*Review*

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**Focus: Metabolism**

# Cancer cell metabolism: the essential role of the nonessential amino acid, glutamine

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## Abstract

**Biochemistry textbooks and cell culture experiments seem to be telling us two different things about the significance of external glutamine supply for mammalian cell growth and proliferation. Despite the fact that glutamine is a nonessential amino acid that can be synthesized by cells from glucose-derived carbons and amino acid-derived ammonia, most mammalian cells in tissue culture cannot proliferate or even survive in an environment that does not contain millimolar levels of glutamine. Not only are the levels of glutamine in standard tissue culture media at least tenfold higher than other amino acids, but glutamine is also the most abundant amino acid in the human bloodstream, where it is assiduously maintained at approximately 0.5 mM through a combination of dietary uptake, *de novo* synthesis, and muscle protein catabolism. The complex metabolic logic of the proliferating cancer cells' appetite for glutamine—which goes far beyond satisfying their protein synthesis requirements—has only recently come into focus. In this review, we examine the diversity of biosynthetic and regulatory uses of glutamine and their role in proliferation, stress resistance, and cellular identity, as well as discuss the mechanisms that cells utilize in order to adapt to glutamine limitation.**

**Keywords** cancer; glutamine metabolism; proliferation; stress response
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## Introduction

With the exception of a few instances, such as cleavage divisions of a fertilized zygote in early embryogenesis, cell proliferation is linked to the biomass accumulation. In the past decade, studies in cancer cell metabolism revealed the central role of numerous metabolic pathways and metabolites in facilitating biosynthesis and bioenergetics required for cell growth and proliferation. Thus, to ensure accumulation of biomass necessary for proliferation, deregulated pro-proliferative and pro-survival signals of cancer cells rewire metabolism to support biosynthesis of proteins, nucleotides, glycans, and lipids, as well as production of energy and NADPH.

In this review, we will first describe the many uses of glutamine and its products in proliferating cells, including its role in supplying carbon and nitrogen atoms for construction of a variety of macromolecular precursors, as well as its significance as a regulator of biosynthesis and bioenergetics, anti-oxidative defense, and gene expression. The consequence of the high demand of proliferating cells for glutamine is the disproportionate depletion of the latter from the surrounding environment. To this end, we will discuss the adaptations that cells use to deal with glutamine limitation, including *de novo* biosynthesis and proteolytic scavenging.

## Glutamine utilization beyond protein synthesis

Along with the rest of the proteinogenic amino acids, glutamine is incorporated into proteins. It is estimated that glutamine accounts for approximately 4.7% of all amino acid residues in human proteome, while in select proteins, for instance, in the structural component of skin epidermal barrier, involucrin, the representation of glutamine residues can reach 25%. However, consumption of glutamine in proliferating cells far exceeds the demands imposed by protein synthesis.

Amino acids contribute to the majority of biomass accumulation in proliferating mammalian cells (Hosios *et al*, 2016). In contrast to unicellular organisms, mammals cannot synthesize all the necessary amino acids for protein synthesis, and must acquire nine out of 21 amino acids from the diet. Notably, the biosynthesis of the rest of the amino acids, which are regarded to as nonessential, is heavily dependent on glutamine. Thus, glutamine deamidation, performed by numerous enzymes in the cells, yields glutamate, which can further be transformed into proline through a series of reductive steps, as well as into aspartate and asparagine, via the utilization of oxidative reactions of the tricarboxylic acid (TCA) cycle. Glutamine-derived glutamate also donates its amine nitrogen toward the biosynthesis of alanine and serine and, by extension, glycine.

### *Glutamine as a nitrogen donor and amino acid precursor*

Another dominant class of nitrogenous compounds that are required for cell proliferation is nucleotides. Notably, glutamine is an indispensable donor of reduced nitrogen for building both purine and pyrimidine bases (Wise & Thompson, 2010). In purine biosynthesis, two glutamine nitrogens are consumed in the biosynthesis of

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inosine monophosphate (IMP), which gives rise to both AMP and GMP. A third nitrogen from glutamine is required to produce guanosine monophosphate (GMP) from inosine monophosphate (IMP). Likewise, the initiating step of pyrimidine biosynthesis involves condensation of glutamine-derived nitrogen with bicarbonate and ATP to generate carbamoyl phosphate. Finally, one more glutamine is consumed in the synthesis of cytidine triphosphate (CTP) from uridine triphosphate (UTP).

Notably, the increased utilization of glutamine nitrogen in nucleotide production is facilitated by the growth-promoting signals. For instance, elevated levels of c-Myc induce the expression of a number of enzymes in the nucleotide biosynthetic pathways, including phosphoribosyl pyrophosphate synthetase 2 (PRPS2), carbamoyl phosphate synthetase II (CAD), thymidylate synthase (TS), inosine monophosphate dehydrogenase 1/2 (IMPDH1/2), and others (Eberhardy & Farnham, 2001; Liu *et al*, 2008; Mannava *et al*, 2008; Cunningham *et al*, 2014). Similarly, loss of Rb and E2F upregulation induces nucleotide biosynthesis enzymes as well (Nicolay & Dyson, 2013). In addition, cancer cells display increased expression of phosphoribosyl amidotransferase (PPAT), the enzyme that transfers amide nitrogen from glutamine to 5-phosphoribosyl pyrophosphate (PRPP), a key reaction in purine biosynthesis (Goswami *et al*, 2015). In addition to being a c-Myc target gene, the CAD enzyme, which generates carbamoyl phosphate in the initiating reaction in the pyrimidine biosynthesis cascade, is positively regulated via phosphorylation by MAP kinase or by S6 kinase downstream of mTORC1 (Graves *et al*, 2000; Ben-Sahra *et al*, 2013; Robitaille *et al*, 2013). The transcriptional induction of genes induced in nucleotide biosynthesis is also observed in cancer cells harboring mutant p53 (Kollareddy *et al*, 2015).

The above results suggest a potential mechanism that proliferating cells use to coordinate growth-promoting signals with glutamine utilization to drive nucleotide biosynthesis. Interestingly, the five reactions in nucleotide biosynthesis that directly utilize glutamine as a substrate exclusively use the γ-nitrogen (amide group) of glutamine (Fig 1A). In addition to nucleotide biosynthesis, the γ-nitrogen of glutamine in mammalian cells is also required to synthesize NAD, glucosamine-6-phosphate, a precursor for protein glycosylation, and asparagine, another nonessential amino acid (Richards & Schuster, 1998; Wellen *et al*, 2010; Fig 1B).

In addition to being incorporated into nucleotides, amino acids, and glucosamine-6-phosphate, the γ-nitrogen of glutamine is subject to a direct cleavage by glutaminase (GLS) enzymes, producing glutamate and free ammonia. GLS is frequently deregulated in cancer (Gao *et al*, 2009; Hu *et al*, 2010; Suzuki *et al*, 2010; Lukey *et al*, 2016), and loss of a single copy of *Gls1* delays tumorigenesis in a mouse model of hepatocellular carcinoma (Xiang *et al*, 2015). It was proposed that the deamidation of γ-nitrogen by GLS contributes to the major intracellular pool of glutamate, another NEAA that can continue to supply both nitrogen and carbon for other biosynthetic reactions. The existence of both mitochondrial and cytosolic isoforms of GLS suggests the importance of compartmentalized production of glutamate, such that the two segregated pools can be used for distinct biosynthetic processes (Cassago *et al*, 2012). In this regard, a number of glutaminase inhibitors have been developed and show tumor-suppressive activities in preclinical models (Wang *et al*, 2010; Gross *et al*, 2014; Shroff *et al*, 2015; Xiang *et al*, 2015). However, in certain cases GLS inhibitors do not have a therapeutic effect (Davidson *et al*, 2016). One possible interpretation is that beside GLS-catalyzed reaction, other biochemical reactions that use γ-nitrogen of glutamine, described above, release glutamate as a product as well. Thus, as GLS activity is inhibited, more glutamine may become funneled into other glutamine-utilizing pathways. Another explanation is that in certain cases, cancer cells may use glucose-derived carbon to maintain TCA cycle intermediates and produce glutamate, therefore diminishing the contribution of the GLS-catalyzed reaction for these processes.

