).

Next, to test whether asparagine availability is the limiting factor for cell proliferation under glutamine depletion, we performed LC-MS analysis of intracellular glutamine and asparagine. Supplementing glutamine-depleted cells with 0.1 mM asparagine modestly restored glutamine but not asparagine in zASPG-transduced cells (Fig. 4B and Fig. S4K), which indicates that asparagine is the limiting amino acid for cell proliferation under these conditions (Fig. S4L). However, to recover maximal proliferation, a supraphysiologic level of exogenous asparagine was required (2 mM) to both restore glutamine levels and overcome zASPG-driven asparagine catabolism (Fig. 4B and Fig. S4K). Consistent with the enhanced proliferation, 0.1 mM asparagine was sufficient for translation rescue in glutamine-depleted conditions in control cells, while zASPG-expressing cells required supraphysiologic asparagine (2 mM) to restore translation (Fig. 4C). The inhibition of protein synthesis at physiologic levels of asparagine (0.1 mM) in zASPG-transduced cells was also manifested by the defect in accumulation of short-lived proteins, such as HIF1α and MYC (Fig. 4D). Constitutive expression of zASPG in MDA-MB-468 breast cancer cell line suppressed its ability to form xenografts when injected subcutaneously into immunocompromised mice (Fig. 4E), compared to control vector-expressing cells. In agreement with the results from cells cultured in vitro (Fig 4B and S4A), zASPG-expressing xenografts displayed a marked depletion of asparagine, while glutamine levels remained largely unaffected (Fig. 4F).

Discussion

How tumor cells adapt to glutamine limitation is an important topic for several reasons. First, despite the fact that tumor cells demand large quantities of glutamine that surpass their need for protein synthesis, the levels of glutamine in the tumor environment are frequently depleted when compared to the levels found in plasma or in normal tissues (Marquez et al., 1989; Rivera et al., 1988; Roberts et al., 1956). This shortage appears to be specific to glutamine and a number of other NEAAs, whereas the EAA levels did not decrease in human pancreatic tumor tissues or in the core regions of xenograft tumors (Kamphorst et al., 2015; Pan et al., 2016). Second, it was shown that in mouse models of brain and lung cancer, tumor cells prefer to use glucose to supply the anaplerotic substrate of the TCA cycle; while glutamine's contribution to the TCA cycle is less important under physiological conditions in vivo (Davidson et al., 2016; Marin-Valencia et al., 2012).

Here we provide evidence that asparagine supports the adaptation of tumor cells to glutamine limitation at levels well below asparagine's physiological concentration (< 0.1 mM) (Stegink et al., 1991). The restoration of proliferation in the absence of exogenous glutamine correlates with an asparagine-dependent induction of GLUL protein. We found that GLUL-dependent glutamine production is required for cell survival and proliferation in the setting of glutamine starvation. Thus, tumor cells adapt to glutamine limitation by switching to de novo glutamine biosynthesis. De novo glutamine synthesis appears to be able to maintain all glutamine-dependent biosynthetic reactions required for cell growth except one, the biosynthesis of asparagine. Even though most glutamine-depleted cells including those studied here upregulate asparagine synthetase (ASNS) (Ye et al., 2010), they are unable to generate sufficient asparagine from endogenously synthesized glutamine to maintain either cell viability or proliferation.

In contrast to lower organisms, most mammalian cells do not express cytosolic asparaginase (Su et al., 2002; Uhlen et al., 2015) and cannot catabolize asparagine to aspartate to maintain non-essential amino acid biosynthesis or TCA cycle anaplerosis. Instead, exogenous asparagine is required for cells to maintain translation when extracellular glutamine is limited. To explore this notion, we engineered the cells capable of catabolizing asparagine via ectopic expression of zebrafish asparaginase (zASPG). In the setting of physiological levels of asparagine (< 0.1 mM), asparaginase prevented the intracellular accumulation of asparagine, effectively blocking the ability of the cells to engage in protein synthesis and proliferate in the absence of exogenous glutamine. Furthermore, zASPG-expressing cells displayed a significant growth defect and reduced asparagine levels in a xenograft assay, thus corroborating our findings from modeling glutamine depletion in vitro. The present data suggest a further explanation for the recent findings of Krall et al. (2016) and Yang et al. (2016). It is likely that stromal cells faced with declining amino acid levels of the tumor environment turn on autophagy to sustain their survival. Unlike the other amino acids, asparagine is not catabolized, but used in countertransport to take up essential amino acids as suggested by Krall et al. (2016). The release of asparagine into the tumor environment then can support tumor cell survival and growth at subphysiologic levels of glutamine by helping restore the translation of GLUL and other adaptive proteins.

Limitations of Study

The in vitro glutamine depletion conditions studied here were in the presence of defined levels of other nutrients and amino acids. However, the concentrations of amino acids and other nutrients in the standard culture medium may well differ from those encountered by cells in vivo. Yet, we did find that expressing a functional asparaginase is markedly inhibitory for xenograft formation in vivo. This adds to a growing body of work that extracellular asparagine can be essential for cancer cell growth in vivo.

