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Perspective

Glutamine Synthetase: Diverse Regulation and Functions of an Ancient Enzyme

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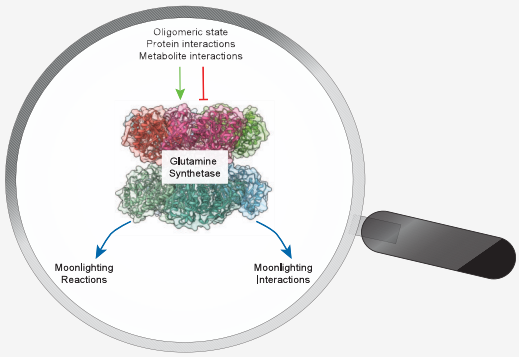
ABSTRACT: Glutamine synthetase (GS) is a ubiquitous enzyme central to nitrogen metabolism, catalyzing the ATP-dependent formation of glutamine from glutamate and ammonia. Positioned at the intersection of nitrogen metabolism with carbon metabolism, the activity of GS is subject to sophisticated regulation. While the intricate regulatory pathways that govern *Escherichia coli* GS were established long ago, recent work has demonstrated that homologues are controlled by multiple distinct regulatory patterns, such as the metabolite induced oligomeric state formation in archaeal GS by 2-oxoglutarate. Such work was enabled in large part by advances in cryo-electron microscopy (cryoEM) that allowed greater structural access to this large enzyme complex, such as assessment of the large heterogeneous oligomeric states of GS and protein-interactor-GS complexes. This perspective highlights recent advances in understanding GS regulation, focusing on the dynamic interplay between its oligomeric state, metabolite binding, and protein interactors. These interactions modulate GS activity, influencing cellular processes such as nitrogen assimilation, carbon metabolism, and stress responses. Furthermore, we explore the emerging concept of GS "moonlighting" functions, revealing its roles in palmitoylation, cell cycle regulation, and ion channel modulation. These diverse functions highlight a newfound versatility of GS beyond its primary catalytic role and suggest complex roles in health and disease that warrant further study.



Glutamine synthetase (GS) is an ancient enzyme present in all domains of life.[1,2] GS catalyzes the condensation of glutamine from glutamate and ammonia using the chemical energy from ATP. Glutamine produced by this reaction contributes not only to amino acid homeostasis but also acts as a common nitrogen and carbon donor. Thus, GS is particularly important for organisms that rely on ammonia as their primary nitrogen source for glutamine synthesis (diazotrophs). Moreover, glutaminase and glutamine amidotransferase domains within metabolic enzymes mobilize glutamine's carbon and nitrogen for various anabolic pathways like nucleotide biosynthesis and replenishing intermediates of the TCA cycle (anaplerosis). Beyond central metabolism, glutamine can be directed toward glutathione and neurotransmitter biosynthesis. Given its prominent role in multiple metabolic pathways, GS is tightly regulated by various sophisticated mechanisms. Many of these mechanisms have only recently been uncovered, fueled in large part by advances in cryoelectron microscopy (cryoEM) techniques, and are detailed below.

In humans, liver expression of GS plays a fundamental role in health and disease by regulating glutamine, the most abundant amino acid in the blood. In fact, estimates suggest that a majority of glutamine in the body is GS-derived.[3] In addition to glutamine formation, GS provides a crucial supplement to the urea cycle toward clearing ammonia from the body.[4−6] Buildup of ammonia is particularly toxic to the brain where ammonia's physiologically relevant protonated form, ammonium, can mimic K^+ and hinder neuronal function.[7] Moreover, high ammonia levels can result in brain swelling, breakdown of the blood-brain barrier, and has been associated with cognitive decline.[8] Importantly, ammonia buildup (hyperammonemia) can result from defect variants of GS,[9] underscoring the importance of GS activity to maintaining overall nitrogen homeostasis. Furthermore, high GS expression in astrocytes is responsible for detoxifying ammonia and regulating levels of the excitatory neurotransmitter glutamate[10] and GS expression levels are important during neurodevelopment.[11] Furthermore, glutamine is crucial for immune cells and GS expression can impact macrophage differentiation into various polarization states.[12,13] Thus, the canonical enzymatic activity of GS is present in many tissues