One advantage that proliferating cells that rely on GLS to produce glutamate from glutamine may have is the ability to maintain a high ratio of glutamate to α-ketoglutarate, which is necessary for driving the biosynthesis of other NEAAs. To this end, the remaining nitrogen of glutamate, which resides at the α-position of glutamine carbon chain (the amine group, Fig 1A), can be transferred to different α-ketoacids by a family of aminotransferases to produce other NEAAs, among which are alanine, aspartate, serine, and ornithine. Recently, alanine aminotransferase 2 (GPT2) has been found to be upregulated by PIK3CA mutation in colorectal cancer, as well as by liver receptor homolog 1 (LRH-1) in liver cancer (Hao *et al*, 2016a; Xu *et al*, 2016). Hao *et al* (2016a) show that PIK3CA mutation causes elevated expression of GPT2, an aminotransferase that transfers amino group from glutamate to pyruvate to generate alanine. Similarly, in liver cancer, both GPT2 and aspartate aminotransferase 1 (GOT1), which transfers an amino group from glutamate to oxaloacetate to generate aspartate, are transcriptionally induced by LRH-1 (Xu *et al*, 2016). Furthermore, phosphoserine aminotransferase (PSAT1) transfers amino group from glutamate to 3-phosphohydroxypyruvate to generate 3-phosphoserine, the precursor of serine. Overexpression of PSAT1 has been found to confer growth advantage and resistance to chemotherapy in colorectal cancer (Vie *et al*, 2008). In addition, elevated expression of PSAT1 correlates with poor prognosis in patients with esophageal squamous cell carcinoma (ESCC) (Liu *et al*, 2016). Indeed, multiple studies show that aminooxyacetate (AOA), a general inhibitor of cellular aminotransferases, profoundly inhibits tumor growth *in vitro* and *in vivo* (Thornburg *et al*, 2008; Korangath *et al*, 2015; Hao *et al*, 2016a). Finally, glutamate supplies both the carbon backbone and the nitrogen for proline biosynthesis. Notably, oncogenic c-Myc induces the expression of proline biosynthesis enzymes while suppressing proline dehydrogenase (POX/PRODH), the first enzyme for proline catabolism (Liu *et al*, 2012). These studies implicate glutamate as a critical product of glutamine catabolism, which is used to synthesize several other NEAAs.

#### *Glutamine as a carbon donor*

Beyond its role as a nitrogen donor, glutamine serves as an important source of carbon for cellular bioenergetic and biosynthetic needs. Indeed, cell proliferation is associated with the high influx of glutamine-derived carbon into the TCA cycle (DeBerardinis *et al*, 2007). Why do proliferating cells require the continuous replenishing of the TCA cycle? Indeed, as demonstrated by DeBerardinis *et al*, proliferating cells utilize the TCA cycle as the source of not only bioenergetic NADH and $FADH_2$ equivalents, but also biosynthetic precursors as well. Thus, most of the citrate generated in the TCA cycle in proliferating cells becomes exported into cytosol, where it is converted into acetyl-CoA, a precursor for the biosynthesis of fatty acids and cholesterol. Furthermore, TCA

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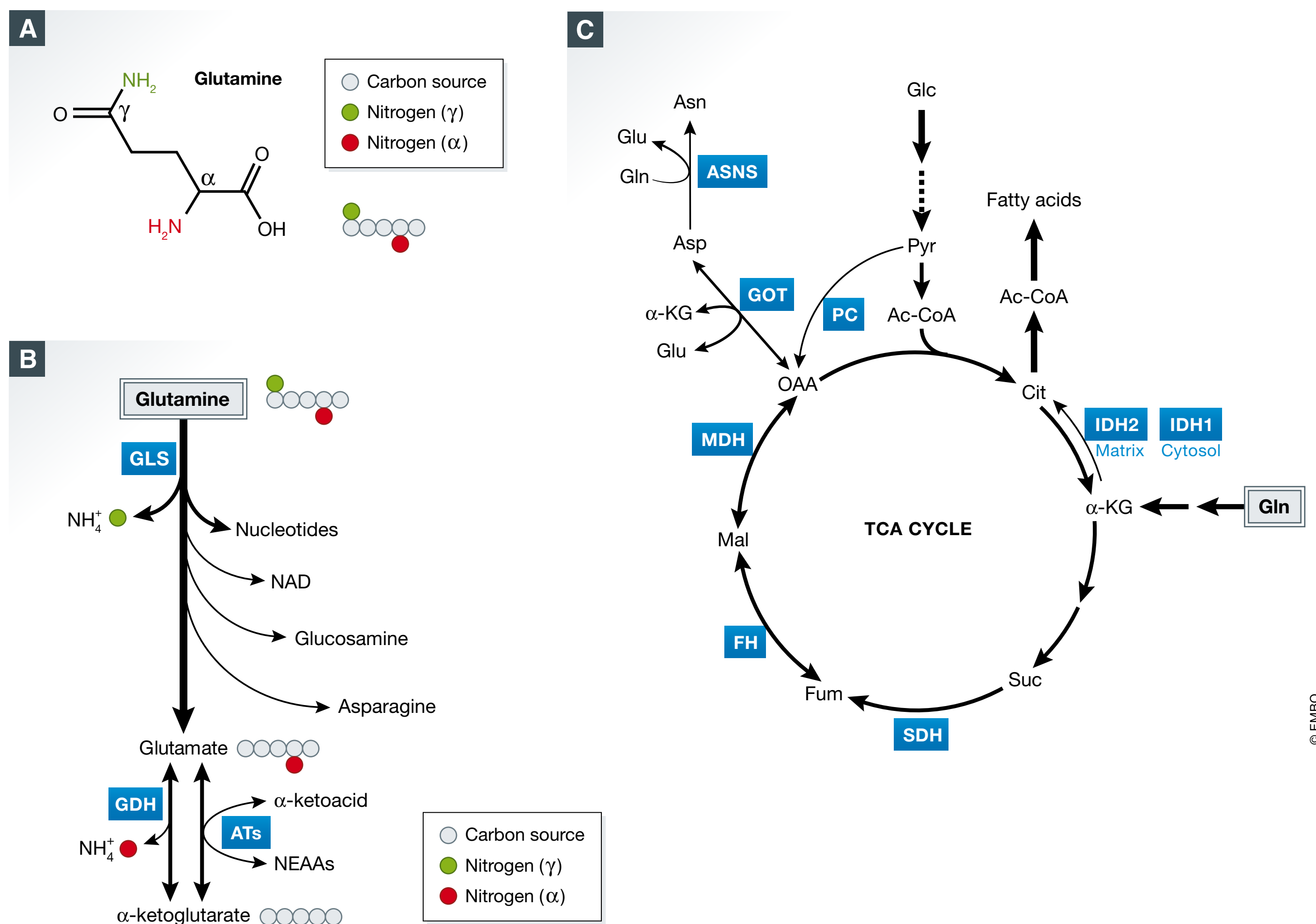