Bacterial L-asparaginases have long been used as a first line of treatment against pediatric acute lymphoblastic leukemia (ALL), and newer formulations have recently shown promising results in adult leukemia (Koprivnikar et al., 2017). Historically, the efficacy of L-asparaginase has been correlated with asparagine synthetase levels. Our study suggests that glutamine deficiency can also contribute to increased tumor dependency on exogenous levels of asparagine. However, it remains to be fully explored how variations in aspartate and glutamate availability and ASNS expression modulate a cancer cell's ability to synthesize sufficient levels of asparagine to support cell survival and growth in vivo.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Craig B. Thompson (thompsonc@mskcc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines—All mammalian cell lines were cultured at 37°C in 5% CO_2 in high glucose DMEM supplemented with 10% FBS. ZMEL-1 cells were cultured at 28°C in 5% CO_2 in high glucose DMEM supplemented with 10% FBS. S2R+ cells (RRID: CVCL_Z831) were cultured at 25°C in Schneider's Drosophila Media. T47D (RRID: CVCL_0553), MCF-7 (RRID: CVCL_0031), ZR-75-1 (RRID: CVCL_0588) and MDA-MB-468 cells (RRID: CVCL_0419) are female breast adenocarcinoma cell lines. SF188 cell line (RRID: CVCL_6948) is a male glioblastoma cell line. Cell lines were authenticated via STR repeat assay performed by Integrated Genomics Operations (MSKCC), and verified to be mycoplasma-free by MycoAlert Mycoplasma Detection Kit (Lonza).

Animals—All animal experiments were performed after approval from the MSKCC Institutional Animal Care and Use Committee or Princeton University Institutional Animal Care and Use Committee and were conducted as per NIH guidelines for animal welfare. 6–8-week old athymic nude female mice (Envigo) were used for xenograft assays. Mice were housed at 4–5 mice per cage at 24°C and at a 12-hour light/dark cycle, and were allowed access to food and water ad libitum. Animals were allowed an acclimatization period of one week prior to the experiment. At the beginning of each experiment, mice were randomly assigned to either control or experimental group. For in vivo infusions with [U-^{13}C]-L-asparagine, 10-week old female C57BL/6 mice pre-catheterized on the right jugular vein (Charles River Laboratories) were used.

METHOD DETAILS

Amino acid deprivation experiments—Cells were rinsed once, followed by the addition of 10% dialyzed FBS-supplemented media containing indicated levels of amino acids. For cell proliferation experiments, cell numbers were counted in triplicates using the Multisizer Coulter Counter (Beckman). For the assessment of the levels of short half-life proteins, cells were cultured in presence or absence of indicated amino acids for 6 hours for SF188 cells or 24 hours for T47D cells and harvested for Western blot analysis. Where indicated, proteasome inhibitor MG132 (10 μM) or lysosome inhibitor chloroquine (20 μM) was added 6 hours prior to harvesting. For puromycin labeling assay, cells were cultured in presence or absence of indicated amino acids for 24 hours. Puromycin (90 μM) was added to cells for 10 minutes, after which cells were harvested for Western blot analysis. Where indicated, thapsigargin (200 nM) was added 30 minutes prior to puromycin pulse.

Western Blotting—Protein extracts were prepared by using 1× RIPA buffer including protease inhibitors (Thermo # 1860932) and phosphatase inhibitors (Thermo # 78428). Equal amount of total protein was separated on NuPAGE Bis-Tris gels (Life Technologies), transferred to nitrocellulose membrane and subjected to the incubation with indicated primary antibodies.

Asparaginase activity assay—Cells were lysed in PBS with 0.1% Triton and sonicated at maximal intensity for 30 seconds per cycle for 3 cycles (Bioruptor-300). Three replicates per condition were harvested. Soluble fractions were incubated with 20 mM asparagine or glutamine for 3 hours. Ammonium was quantified by the Ammonia Assay Kit (K370-100, BioVision). For the ammonium in the culture media, the results were normalized to the total number of cells.