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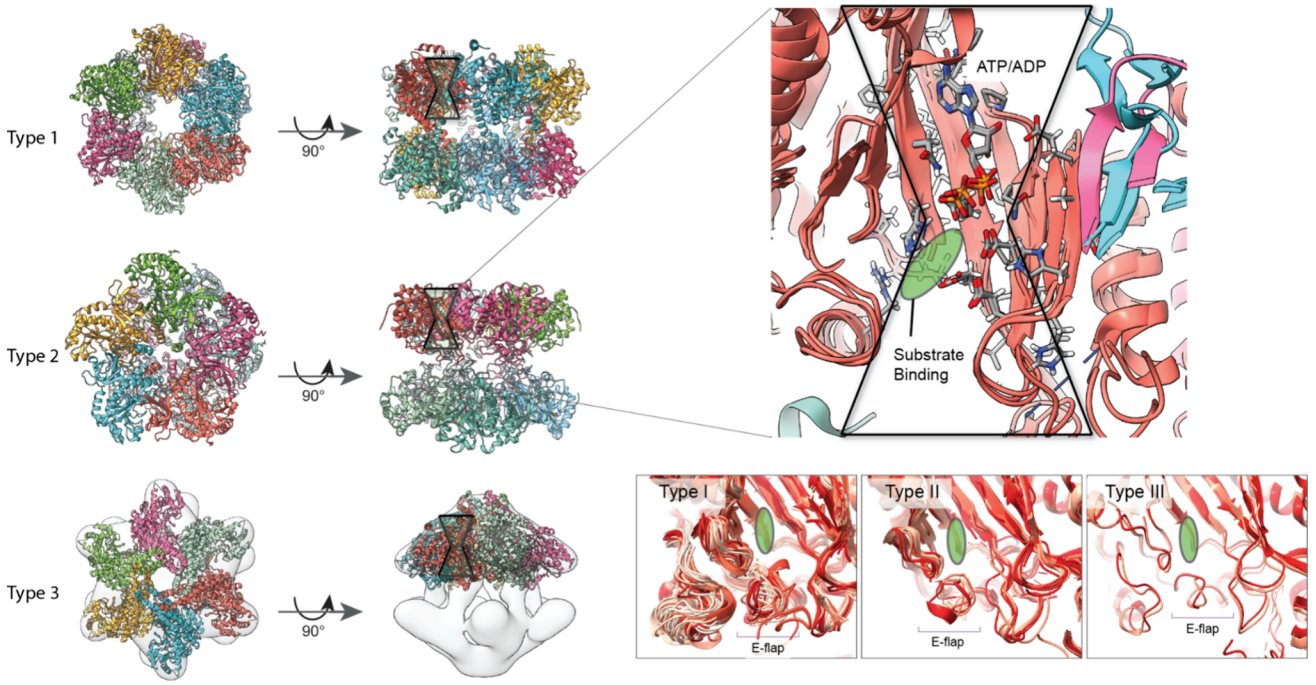

Figure 1. Architecture of glutamine synthetases. Crystal structures of a representative Type I GS (*Bacillus subtilis*, PDB 4LNI; top), Type II GS (*Homo sapiens*, PDB 2QC8; middle), and Type III GS (*Bacteroides fragilis*, PDB 3O6X fit into EMD 1204; bottom) with individually colored subunits. The active site bifunnel is highlighted in the side view (left). At right is an overlay of a single active site bifunnel for each GS model shown at left. Substrate binding site is highlighted in green. ATP or ADP nucleotides are in stick representation with active site residues demonstrating conserved architecture. Bottom right: overlay of extant structures of GS (from PDB on October 31, 2024 that contained E.C. 6.3.1.2) demonstrates conformational flexibility about the E-flap and adenylation loop.

and cell types and is likely subject to different GS regulatory needs.