**Figure 1. Glutamine supplies nitrogen and carbon for biosynthetic reactions.**
(A) Chemical structure of glutamine. (B) Usage of γ- and α-nitrogen of glutamine in mammalian cells. GLS: glutaminase; GDH: glutamate dehydrogenase; ATs: aminotransferases. (C) Glutamine-derived carbon enters the TCA cycle through α-KG to supply anaplerotic substrates. Glucose-derived pyruvate can enter the TCA cycle through OAA. This reaction is mediated by PC, which is suppressed when glutamine-derived carbon enters the TCA cycle. Gln: glutamine; α-KG: α-ketoglutarate; Suc: succinate; Fum: fumarate; Mal: malate; OAA: oxaloacetate; Cit: citrate; Glu: glutamate; Asp: aspartate; Asn: asparagine; Glc: glucose; Pyr: pyruvate; Ac-CoA: acetyl-CoA; SDH: succinate dehydrogenase; FH: fumarase; MDH: malate dehydrogenase; GOT: aspartate aminotransferase; ASNS: asparagine synthetase; PC: pyruvate carboxylase; IDH1/2: isocitrate dehydrogenase 1/2. IDH1 is localized in cytosol.

cycle-derived oxaloacetate is used to synthesize aspartate and asparagine.

In many cell types, pyruvate carboxylation to oxaloacetate is suppressed, thereby rendering them reliant on glutamine catabolism to replenish the oxaloacetate that can condense with acetyl-CoA to produce citrate and drive the TCA cycle (DeBerardinis *et al*, 2007). The efflux of carbon precursors away from the TCA cycle must be balanced by the influx of carbons elsewhere. A route of entry for the glutamine-derived carbon into the TCA cycle is via the conversion of glutamate into its α-ketoacid form, α-ketoglutarate (α-KG; Fig 1C). In agreement with this notion, GLS1 expression itself is under positive control by c-Myc (Gao *et al*, 2009). In addition, a cell-permeable form of α-KG (dimethyl-α-KG) can completely suppress glutamine-depletion-induced apoptosis in MYC-transformed cells (Yuneva *et al*, 2007; Wise *et al*, 2008). As far as the conversion of glutamate to α-ketoglutarate goes, this reaction can be catalyzed either by glutamate dehydrogenase (GDH), which releases free ammonia, or by a family of aminotransferases, which transfer the α-amine to α-ketoacids, expanding the nonessential amino acid pool. These aminotransferases may play a dominant role to replenish the cellular pool of α-KG, as their inhibition induces cell death, which can be rescued by dimethyl-α-KG (Wise *et al*, 2008). However, when glycolysis is perturbed, GDH is required for cell survival, suggesting a potential compensation due to the lack of glucose-derived carbon influx into the TCA cycle (Yang *et al*, 2009).

In addition to its role in providing carbon and nitrogen toward the biosynthesis of diverse biosynthetic precursors, glutamine carbons also contribute to ATP production through their oxidation in the TCA cycle. Indeed, in proliferating cells, glutamine depletion markedly reduces NADH/$NAD^+$ ratio and inhibits oxygen consumption (Fan *et al*, 2013). This study concludes that glycolysis alone is not sufficient to sustain ATP production, even though the inhibition of complex I of the respiratory chain is not able to alter the cellular ATP level when glucose is unlimited (Javeshghani *et al*, 2012). However, whether ATP is a limiting factor for cell proliferation has been controversial until recently. Two recent studies shed light on

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this question. Birsoy *et al* (2015) and Sullivan *et al* (2015) have demonstrated that the inhibition of cell proliferation by oligomycin, an ATP synthase inhibitor, can be rescued by an uncoupling agent FCCP, which dissipates the electrochemical gradient across the mitochondrial membrane, resetting the NADH/NAD$^+$ ratio to normal. In this study, the authors show that it is not the mitochondrial ATP production, but the uninterrupted flux through the TCA cycle, enabled by the low NADH/NAD$^+$ ratio, is the major limiting factor for cell proliferation. Thus, continued transfer of electrons from NADH to molecular oxygen is necessary for maintaining the low NADH/NAD$^+$ ratio, which, in turn, allows the TCA cycle flux toward the biosynthesis of oxaloacetate. Oxaloacetate is an immediate precursor of aspartate, a proteinogenic amino acid, as well as a precursor for the biosynthesis of nucleotides and asparagine. Together, these results suggest that despite the fact that glutamine carbons contribute to a significant fraction of cellular energy production, the remaining ATP production via glycolysis is sufficient to maintain the energy needed for a cell to proliferate even when the electron transport is compromised.

#### *Therapeutic application of glutamine catabolism in cancer*

Given a versatile usage of glutamine in proliferating cells, a number of glutamine-mimetic compounds, including 6-diazo-5-oxo-L-norleucine (DON), acivicin, and azaserine, have been evaluated in preclinical and clinical settings for their anti-tumor activities. Despite their promising tumor-suppressive activities *in vitro*, all of these compounds displayed significant toxicity toward the gastrointestinal tract, immune cells, and central nerve system due to their nonselective inhibition of glutamine metabolism (Ahluwalia *et al*, 1990). In search for more selective inhibitors of glutamine catabolism, extensive efforts have been focused on glutaminase (GLS), the activity of which is dysregulated in variety of cancers. In this regard, several glutaminase inhibitors have been developed, including 968, BPTES, and CB-389 (Le *et al*, 2012; Gross *et al*, 2014; Stalnecker *et al*, 2015). Among these, CB-389 is currently being tested in a number of phase I clinical trials, where its efficacy is being evaluated in patients with both solid tumors and hematological malignancies.

In addition to the GLS-targeted compounds, inhibitors that target the conversion of glutamate to α-KG have also been tested in preclinical models of breast cancer and neuroblastoma (Qing *et al*, 2012; Korangath *et al*, 2015). In this regard, green tea catechin EGCG, which is a GDH inhibitor, and aminooxyacetate (AOA), a nonselective aminotransferase inhibitor, have been evaluated. The anti-tumor efficacy of these inhibitors may depend upon the relative dependency of a tumor on the activity of GDH or on aminotransferases as means of replenishing the intracellular pool of α-KG from glutamate. Indeed, AOA has been approved for the treatment of tinnitus (Guth *et al*, 1990). Its application in cancer deserves further exploration in light of the critical role of aminotransferases in mediating glutamine-dependent biosynthesis.

#### *Role of glutamine in redox control*

Tumor cells encounter oxidative stress during their initiation, progression, metastatic colonization, and following the exposure to anti-tumor therapeutics, which increases their dependence on anti-oxidative responses (Gorrini *et al*, 2013). Products of glutamine metabolism in particular play an essential role in facilitating cellular anti-oxidative defenses. First, glutamine-derived glutamate is utilized in the *de novo* biosynthesis of glutathione, a primary cellular antioxidant. Glutathione is a tripeptide comprised of three NEAAs: glutamate, cysteine, and glycine (Lu, 2009). Glutamate–cysteine ligase (GCL) condenses glutamate with cysteine in an ATP-dependent manner to generate γ-glutamylcysteine, which is further condensed with glycine through glutathione synthetase (GS), generating glutathione (Fig 2). In agreement with glutamine being the primary source of glutamate in the cell, exposure of cells to the uniformly labeled $^{13}$C-glutamine shows a pattern of enrichment of five $^{13}$C carbons in glutathione. Glutamine starvation of transformed cells reduces their glutathione pool (Yuneva *et al*, 2007). Human primary acute myeloid leukemia (AML) and metastatic liver cancer are characterized by significant elevation of the enzymes for glutathione biosynthesis, including GCL and GS (Pei *et al*, 2013; Nguyen *et al*, 2016a), suggesting that these tumors might be sensitive to inhibitors of glutathione biosynthesis.