GC-MS and LC-MS analysis of metabolites—All of the mammalian cells and ZMEL-1 cells were cultured in DMEM without glutamine (−Q) or without glutamine but with asparagine (−Q + N) for 16 hours prior to metabolite extraction. S2R+ cells were maintained at 25°C in the Schneider's Drosophila medium. For the 16 hours incubation prior to metabolite extraction, cells were incubated in a modified version of Schneider's Drosophila medium that does not contain aspartic acid, glutamic acid, α-ketoglutaric acid, fumaric acid, malic acid and succinic acid. For harvesting, cells were rinsed with PBS once and incubated with 80% methanol pre-chilled to −80°C for 30 minutes at −80°C. Three replicates per condition were harvested. Supernatant was collected by centrifugation. For GC-MS, the supernatant was dried by spin vacuum, dissolved in 40 mg/mL methoxyamine in pyridine and derivatized with MSTFA+1% TMCS. One microliter of trimethylsilyl-derivatized organic acids was analyzed through an Agilent 7890A GC equipped with an HP-5MS capillary column and connected to an Agilent 5975 C mass selective detector. The LC-MS method involved reversed-phase ion-pairing chromatography coupled by negative mode electrospray ionization to a stand-alone orbitrap mass spectrometer (Thermo Scientific) scanning from m/z 85–1000 at 1 Hz at 100,000 resolution with LC separation on a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 μm particle size, Phenomenex, Torrance, CA) using a gradient of solvent A (97:3 H_2O/MeOH with 10 mM tributylamine and 15 mM acetic acid), and solvent B (100% MeOH). The gradient was 0 min, 0% B; 2.5 min, 0% B; 5

min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Injection volume was 10 μL, flow rate 200 μl/min, and column temperature 25 °C(Lemons et al., 2010; Lu et al., 2010; Munger et al., 2008). Data were analyzed using the MAVEN software suite (Melamud et al., 2010). All the values of the compounds from mass spectrum were corrected to the internal spiked control (D-2-hydroxyglutaric-2,3,3,4,4-d5 acid, d5-2HG at 2 μM) before further normalization.

Intravenous infusion of [U-^{13}C] asparagine—In vivo infusion was performed on 10-week old female C57BL/6 mice pre-catheterized on the right jugular vein (Charles River Laboratories, Wilmington, MA), following protocols approved by the Princeton University Institutional Animal Care and Use Committee. The mice were maintained on a normal light cycle (8 AM – 8 PM). The mouse infusion setup (Instech Laboratories, Plymouth Meeting, PA) included a tether and swivel system so that the animal has free movement in the cage. [U-^{13}C] asparagine (Cambridge Isotope Laboratories, Cambridge, MA) was prepared as 0.1 M solution in saline and infused via the catheter at a constant rate of 0.1 μl/min/g for 150 min. A few minutes before the end of the infusion, the blood sample (~20 μl) was collected by tail bleeding. Blood samples were kept on ice before centrifugation at 4°C for serum. At the end of the infusion, the mouse was euthanized by cervical dislocation and tissues were quickly dissected and snap frozen in liquid nitrogen with pre-cooled Wollenberger clamp (Wollenberger et al., 1960). Serum and tissue samples were kept at −80°C before mass spectrometry analysis.

Metabolites were extracted from serum samples (thawed on ice) by adding 100 μl 80% methanol at −80°C followed by vortexing and centrifugation at 16,000 × g for 10 min. Insoluble pellets were re-extracted with 100 μl −20°C methanol:acetonitrile:H2O solution at 2:2:1 ratio. The supernatants were combined. Frozen tissue samples were weighed (~20 mg each sample), ground with Cryomill (Retsch, Newtown, PA), and extracted for two rounds by adding −20°C methanol:acetonitrile:H2O (2:2:1) solution followed by vortexing and centrifugation at 16,000 × g for min. The volume of the extraction solution (in μl) in each round was 20 times of the weight of tissue (in mg). The supernatants were combined. Serum and tissue extractions were directly run on a LC-MS system. Briefly, a stand-alone quadrupole orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, Waltham, MA) operating in negative ion mode was coupled to hydrophilic interaction chromatography and used to scan from m/z 70 to 1000 at 1 Hz and 140,000 resolution. LC separation was on a XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters, Milford, MA) using a gradient of solvent A (20 mM ammonium acetate plus 20mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 150 μl/min. The gradient was: t=0, 85% B; t=3 min, 80% B; t=6, 75% B; t=8 min, 70% B; t=10 min, 50% B; t=12 min, 25% B; t=14 min, 0% B; t=24 min, 85% B; t=30 min, 85% B. Data were analyzed using the MAVEN software (Clasquin et al., 2012). Isotope labeling was corrected for natural abundances of ^{13}C, ^{2}H, and ^{15}N.

MDA-MB-468 xenograft experiment—MDA-MB-468 cells were infected with retroviruses expressing GFP only or zASPG with GFP linked by internal ribosomal entry site. GFP-positive cells were sorted and maintained in standard DME medium. 5×10^{6} cells

were collected, mixed with 50 μL PBS and 50 μL Matrigel (BD 356273) and injected subcutaneously into each flank of the 6–8 weeks old athymic nude female mice (Envigo). Eight animals per condition were injected. Tumor volumes were calculated by $l \times w^2 \times \pi/6$. (l: tumor length; w: tumor width). At the end point of the experiment, mice were euthanized by CO_2 inhalation. Tumor tissues from one flank of each individual mouse were collected and subjected to metabolite extraction as described above. Asparagine and glutamine were quantified by LC-MS as described in the previous paragraph.