Glutamine synthetases have a common reaction mechanism.[1,2,14] Glutamate is activated in the first step by ATP phosphorylation of the γ-carbon. The activated γ-glutaryl phosphate electrophile reacts with an ammonia nucleophile to generate glutamine and release phosphate. All diverged sequence and structural types of GS bear a bifunnel active site architecture to perform this reaction. In the forward reaction, ATP binds one side of the active site and glutamate and ammonia bind the opposite funnel. Divalent metal cations are found to facilitate ligand orientation and ion coordination. Additionally, there is a common "E-flap" motif in GS enzymes which is a conformationally variable loop that contains a catalytic glutamate at loop turn that is presumed to deprotonate ammonium ions to generate active ammonia (Figure 1). Every structural type of GS presents a composite active site where catalytic residues are contributed from two different protomers of the complex (Figure 1).

The important biological niches where GS activity is required across all domains of life makes it a common drug target. In cancer, GS expression has pleiotropic effects where GS expression can inhibit cell growth as demonstrated in hepatocellular carcinoma[15] and gastric cancers[16] or support growth in glutamine limited conditions in glioblastoma[17] and sarcomas.[18] Given that certain cancers may be dependent or bolstered by GS activity, GS has been proposed as a drug target.[18,19] Mechanism-based inhibitors have been developed and uncovered for GS, including methionine sulfoximine, phosphinothricin (a current herbicide), and tabtoxinine-β-lactam.[2,20] These inhibitors are orthosteric meaning that they bind at the active site. However, owing to the largely conserved reaction mechanism of GS and by extension, largely conserved active site residues (Figure 1), many of these inhibitors act broadly on GS homologues or isoforms suggesting that future development of nonorthosteric inhibitors may be fruitful and may be inspired by natural mechanisms that regulate GS activity.[2]

GS homologues display many structural types that confer different regulatory features (Figure 1). Recent advances in cryoEM structural methodology have enabled unique insight into these regulatory features and mechanisms revealing multiple quaternary states, protein binding partners, and metabolite binding sites that confer changes to GS activity in various homologues. While the active site of these GS homologues is largely conserved (Figure 1) their mechanisms for tuning activity have diverged. Multiple reviews have explored these structural features and physiological roles in detail[2,21−25] and this perspective will focus on the most recent findings.

Furthermore, beyond regulation of canonical reaction catalysis, metabolic enzyme moonlighting is a phenomenon where enzymes adopt activities that are outside their canonical role as catalysts. Multiple unique moonlighting functions have been reported for human GS which will be reviewed here. GS structure and function have been studied for decades but new features and functions are still being uncovered and we hope to offer an update to these studies in this work.

GS Regulation through Quaternary State Remodeling. As of the writing of this perspective, there are 77 deposited structures in the Protein Data Bank. Of solved structures, all GS enzymes have a composite active site with residues contributed from two adjacent protomers. Therefore, catalysis can only be supported by homooligomers and various