A second way in which glutamine-derived glutamate contributes to glutathione biosynthesis is through facilitating the uptake of cystine via the xCT transporter, which is coupled to the efflux of glutamate (Fig 2). Once inside the cell, cystine is converted to cysteine, which can then be incorporated into glutathione. Glutamine-starved breast cancer cells display a defect in cystine uptake through the xCT antiporter (Timmerman *et al*, 2013). In addition, pharmacological inhibition of xCT elevates cellular reactive oxygen species (ROS) levels and suppresses tumor growth, making it a potential therapeutic target (Timmerman *et al*, 2013; Lanzardo *et al*, 2016; Tsuchihashi *et al*, 2016). Interestingly, cysteine itself can be synthesized in the cell from homocysteine, an intermediate of methionine catabolism (Lu, 2009). It will be interesting to know whether the resistance of some tumor cells to xCT inhibitors is at least partially due to their capacity to synthesize cysteine *de novo*.

The third way in which glutamine contributes to the cellular redox balance is via support of NADPH production. In a proliferating cell, reducing equivalent donor NADPH is utilized not only in the biosynthesis of fatty acids and cholesterol, but also to revert oxidized glutathione (GSSG), as well as thioredoxins, a class of cysteine-containing antioxidant proteins, back to their reduced states. Son *et al* showed that pancreatic ductal adenocarcinoma (PDAC) cells rely on glutamine to maintain the cytosolic NADPH pool (Son *et al*, 2013). In this paper, the authors show that glutamine-derived aspartate is converted to oxaloacetate (OAA) in the cytosol through GOT1, which is transcriptionally induced by mutant KRas, a common oncogenic lesion in PDAC. Subsequently, OAA is converted to malate by malate dehydrogenase 1 (MDH1), and malate is converted to pyruvate to generate NADPH through malic enzyme 1 (ME1).

#### *Glutamine metabolism contributes to chromatin organization*

Multiple lines of evidence indicate that select cellular metabolites are not only used to generate macromolecular building blocks or extract energy, but also serve as co-factors or substrates in a variety of cellular regulatory cascades, including those that directly modify histones and DNA. Thus, levels of certain metabolites are continuously monitored by a cell, directly informing cellular decisions on gene expression, affecting cellular differentiation as a result (Pavlova & Thompson, 2016). The glutamine-derived metabolite α-KG has been implicated in modulating cellular histone and DNA methylation levels. α-KG serves as a co-substrate for a class of dioxygenase enzymes, among which are Jumonji C

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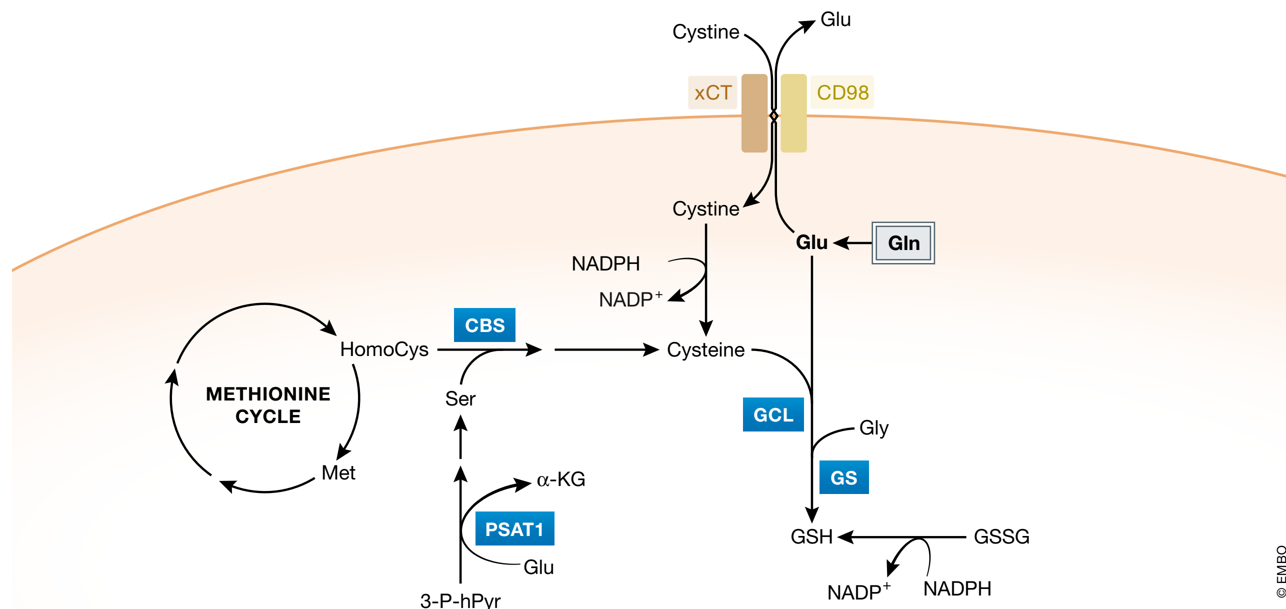


**Figure 2. The key role of glutamine-derived glutamate in glutathione biosynthesis.**
Glutamine-derived glutamate is a necessary substrate to synthesize glutathione. In addition, glutamate functions as an exchanging counter ion to import extracellular cystine through the xCT transporter. In the cell, cystine is converted to cysteine that is used as a second substrate for glutathione biosynthesis. Gln: glutamine; Glu: glutamate; Gly: glycine; GSH: glutathione; GSSG: glutathione disulfide; α-KG: α-ketoglutarate: Ser: serine; Met: methionine; HomoCys: homocysteine; GCL: glutamate–cysteine ligase; GS: glutathione synthetase; CBS: cystathionine beta-synthase; PSAT1: phosphoserine aminotransferase 1.

domain-containing histone demethylases and TET family DNA demethylases, which catalyze the oxidation of methyllysine residues of histones to hydroxymethyllysine and oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, respectively (Fig 3). In these reactions, α-KG itself is oxidized to succinate, and the rising levels of the latter can inhibit the progression of α-KG-dependent histone or DNA demethylase reactions. Indeed, in murine embryonic stem cells (mESC), elevated α-KG/succinate ratios are associated with the naïve pluripotent state, and the direct manipulation of intracellular α-KG by either addition of cell-permeable form of α-KG or via glutamine withdrawal is sufficient to modulate H3K27me3 and TET-dependent DNA methylation and affect differentiation (Carey *et al*, 2015). Glutamine depletion can also promote differentiation of naïve $CD4^+$ T cells into immunosuppressive $Foxp3^+$ regulatory T ($T_{reg}$) cells even in the presence of cytokines that typically promote the induction of T helper 1 ($T_H1$) cells (Klysz *et al*, 2015). Accordingly, addition of dimethyl-α-KG restores the intracellular α-KG levels and enables $T_H1$ cell differentiation under glutamine deprivation. In cancer cells, loss-of-function mutations of succinate dehydrogenase (SDH) subunits have been found in familial paragangliomas and pheochromocytomas, as well as in a subset of sporadic gastrointestinal stromal tumors (Astuti *et al*, 2001; Janeway *et al*, 2011). Accumulation of intracellular succinate in these tumors as a consequence of SDH loss is associated with a global inhibition of DNA demethylation, which contributes to their tumorigenic state (Xiao *et al*, 2012; Killian *et al*, 2013; Letouze *et al*, 2013). Recently, Pan *et al* (2016) showed that glutamine deficiency in the core region of solid tumors correlates with histone hypermethylation, dedifferentiation, and therapeutic resistance in a melanoma model.