[U-^{13}C]-labeling experiment—For labeling with [U-^{13}C]-asparagine and [U-^{13}C]-glutamine, cells in triplicates were treated with doxycycline (1 μg/mL) for 48 hours, and then incubated with the conditional media containing 4 mM [U-^{13}C]-asparagine or [U-^{13}C]-glutamine for 16 hours. Metabolites were extracted and subjected to GC-MS analysis as described above. Enrichment of ^{13}C was assessed by quantifying the abundance of the following ions: aspartate, *m/z* 349–353; malate, *m/z* 335–339; citrate, *m/z* 465–471; glutamate, *m/z* 363–368. For [U-^{13}C]-glucose labeling, cells were cultured in indicated amino acid conditions for 18 hours, after which culture medium was substituted to an equivalent formulation, but with 25 mM [U-^{13}C]-glucose for 6 hours. The results were corrected to the internal spike-in control (d5-2HG), adjusted for natural abundance by using IsoCor software (Millard et al., 2012) and then normalized to packed cell volume.

Molecular cloning and virus production—A single FLAG-tag was added to the N-terminus of the cDNAs of yeast ASP1 or zebrafish zASPG. The cDNAs were inserted into a modified pTRIPZ lentiviral vector (Dharmacon) or pMSCV-IRES-GFP (Addgene). Guide RNAs against human glutamine synthetase (sgGLUL-1, sgGLUL-2 and sgGLUL-3) were cloned into pLentiCRISPR V2 vector. For CRISPR rescue experiment, mouse Glul cDNA was cloned into pTURN-hygro retroviral expression vector, and HA tag was added to the C-terminus. Human ASRGL1 cDNA was cloned into pMSCV-puro retroviral expression vector (Clontech). Lentiviral particles were produced in 293T cells by using psPAX2 and pMD.2 packaging plasmids (Addgene). Retroviral particles were produced in 293T cells by using pcleo and pCMV-VSV-G packaging plasmids (gift from Scott Lowe). Cells were infected with viral supernatant in presence of 6 μg/mL of polybrene overnight and subjected to puromycin (2 μg/mL) or hygromycin (200 μg/mL) selection, or GFP sorting.

mRNA quantification—Cells were cultured overnight in indicated media conditions. Total RNA was isolated using TRIzol according to the manufacturer's instructions. 1 ug of total RNA was used for cDNA synthesis with oligo-dT primer, using SuperScript RT III reverse transcriptase (Thermo Fisher Scientific). qPCR was performed on triplicates using Taqman probes against human GLUL (Hs00365928_g1) and ACTB (4333762T).

^{35}S-Methionine assay—Cells were pre-cultured in indicated media conditions for 4 hours, and then washed once with PBS. Conditional media but lacking unlabeled methionine and cysteine were applied to the cells in the presence of 0.1 mCi ^{35}S-labeled methionine/cysteine mixture for 30 minutes. Cells were collected for protein extraction with RIPA buffer in the presence of protease inhibitors. 2 microliters of radio-labeled protein were spotted onto small pieces of filter papers and air-dried. Filter papers were boiled in 10% TCA

(Trichloroacetic acid, T0699, Sigma) for 5 minutes, followed by 2× washes in ice-cold water, 2× washes in ice-cold 95% ethanol, and 1× wash in acetone. Individual filter papers were added to scintillation vials and counted with scintillation counter.

QUANTIFICATION AND STATISTICAL ANALYSIS

P values were calculated using Student's two-tailed paired t-test, except for the xenograft experiment depicted in Fig. 4E, for which a two-way ANOVA test was used (GraphPad Prism 6). Error bars represent standard deviation between experimental replicates. P values are provided in the figure labels, while the number of replicates is indicated in figure legends. P values below 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Glutamine-deprived cells sustain proliferation via *de novo* glutamine biosynthesis
- Proliferation and protein synthesis in the absence of glutamine requires asparagine
- Mammalian cells do not catabolize asparagine
- Asparaginase blocks the ability of cells to adapt to glutamine deprivation