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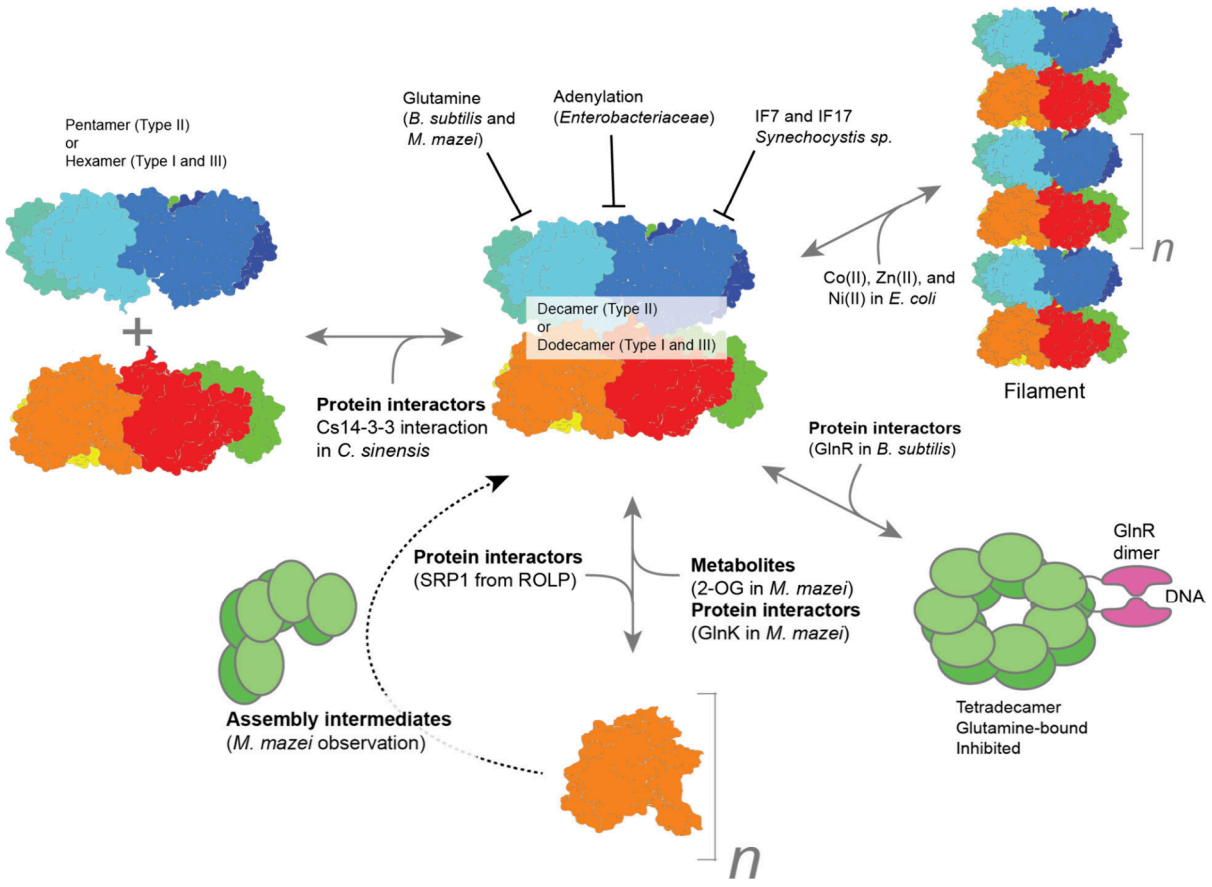

Figure 2. Complex regulation of glutamine synthetase and quaternary state remodeling. Outlined are the protein interactors, cofactors, and metabolites that promote different oligomeric states of GS including monomers, pentamers, hexamers, octamers, decamers, dodecamers, tetradecamers, and higher order filaments.

oligomeric states can support the required active site architecture (Figure 1). GS is currently classified into three main types (1, 2, and 3) as well as GS1 subtypes α, β, and γ following the classification defined in Fernandes et al. 2022.[26] Type 1 has been observed in a primarily dodecameric architecture formed as a dimer of hexamers and type 2 is primarily decameric as a dimer of pentamers.[1] Type III was crystallized in a hexameric form[27] but has been observed in dodecameric states as well.[28] In all cases, active sites are formed between monomers within pentamers and hexamers (Figure 1).

Recent phylogenetic tracing and GS homologue characterizations have revealed that there is a diversity of homologues where regulation, and even catalytic function, cannot always be predicted.[26,29] Indeed, many Type I-γ do not display canonical glutamine synthesizing activity, but rather polyamine biosynthetic capabilities as discussed in Fernandes et al. 2022.[26] This is likely due to the absence of the common β-grasp domain found in Type I-α, I-β, and II enzymes. Further biochemical work is required to fully understand the putative neofunctionalization of GS in polyamine biosynthesis. Additionally, given that GS can accommodate other nitrogen substrates, like hydroxylamine (common *in vitro* assay for activity[2]) and methylamine (physiologically provided by host microbiome[30]), it is possible that various GS homologues may harbor different catalytic functions beyond or in addition to glutamine synthesis. Despite the functional and regulatory heterogeneity, the sequence (sub)type of GS can indicate certain canonical regulatory features.