The central role of glutamine-derived α-KG in modulating histone and DNA methylation is also exemplified by the gain-of-function mutations in IDH1 and IDH2, which have been identified in glioma, chondrosarcoma, cholangiocarcinoma, acute myeloid leukemia (AML), and a small portion of adult T-cell acute lymphoblastic leukemia (T-ALL) (Balss *et al*, 2008; Parsons *et al*, 2008; Mardis *et al*, 2009; Paschka *et al*, 2010; Borger *et al*, 2012; Cohen *et al*, 2013; Van Vlierberghe *et al*, 2013). The mutations of IDH1 or IDH2 exhibit a neomorphic activity by converting glutamine-derived α-KG to 2-hydroxyglutarate (2-HG), which competitively inhibits α-KG-dependent histone and DNA demethylases (Dang *et al*, 2010; Figueroa *et al*, 2010; Ward *et al*, 2010; Lu *et al*, 2012; Turcan *et al*, 2012; Fig 3). Consistently, glioma cells with mutant IDH1 display an elevated dependency on glutamine, rerouting the entry of pyruvate into the TCA cycle through PC (Seltzer *et al*, 2010; Izquierdo-Garcia *et al*, 2014). In a mouse model of T-ALL, mutation of IDH1 increases the sensitivity of leukemic cells to glutamine depletion (Hao *et al*, 2016b), indicating a potential therapeutic vulnerability in tumors harboring IDH mutations.

In addition to dioxygenase enzymes that drive histone and DNA demethylation, α-KG also serves as a substrate for a class of prolyl 4-hydroxylase enzymes, which mediate the ubiquitination and degradation of hypoxia-inducible factor 1 α (HIF1α), a key transcription factor that facilitates the cellular adaptation to low oxygen levels (Fig 3). Despite the fact that IDH1 mutations were originally found to result in an increased level of HIF1α (Zhao *et al*, 2009), Kuivonen *et al* showed that prolyl 4-hydroxylases are only sensitive to the L-enantiomer (Koivunen *et al*, 2012). Further exploration is necessary to determine the contribution of glutamine to the gene expression as a consequence of the alteration of intracellular α-KG,

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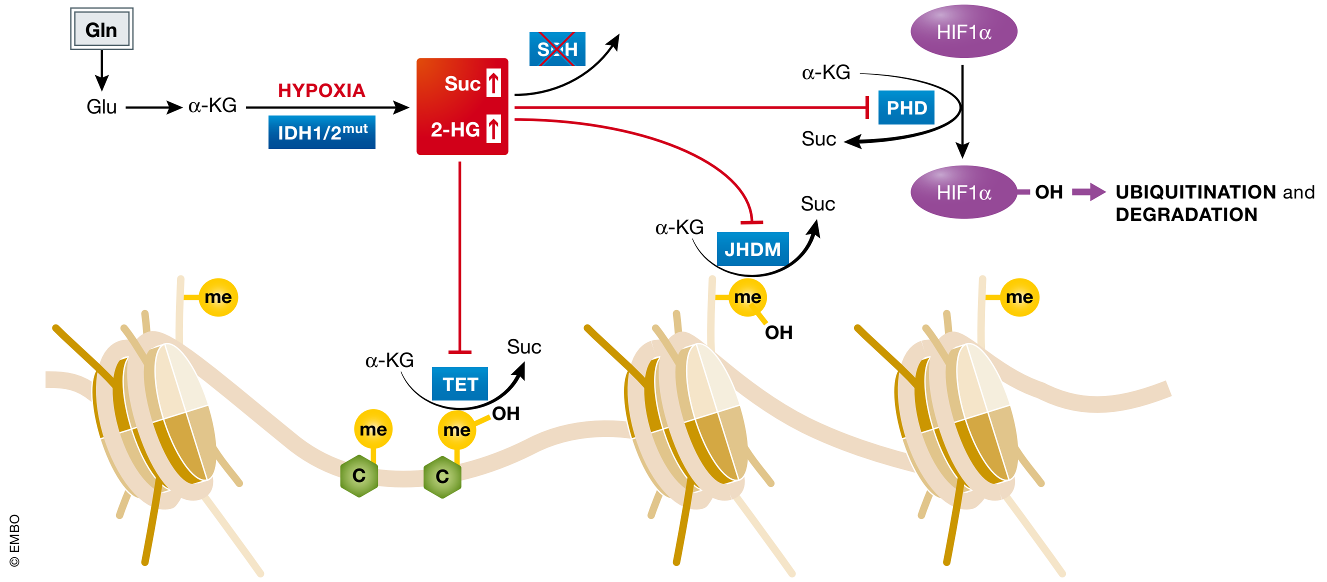


**Figure 3. Glutamine-derived α-KG is a substrate for α-KG-dependent dioxygenases.**
α-KG is the substrate of Jumonji C histone demethylases (JHDM) and TET DNA demethylases and therefore mediates histone and DNA demethylation. In addition, α-KG is the substrate of prolyl hydroxylase (PHD) that mediates HIF1α ubiquitination and degradation. These α-KG-dependent dioxygenases convert α-KG to succinate that can feedback inhibit their dioxygenase activity. Either cancer-associated IDH1/2 mutations or oxygen limitation can cause accumulation of 2-HG, which can competitively inhibit α-KG-dependent dioxygenases.

2-HG, and succinate, which can affect global chromatin modification patterns or levels of specific transcriptional regulators, such as HIF, depending on the cellular context.

## Cellular adaptations to glutamine limitation

Increased consumption of glutamine by proliferating tumor cells, coupled with inadequacies of tumor vascular supply, results in selective depletion of glutamine from the microenvironment (Vaupel *et al*, 1989). In tumors, glutamine levels can be profoundly reduced when compared to the surrounding normal tissues and plasma (Roberts *et al*, 1956; Rivera *et al*, 1988; Marquez *et al*, 1989). In addition, a spatial examination of amino acid levels in cancer cell xenografts has found glutamine to be among the most depleted in the xenograft core, when compared to the periphery of the tumor (Pan *et al*, 2016). Furthermore, metabolomic analysis from primary human pancreatic ductal adenocarcinoma (PDAC) tissue revealed a significant reduction of glutamine and several other NEAAs in the tumor tissues relative to the adjacent normal tissues as well (Kamphorst *et al*, 2015). These findings warrant further investigation of the adaptations that various tumors may employ to augment limited glutamine levels. Understanding these adaptations may not only uncover tumor metabolic vulnerabilities that can be exploited in anti-tumor therapy, but also provide guidance for more effective immunotherapy as well, as the tumor-associated T lymphocytes may compete with tumor cells for glutamine.

### *Glutamine uptake*

One way to increase glutamine acquisition within the tumor environment is through the induction of glutamine uptake. In most cells, ASCT2 is the major transporter for glutamine uptake. Its expression is upregulated by oncogenic MYC or E2F3, consistent with the role of these signaling molecules in directly increasing glutamine uptake (Wise *et al*, 2008; Reynolds *et al*, 2014). Elevated expression of ASCT2 was found in triple-negative breast cancer patients, correlating with poor survival in xenograft mouse models (van Geldermalsen *et al*, 2016). Gamma-L-glutamyl-*p*-nitroanilide (GPNA), an ASCT2 inhibitor, has been shown to suppress glutamine uptake and cell growth in lung cancer cells (Hassanein *et al*, 2013). In mice, ASCT2 deficiency impairs the induction of $T_H1$ and $T_H17$ cells, due to a defect of glutamine uptake and mTORC1 activation (Nakaya *et al*, 2014). However, ASCT2 is not the only transporter for glutamine uptake. It was recently reported that depletion of ASCT2 leads to the induction of SNAT1 and SNAT2, two other sodium-neutral amino acid transporters, which is sufficient to compensate for glutamine uptake (Broer *et al*, 2016; Fig 4A). Glutamine transporters are potential cancer therapeutic targets, but the therapeutic effects associated with their inhibition may be affected by the effects of this inhibition on the transport of other amino acids as well.