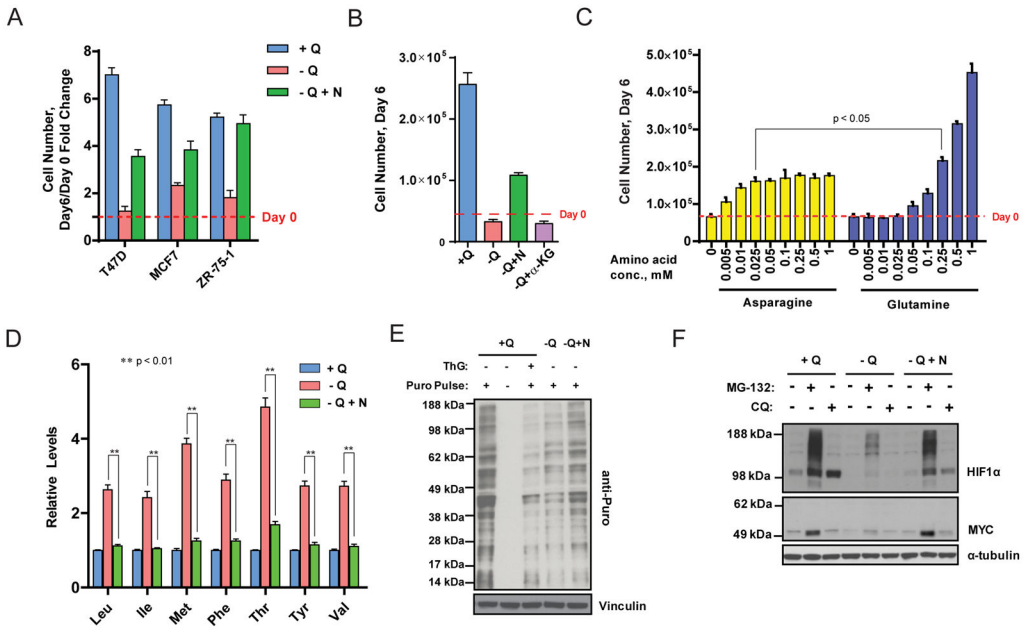

Figure 1. Asparagine sustains protein translation to support cell proliferation in the absence of exogenous glutamine

A. Cell proliferation was measured in indicated cell lines cultured in the presence of 2 mM glutamine (+Q), in the absence of glutamine (−Q) or in the absence of glutamine supplemented with asparagine (−Q+N). Asparagine was supplemented at 2 mM. **B.** A cell-permeable form of α-KG (5 mM) is unable to restore proliferation of glutamine-deprived T47D cells. **C.** T47D cell proliferation was assayed in various concentrations of asparagine or glutamine. **D.** GC-MS analysis of intracellular EAAs after 16-hour culture of T47D cells in indicated media conditions. Relative metabolite levels are shown. **E.** T47D cells were cultured in the indicated media for 24 hours. Puromycin (90 μM) was added 10 minutes before sample collection. Whole cell lysates were subjected to Western blotting with puromycin antibody. Thapsigargin (ThG), a general translation inhibitor, was used as a control. **F.** T47D cells were cultured in the indicated media for 24 hours. A proteasome inhibitor, MG-132 (10 μM) or a lysosome inhibitor, CQ (20 nM) was added 6 hours before harvesting for Western blot analysis. Data in **A–D** are presented as the mean ± SD of triplicates from a representative experiment. P-values are shown as indicated. See also Figure S1 and Table S1.