Type 1-β contains a regulatory "adenylation loop" that is posttranslationally adenylated by a bifunctional adenyltransferase enzyme which lowers enzyme activity in the adenylated state.[2] The "adenylation loop" abuts the "E-flap" moiety, which is a conserved loop containing a catalytic glutamate that can close over the amino acid and ammonia half-site of the active site (Figure 1). The "E-flap" is predicted to be flexible to open and close over the active site and indeed appears to display conformational heterogeneity in known structures (Figure 1). The "adenylation loop" is missing from Type 1-α, 1-γ, and Type II homologues but there is a loop corresponding to this position in Type III GS. However, the role of this loop in Type III GS is of unknown significance. Given the chemical breadth of small molecule inhibitors that target the amino acid binding site, it is perhaps unsurprising that GS homologues may have evolved toward other substrate specificities.[2]

The most studied example of Type I-α GS is from studies of *Bacillus subtilis* GS which displays a strict product inhibition mechanism. Glutamine is both retained in the active site for this GS homologue to inhibit further catalysis and additionally, it was recently found that glutamine-bound GS is primed for binding to the glutamine responsive transcription factor, GlnR.[31,32] Schumacher and colleagues (2022)[31,32] demonstrated that *B. subtilis* GS could bind to a transcriptional repressor protein GlnR and that when bound, GS preferentially formed a tetradecameric (14 subunit) assembly (Figure 2) that

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is at least partially catalytically inhibited.[32] The tetradecameric form of GS is not optimal for activity and forms a cavity that is bound by GlnR. Additionally, binding of the C-terminal tail of GlnR leads to formation of an active dimer of GlnR that promotes its DNA-binding function. Thus, GlnR*GS represents a transcriptional repressor complex that silences the genes involved in nitrogen assimilation. All together, under high glutamine conditions, GS catalytic activity is product inhibited and GlnR/oligomeric state inhibited, and transcription of the *glnRA* operon is halted. The authors further go on to show that this phenomenon is not restricted to *B. subtilis* but extends to the other organisms tested.

In methanogenic archaea, GS regulation involves regulating changes to GS oligomeric state to tune enzyme activity. The TCA cycle intermediate 2-oxoglutarate (2-OG, also known as α-ketoglutarate, αKG) is presumed to be a master indicator of carbon availability in these organisms and was found in two studies to be obligate for dodecameric assembly of this GS these type Iα GS homologues from *Methanothermococcus thermolithotrophicus*, *Methermicoccus shengliensis*, and *Methanosarcina mazei*.[33−35] Both X-ray crystal and cryoEM structures of 2-OG bound dodecameric GS revealed that 2-OG filled a critical pocket in between monomers within the hexamer and enforce a mechanism whereby glutamine synthesis only occurs under carbon replete conditions. Such a metabolite-protein interaction seeding and stabilizing an assembled oligomeric state is a potent mode of regulation for GS given the nature of a composite active site that is completely inactive in the monomeric state (Figure 2).

These methanogenic archaeal GS homologues were also investigated for product-based feedback inhibition. The homologues from *M. shengliensis* and *M. mazei* could be completely inhibited by glutamine which was defined by the presence of a regulatory arginine residue that closes over the glutamine bound to the active site. Removal by natural or artificial mutation of this arginine residue completely abolishes glutamine feedback inhibition in these GS homologue similar to the *Bacillus subtilis* GS homologue.[33]

In addition to these modes, another regulatory protein in the ammonia assimilation, GlnK, has been structurally resolved to complex with GS. GlnK is a PII protein that is commonly involved in protein−protein interactions,[36,37] is induced under nitrogen starvation, and recently reported to bind to Type I GS.[38,39] A trimer of GlnK was found to bind to the top central cavity of the GS dodecamer on both faces by cryoEM.[39] Interestingly, this study also resolved an apparent assembly intermediate corresponding to an octameric complex that resembled a "crescent moon" shape of an incomplete dodecamer (Figure 2). The authors proposed a model wherein GlnK stabilizes the dodecameric protein, disfavoring assembly intermediates, to facilitate GS activity by promoting the highly active dodecamer. However, the full interplay between effector proteins, like GlnK, with the metabolite interaction[39] with 2-OG to stabilize the active dodecameric form of GS is not yet fully clear.