In addition to its therapeutic implications, the increased uptake of glutamine by some tumors provides a unique potential for tumor imaging. Indeed, glucose-based [$^{18}$F] fluorodeoxyglucose positron emission tomography (FDG-PET) has been used for thirty years to detect and monitor tumors in clinic, exploiting the phenomenon of the markedly elevated uptake of glucose by a wide variety of tumors (Kelloff *et al*, 2005). However, FDG-PET imaging is not effective in brain tumors, as normal brain tissue takes up large quantities of glucose as well. In contrast to FDG-based imaging, [$^{18}$F] fluorinated glutamine ($^{18}$F-FGln) displays low background uptake in brain tissues, and has been successfully used for tumor detection in mouse models of glioma and in glioma patients (Lieberman *et al*, 2011; Ploessl *et al*, 2012), validating it as a diagnostic tool (Venneti *et al*, 2015).

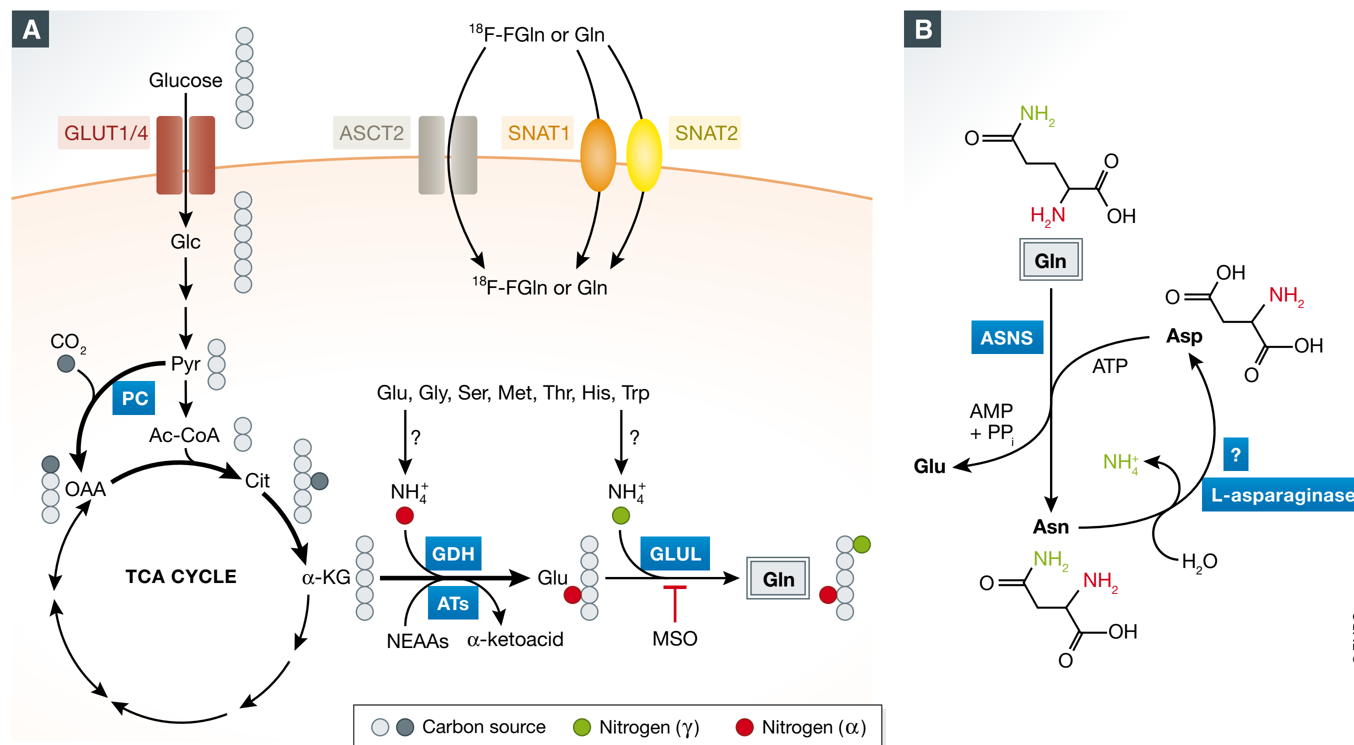


**Figure 4. Glutamine uptake and *de novo* biosynthesis.**

(A) Glutamine uptake can be mediated by ASCT2 and SNAT1/2 transporters, allowing the application of $^{18}$F-FGln-based PET imaging as a diagnostic tool for brain tumors. Furthermore, glucose-derived carbon can be used as precursors to synthesize glutamine *de novo*. (B) A potential mechanism for asparagine as the nitrogen source for glutamine biosynthesis. However, this activity has only been reliably observed in unicellular organisms thus far. GLUL: glutamate-ammonia ligase (glutamine synthetase); MSO: methionine sulfoximine.

*Glutamine biosynthesis* de novo

The fact that most cancer cells cannot proliferate or even survive in the absence of exogenous glutamine is surprising, because mammalian cells possess all the necessary enzymatic machinery to synthesize glutamine *de novo*. In particular, glutamine synthetase (GLUL) enzyme catalyzes the condensation reaction between glutamate and ammonia in an ATP-dependent manner, which generates glutamine. In mammalian tissues, GLUL is ubiquitously expressed but is particularly enriched in the liver, brain, and muscle (Haberle *et al*, 2006). Multiple growth factors and oncogenic signals positively regulate GLUL transcription (van der Vos *et al*, 2012; Bott *et al*, 2015; Cox *et al*, 2016), and exogenous glutamine was found to directly destabilize GLUL protein through facilitating its ubiquitination and degradation (Nguyen *et al*, 2016b). Elevated GLUL expression was found to be an early marker of hepatocellular carcinoma (Long *et al*, 2010), and is a predictor of poor survival in patients with glioma and liver cancer (Osada *et al*, 2000; Rosati *et al*, 2013). However, despite expressing GLUL, most cancer cells in culture require exogenous glutamine for growth and survival. One simple interpretation is that at least in tissue culture, *de novo* biosynthesis of glutamine is not sufficient to accommodate the demands of the great variety of glutamine-utilizing enzymes. However, the limiting factors for cellular adaptation to the deficit of exogenous glutamine have yet to be elucidated. One potential lead is a recent report demonstrating that mESC can proliferate without exogenous glutamine when treated with both a MEK and a GSK-3β inhibitor (Carey *et al*, 2015). The fact that the MEK pathway and/or GSK-3β are activated in most malignant cells suggests that their activation may be responsible for the inhibition of cancer cell to upregulate glutamine synthesis to levels that support cell growth.