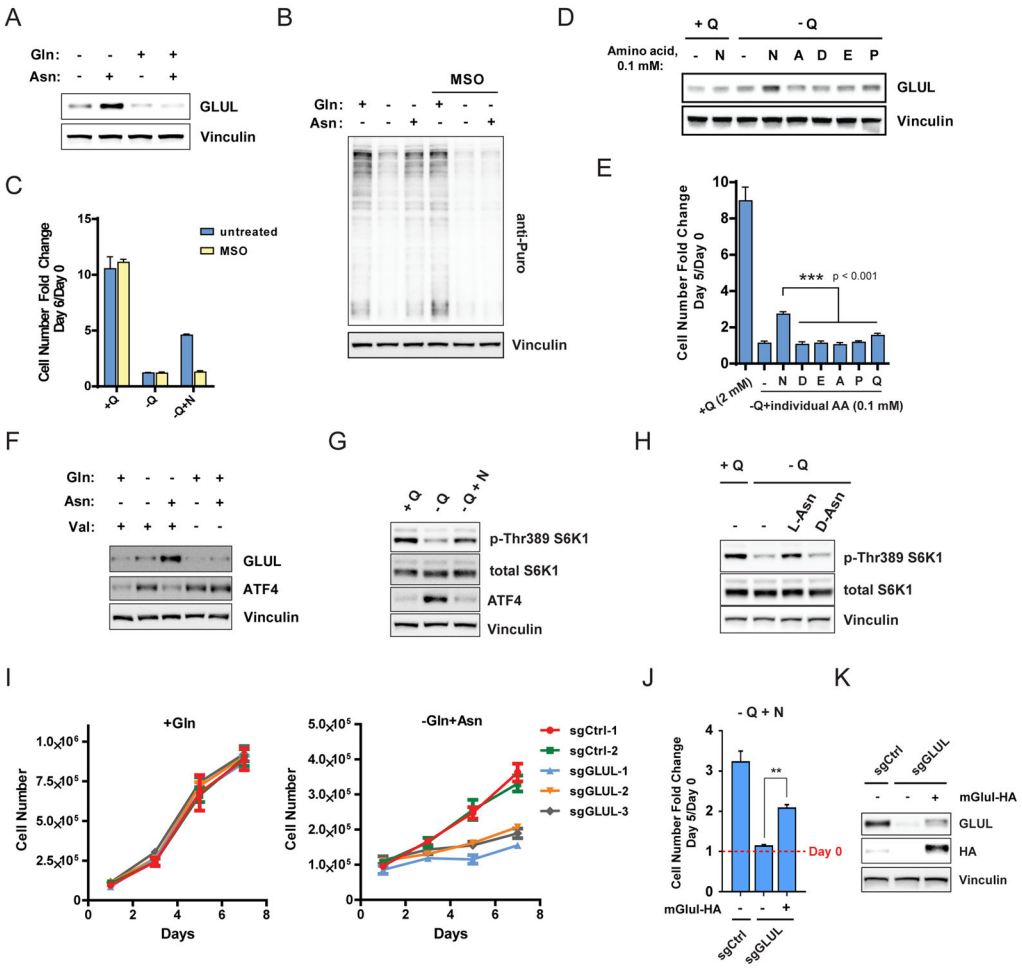

Figure 2. Glutamine synthetase (GLUL) is required for asparagine to rescue cell proliferation in the absence of exogenous glutamine

A. Western blot of GLUL from T47D cells cultured in indicated media conditions for 24 hours. Glutamine (Gln) and asparagine (Asn) were supplemented at 2 mM. **B.** MSO (2 mM) treatment blocks puromycin incorporation into newly synthesized polypeptides when exogenous glutamine is replaced with asparagine. **C.** MSO (2 mM) treatment prevents T47D cell proliferation when glutamine (Q)-deficient medium is supplemented with asparagine (N). **D.** Asparagine (N) specifically induces GLUL protein accumulation under glutamine starvation, as compared to alanine (A), aspartate (D), glutamate (E) and proline (P). **E.** At 0.1 mM, aspartate (D), glutamate (E), alanine (A), proline (P) and glutamine (Q) cannot support T47D cell proliferation as well as the equimolar concentration of asparagine (N). **F.** GLUL and ATF4 levels in T47D cells cultured in indicated media conditions for 24 hours. Glutamine (Gln) and asparagine (Asn) were supplemented at 2 mM, while valine (Val) was supplemented at 0.8 mM. **G.** Activation of mTORC1 is compromised in glutamine-deprived T47D cells, and is restored by asparagine. **H.** L-asparagine, but not D-asparagine, restores mTORC1 activity in glutamine-deprived T47D cells. L-asparagine, D-asparagine and glutamine were used at 2 mM. **I.** Genetic inactivation of GLUL by three independent sgRNAs blocks T47D cell proliferation when glutamine is replaced with asparagine. **J, K.**

Proliferation defect of sgGLUL cells in glutamine-deficient media supplemented with 2 mM asparagine is restored by the ectopic expression of mouse Glul-HA, resistant to targeting by sgGLUL. Data in **C, E, I** and **J** are presented as the mean ± SD of triplicates from a representative experiment. P-values are shown as indicated. See also Figure S2.