Second, tumors have been reported to synthesize some glutamine. Kung *et al* (2011) showed that luminal breast cancer cells are resistant to glutamine-depletion-induced growth inhibition and apoptosis, when compared to the basal breast cancer cells. Consistently, luminal breast cancer cells display high levels of GLUL expression. In a mutant KRas-driven mouse pancreatic tumor model, oncogenic MYC enhances GLUL expression (Bott *et al*, 2015). In this study, the authors show that inhibition of GLUL suppresses tumor growth *in vivo*. Furthermore, glioma cells were shown to utilize glucose carbons to maintain TCA cycle anaplerosis, and consequently, glutamate and glutamine biosynthesis under glutamine limitation (Tardito *et al*, 2015; Fig 4A). However, the source of the free ammonia that is necessary for glutamine biosynthesis through the GLUL remains more of a mystery. Together, these studies suggest that the dependency of tumor cells on glutamine can be dictated both by the oncogenic signals and by the tissue of origin.

In some cell types, asparagine is sufficient to suppress glutamine-depletion-induced apoptosis (Zhang *et al*, 2014). However, despite the fact that most cells synthesize asparagine from glutamine (Balasubramanian *et al*, 2013), its catabolism has only been reliably reported in unicellular organisms (Peterson & Ciegler, 1969; Jones & Mortimer, 1970; Dunlop & Roon, 1975; Fig 4B). Recently, it was

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reported that asparagine may rescue cells by replacing a required role for glutamine as a counter ion in the import of extracellular amino acids, which is essential to maintain mTOR activation and protein translation (Nicklin *et al*, 2009; Krall *et al*, 2016). Whether asparagine also supplies reduced nitrogen for glutamine biosynthesis, or plays other regulatory roles to mediate cellular adaptation to glutamine limitation in mammalian cells remains to be determined.

#### *Glutamine catabolism* in vivo

As mentioned above, the ability of some tumors to synthesize glutamine *de novo* to meet biosynthetic needs suggests that exogenous glutamine may not be required by all proliferating cells *in vivo*. In an orthotopic mouse model of primary human glioma and in a mouse model of lung cancer driven by mutant KRas, it has been shown that the tumor cells can utilize glucose as a preferred anaplerotic entry port into the TCA cycle via the action of pyruvate carboxylase (PC; Marin-Valencia *et al*, 2012; Davidson *et al*, 2016). However, PC was found to be dispensable for the cancer cells *in vitro*, when glutamine is supplied in the culture medium. These two studies challenge the importance of glutamine catabolism in replenishing the TCA cycle intermediates, a phenomenon observed in most of the cultured cells. These results also put into question the broader applicability of the therapeutics that target glutamine entry into the TCA cycle or its catabolism via glutaminase. Further exploration of other *in vivo* tumor models as well as clinical studies is necessary to determine whether the limited dependence of some tumor cells on glutamine anaplerosis is associated with select oncogenic contexts or tissues of origin, or whether it reflects glutamine availability in the specific tissue environments.

As mentioned above, availability of glutamine in the milieu of the tumor is often limited (Roberts *et al*, 1956; Rivera *et al*, 1988). The fact that cancer cells can use glutamine-derived carbons to fuel the TCA cycle, or use glucose-derived carbons to both replenish the TCA cycle and synthesize glutamine may reflect a certain degree of metabolic plasticity of tumor cells, aimed at optimizing their growth in shifting nutrient conditions. Furthermore, investigating the relative degree of tumor dependency on exogenous glutamine at distinct stages of tumorigenesis—that is, in the primary tumor initiation and expansion, local tissue invasion, survival in the circulation and seeding, and colonization of distant organs—may provide further insights into the therapeutic potential of targeting glutamine metabolism in cancer therapy.

To evaluate how tumor glutamine dependency is influenced by the concentration of amino acids and other nutrients that are typically encountered by cells *in vivo*, Tardito *et al* have designed a serum-like modified Eagle's medium (SMEM), which contains nutrient concentrations at levels close to those found in human plasma, and assessed the response to glutamine limitation of glioma cells under these conditions. Indeed, they found that the cell proliferation in SMEM medium was largely unaffected by the presence or absence of exogenous glutamine; furthermore, GLUL was found to be both necessary and sufficient for the proliferation in the absence of exogenous glutamine (Tardito *et al*, 2015). In turn, newly synthesized glutamine was then utilized for nucleotide biosynthesis to support the growth of glioma cells. This study is consistent with the fact that glucose-derived carbon is the main supply of the TCA cycle intermediates *in vivo*, which can be used as precursors to synthesize glutamine and other NEAAs. However, the source of reduced nitrogen for the *de novo* glutamine biosynthesis remains more of a mystery. As glutamine biosynthesis through GLUL enzyme requires free ammonia, which is maintained at rather low levels in plasma (less than 35 μM), and is absent in standard tissue culture media formulations, ammonia can be generated through the catabolism of glutamate, glycine, serine, methionine, threonine, histidine, and tryptophan (Fig 4A); however, the relative contribution of these sources to the ammonia supply for glutamine biosynthesis is in need of further investigation. Insights into the sources of ammonia for the *de novo* glutamine biosynthesis may unveil novel therapeutic targets aimed at the inhibition of glutamine biosynthesis.

#### *Protein and cell corpses can supply glutamine*

In addition to the upregulation of uptake from the extracellular fluid and *de novo* biosynthesis in cell, some tumor cells can use alternative ways to obtain glutamine and other amino acids via the breakdown of engulfed extracellular proteins, apoptotic bodies, or even living cells. Such unconventional nutrient acquisition strategies can play a critical adaptive role in conditions when glutamine is limited. For example, extracellular proteins are abundant in plasma and tumor environment, but are not typically considered as a nutrient source. However, it was demonstrated that extracellular proteins can be taken up by cells through macropinocytosis, a process that involves a nonselective engulfment of the extracellular fluid phase, which gives rise to giant vesicles termed macropinosomes (Kerr & Teasdale, 2009). Engulfed proteins, as well as larger macromolecular structures, can then be digested through the action of lysosomal proteases as means of recovering free amino acids (Fig 5).

Macropinocytosis has been described in normal, growth factor-stimulated cells, but it becomes markedly enhanced by oncogenic Ras signaling (Bar-Sagi & Feramisco, 1986). Indeed, incubation of KRas-transformed cells with $^{13}$C-labeled soluble proteins under low glutamine conditions restored labeled free amino acids and TCA cycle intermediates, indicating a recovery of sufficient quantities of glutamine to replenish the TCA cycle (Commisso *et al*, 2013). In this study, the authors show that soluble albumin can rescue proliferation of KRas-transformed cells in glutamine-limited conditions. Furthermore, 5-(N-ethyl-N-isopropyl) amiloride (EIPA), an inhibitor of macropinocytosis, suppresses xenograft tumor growth, indicating a critical role of macropinocytosis to supply amino acid *in vivo* (Commisso *et al*, 2013). While not specific for glutamine, the catabolism of proteins increases not only glutamine but other amino acids essential and nonessential as well (Kamphorst *et al*, 2015; Palm *et al*, 2015).