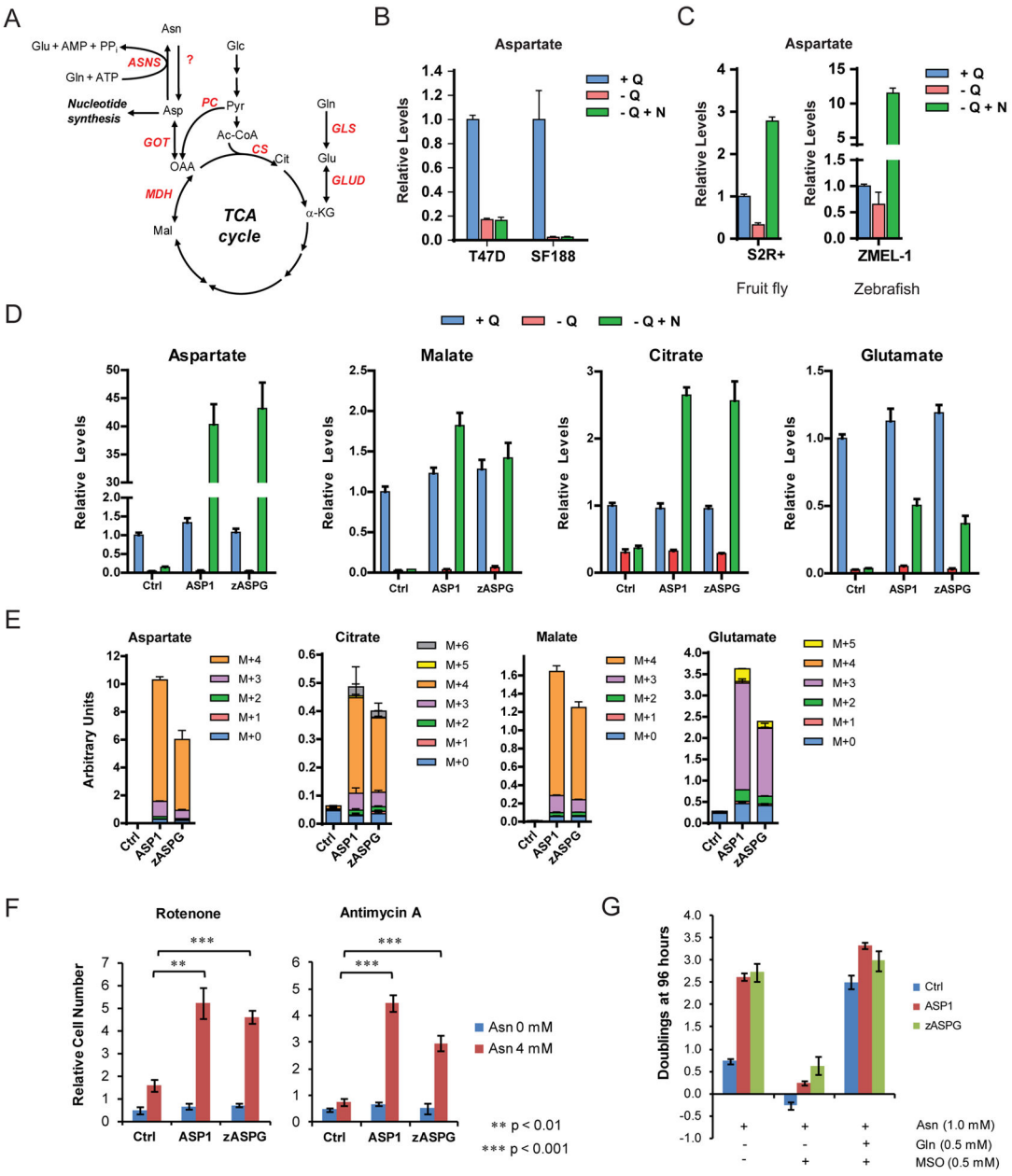

Figure 3. Ectopic expression of a yeast or zebrafish asparaginase restores the capacity of mammalian cells to use asparagine as a biosynthetic substrate

A. Diagram shows the three potential pathways which generate aspartate. **B** GC-MS analysis of intracellular aspartate from T47D and SF188 cells cultured in media as indicated for 16 hours. For T47D cells, glutamine and asparagine were used at 2 mM. For SF188 cells, glutamine and asparagine were used at 6 mM and 4 mM respectively. Relative metabolite levels are shown. **C.** GC-MS analysis of intracellular aspartate from S2R+ (*D. melanogaster*) and ZMEL-1(*D. rerio*) cells cultured in the media as indicated for 16 hours. Glutamine and asparagine were used at 12 mM and 10 mM respectively for S2R+ cells, and at 6 mM and 4 mM for ZMEL-1 cells. Relative metabolite levels are shown. **D.** SF188 cells transduced with doxycycline-inducible lentiviral vectors driving ectopic expression of empty vector (Ctrl),

yeast (ASP1) or zebrafish (zASPG) asparaginase were treated with doxycycline for 48 and then switched to medium ± glutamine (±Q, 6 mM) or –glutamine+asparagine (−Q+N, 4 mM). Relative levels of indicated intracellular metabolites 16 hours after the medium switch were determined by GC-MS. **E.** Cells as described in **D** were treated with doxycycline for 48 hours and then switched to glutamine deficient medium containing 4 mM [U-^{13}C]-asparagine for 16 hours. Indicated intracellular metabolites were quantified by GC-MS. M+0, unlabeled; M+1, M+2, M+3, M+4, M+5 and M+6 represent the degree of *m/z* increase due to labeling, adjusted by natural abundance. The results are presented as absolute ion intensity of each species. Data are presented as the mean ± SD of triplicates from a representative experiment. **F.** Cells, as described in **D**, were treated with doxycycline for 48 hours in complete medium, followed by the addition of an inhibitor of complex I (rotenone) or III (antimycin A) ± asparagine. Cell number was recorded at 96 hours following inhibitor addition and normalized to 0 hour. **G.** Cells were treated with doxycycline for 48 hours and then switched to glutamine-deficient medium containing 0.125× MEM/S/G (amino acids present in standard DME medium). Asparagine, glutamine and/or MSO were added as indicated. Doubling time was calculated at 96 hours post medium switch. Data in **B–G** are presented as the mean ± SD of triplicates from a representative experiment. P-values are shown as indicated. See also Figure S3 and Figure S4.

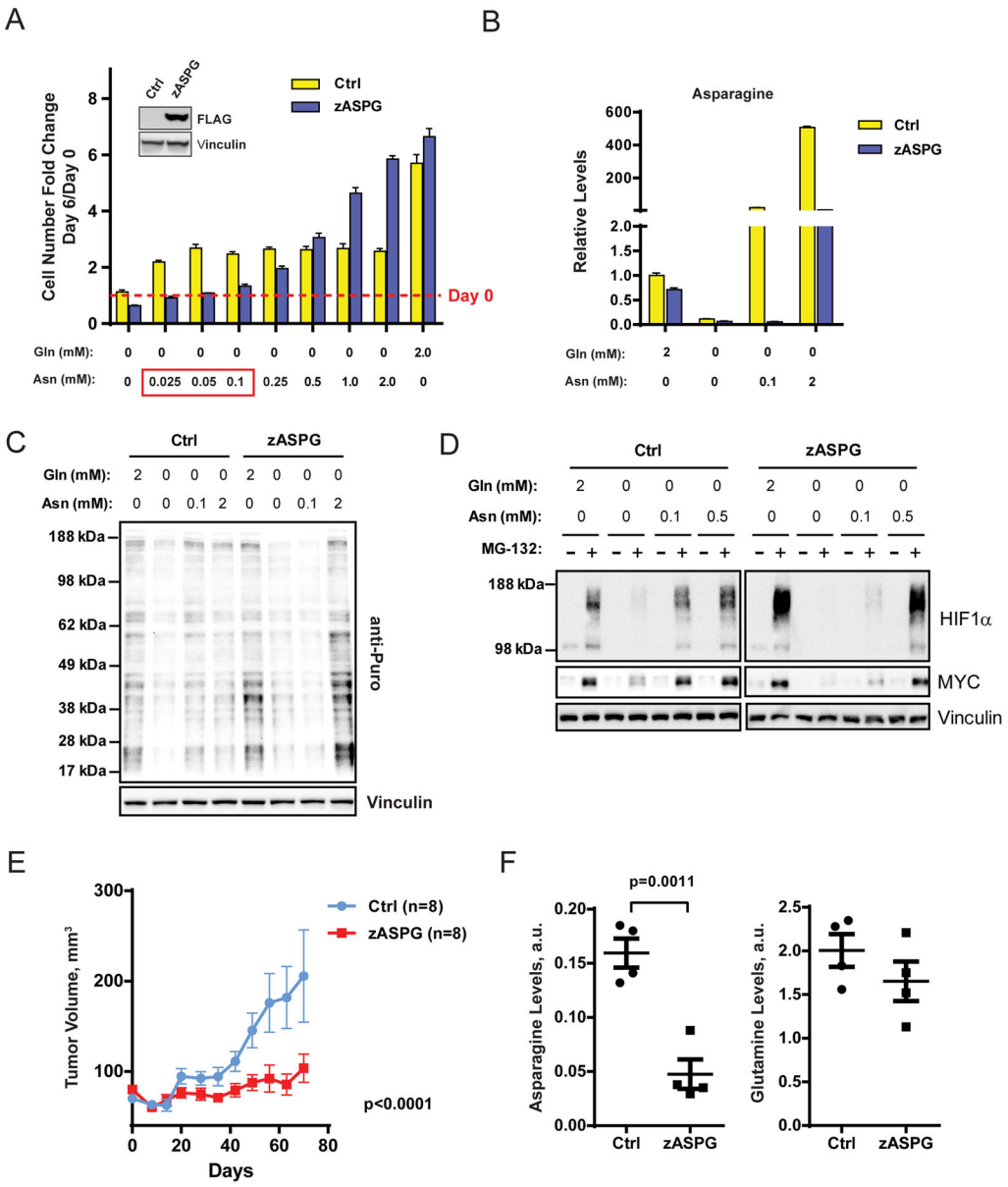

Figure 4. zASPG inhibits protein translation and cell proliferation under glutamine depletion only when exogenous asparagine level is low

A. T47D cells transduced with constitutively expressed empty vector (Ctrl) or zebrafish asparaginase (zASPG) were cultured in various concentrations of asparagine in the absence of glutamine. Cell proliferation was recorded as a cell number fold change 5 days post medium switch. Cells cultured in 2 mM glutamine without asparagine were used as a control. Red box: concentration of asparagine similar to the levels in human plasma. **B.** LC-MS quantification of intracellular asparagine under conditions as indicated for 16 hours. Relative metabolite levels are shown. Data in **A** and **B** are presented as the mean ± SD of triplicates from a representative experiment. **C.** Ctrl- or zASPG-transduced cells were cultured in conditions as indicated for 24 hours. Puromycin was added at 90 μM for 10 minutes before protein harvest. Whole cell lysates were subjected to Western blotting with

puromycin antibody. **D.** Ctrl- or zASPG-transduced cells were cultured in conditions as indicated for 24 hours. MG-132 (10 μM) was added for 6 hours before harvesting for Western blot analysis. **E.** Ctrl- or zASPG-transduced MDA-MB-468 cells were inoculated subcutaneously into both flanks of athymic female nude mice. Tumor volumes were recorded weekly. **F.** Tumor tissues were collected at the end point of the experiment in **E**. Asparagine and glutamine levels were quantified by LC-MS. The results were normalized to the weight of tumor tissues, and then to the median of all amino acids in each tumor sample. P values for **E** and **F** were determined by two-way ANOVA analysis Data in **E** and **F** are presented as the mean ± SD of n = 8 from a representative experiment. See also Figure S4.