In addition to the extracellular proteins, the engulfment and digestion of entire living cells or apoptotic cells can be also employed as a way of recovering free amino acids (Fig 5). Thus, the growth of MCF10A mammary epithelial cells under amino acid-free conditions can sustain through the engulfment of living cells via the process of entosis, and this effect can be reversed by blocking of lysosomal-mediated digestion (Krajcovic *et al*, 2013). In this study, similar effect was observed in macrophages engulfing apoptotic bodies (Krajcovic *et al*, 2013). Notably, entosis represents an interesting example of oncogene-driven cell–cell competition, as KRas-transformed cells were found to be more likely to consume their nontransformed neighbors than be engulfed themselves (Sun *et al*, 2014). Amino acids can also be recovered from the intracellular sources, such as proteins and organelles, via a process of autophagy

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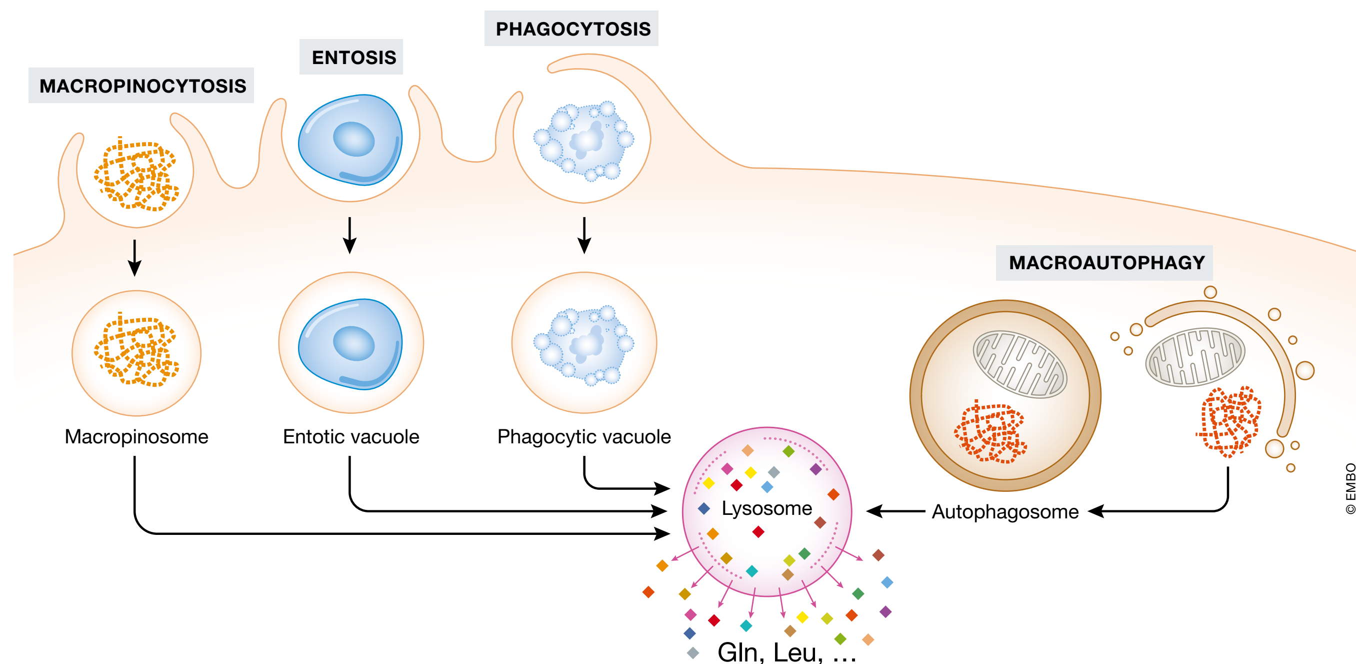


**Figure 5. Glutamine acquisition through proteolytic scavenging.**
Extracellular proteins (macropinocytosis) and live/dead cells (entosis and phagocytosis) can be engulfed and digested in lysosomes to release free amino acids, including glutamine. In addition, intracellular proteins and organelles can also be digested in lysosomes to release free amino acids via macroautophagy.

(Jiang *et al*, 2015). During autophagy, intracellular proteins and organelles are enwrapped by a double-membrane structure that is eventually fused with the lysosome for digestion (Fig 5). Since autophagy recycles intracellular materials, it cannot give rise to the biomass accumulation that is necessary for proliferation; however, it can sustain energy production to support long-term cell survival (Lum *et al*, 2005). Similar to the other amino acid scavenging pathways, autophagy recycles intracellular amino acids nonselectively.

## Summary

Glutamine has been the most widely studied nutrient other than glucose in the field of cancer cell metabolism during the past decade. The central function of glutamine in cell proliferation can be attributed to its role in numerous biological processes, including its role in biosynthesis and bioenergetics, anti-oxidative defense, chromatin modification/gene transcription, facilitation of transport of other amino acids across the plasma membrane, and regulation of cell signaling (Gonzalez & Hall, 2017). The relative effects of glutamine as well as glutamine-derived metabolites can be dictated by both the tissue and the oncogenic context. A number of pharmacological inhibitors of glutamine uptake and catabolism have been developed. Some of them have entered clinical trials or have been FDA-approved to be used in patients with cancer or other diseases (Altman *et al*, 2016). One of the first successful metabolic therapies, L-asparaginase has been used in the clinic to treat acute lymphoblastic leukemia (ALL) for close to three decades. L-asparaginase functions by depleting plasma asparagine and glutamine, such that the ALL cells, which are auxotrophic for asparagine and require large amounts of glutamine, are selectively affected by this treatment (van den Berg, 2011). However, L-asparaginase has only been proven to be effective in ALL and some NK/T-cell lymphomas. Its application in AML, non-Hodgkin's lymphoma (NHL), and solid tumor has not been found to be successful (Clarkson *et al*, 1970; Jaffe *et al*, 1971). Its use in other cancers has been limited by its immunogenicity (van den Berg, 2011).

On the other hand, growing evidence suggests that various tumor types may reside in an environment where glutamine is profoundly limited. Therefore, tumor cells have to develop adaptive strategies that would allow them to sustain their growth and survival. In this regard, induction of *de novo* biosynthesis of glutamine or acquisition of glutamine through catabolism of extracellular and intracellular proteins has been shown to provide a source of missing glutamine for cells. For example, in a $KRas^{G12D}$-driven mouse pancreatic cancer model, inhibition of mTORC1 remarkably enhances the capacity of tumor cells to use extracellular protein to restore their amino acid pools, leading to increase in the tumor burden (Palm *et al*, 2015). Even though the phenotype is unlikely to be solely due to the augmented glutamine acquisition, this study revealed a fundamentally opposing strategy of nutrient acquisition in amino acid-replete and amino acid-starved settings, which warrants reconsideration of the current therapeutic approaches that are based on mTORC1 inhibition in at least some oncogenic contexts.

In addition, tumor cells can adapt to the limitation of glutamine through the inhibition of global protein translation. In yeast and in mammalian cells alike, glutamine was shown to activate mTORC1 in a RagA/B-independent manner (Stracka *et al*, 2014; Jewell *et al*, 2015). Accordingly, depletion of glutamine may result in suppressed mTORC1 signaling, especially when other amino acids are limiting, too. In addition, depletion of glutamine, along with other amino acids, triggers the inhibitory eIF2α phosphorylation through GCN2

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kinase (Harding *et al*, 2000). Both of these signaling modulations result in the inhibition of translation initiation, acting together to preserve the amino acid pools while allowing selective translation of proteins necessary for the adaptive responses. For instance, the eIF2$\alpha$ phosphorylation promotes the translation of the transcription factor ATF4, which functions as a master regulator of a set of genes involved in adaption to starvation. Accordingly, the inhibition of GCN2 or of ATF4 prevents xenograft growth *in vivo* (Ye *et al*, 2010; Horiguchi *et al*, 2012), suggesting a potential to target this pathway as a cancer therapeutic.

In conclusion, glutamine metabolism is key to the survival, proliferation, differentiation state, and stress resilience in normal proliferating cells as well as in the context of tumorigenesis. Further exploration of the ways in which cellular glutamine status affects these diverse processes, as well as the investigation of strategies that cells may adopt to withstand glutamine limitation, may uncover new intersections between metabolism and disease, revealing novel opportunities for therapeutic intervention.

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### Conflict of interest

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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