The assembly status of GS impacting activity has also been observed in eukaryotic Type II GS'. Two Type II plant GS homologues, *Camellia sinensis* (CsGSIb) and *Glycine max* (GmGSβ2) were investigated with a combination of cryoEM, activity analysis, and mutagenesis ultimately finding that assembly into the decameric state was required to attain full activity.[40] The pentameric forms of these enzymes (either native pentamer in CsGSIb or designed by mutation to GmGSβ2) were less active. Additionally, activity was dependent on enzyme concentration, suggesting an equilibrium between pentameric and decameric states. Structurally, their pentameric 3D reconstructed map displayed low local resolution at the periphery of each individual subunit at the tips of the pentagon which was interpreted to be due to higher conformational heterogeneity overall of the particles. Finally, the authors demonstrated interaction between the native GS decamer and a 14-3-3 protein. 14-3-3 proteins are common phosphorylation binding proteins that mediate various protein−protein interactions (PPIs) and implicating this class of protein in oligomeric state maintenance of GS leaves open the possibility that this class of proteins could be playing a chaperone-like function for GS more broadly. The authors propose a model wherein pentamers and decamers are at equilibrium but decameric forms provide a more rigid scaffold to promote intrinsic activity.[40]

Oligomeric state dependent activity changes were also found at play in rice plants, where leaf yellowing induced by the rice orange leaf phytoplasma (ROLP) was found to be a result of disrupted GS Type II decamer formation.[41] This phytoplasma secretes an effector protein, SRP1 (Secreted ROLP Protein 1), which directly targets and binds to GS2 in rice chloroplasts. This interaction disrupts the crucial decameric structure of the GS2 decamer, significantly impairing its enzymatic activity. The lower glutamine availability reduced chlorophyll production causing leaf yellowing and thus increased attractiveness to leafhopper vectors that spread the phytoplasma. This study not only reveals a novel mechanism by which pathogens manipulate host plants but also underscores the critical importance of GS2 decamer structure and function in plant physiology, highlighting alteration of oligomeric state potential as a target for disease control.

GFP screens in *S. cerevisiae* have predicted that upward of ∼50% of metabolic enzymes can coalesce into a form of higher-order structure *in vivo*.[42] Many of these enzymes appear to form filament structures and filament formation has emerged as an important mode of higher-order structural regulation that impacts the structure−function relationships of enzymes.[43,44] Filament formation can confer numerous allosteric effects and is spatially and temporally resolved in the cell.[43] Interestingly, this phenomenon has been reported for glutamine synthetase in *Saccharomyces cerevisiae*,[42] *Methanosarcina mazei*,[34,39] and *Escherichia coli*.[45−48] Where the *M. mazei* filaments were noted but not explored, the filaments from *E. coli* and *S. cerevisiae* have supporting studies. However, the functional importance and regulatory potential of GS filament formation remains incompletely understood.

In *E. coli*, filament formation is seeded by the specific divalent cations such as Co(II), Zn(II), and Ni(II) where the Ni(II)-induced filament structure was obtained by cryoEM.[47] An N-terminal helix coordinates the Ni(II) ion across a "head-to-head" interface between dodecamers in this enzyme. The residues that coordinate the Ni(II) are found in different GS homologues but not conserved across GS types. The physiological relevance of this assembly is incompletely understood however as the inclusion of the typical cellular concentrations of substrates ATP or glutamate dissolved the filaments and millimolar concentrations of Ni(II) were required to fully form filaments.

In *S. cerevisiae*, the first crystal structure, a 20-mer in the asymmetric unit which represented two decameric GS' presented a putative "head-on-head" arrangement of GS

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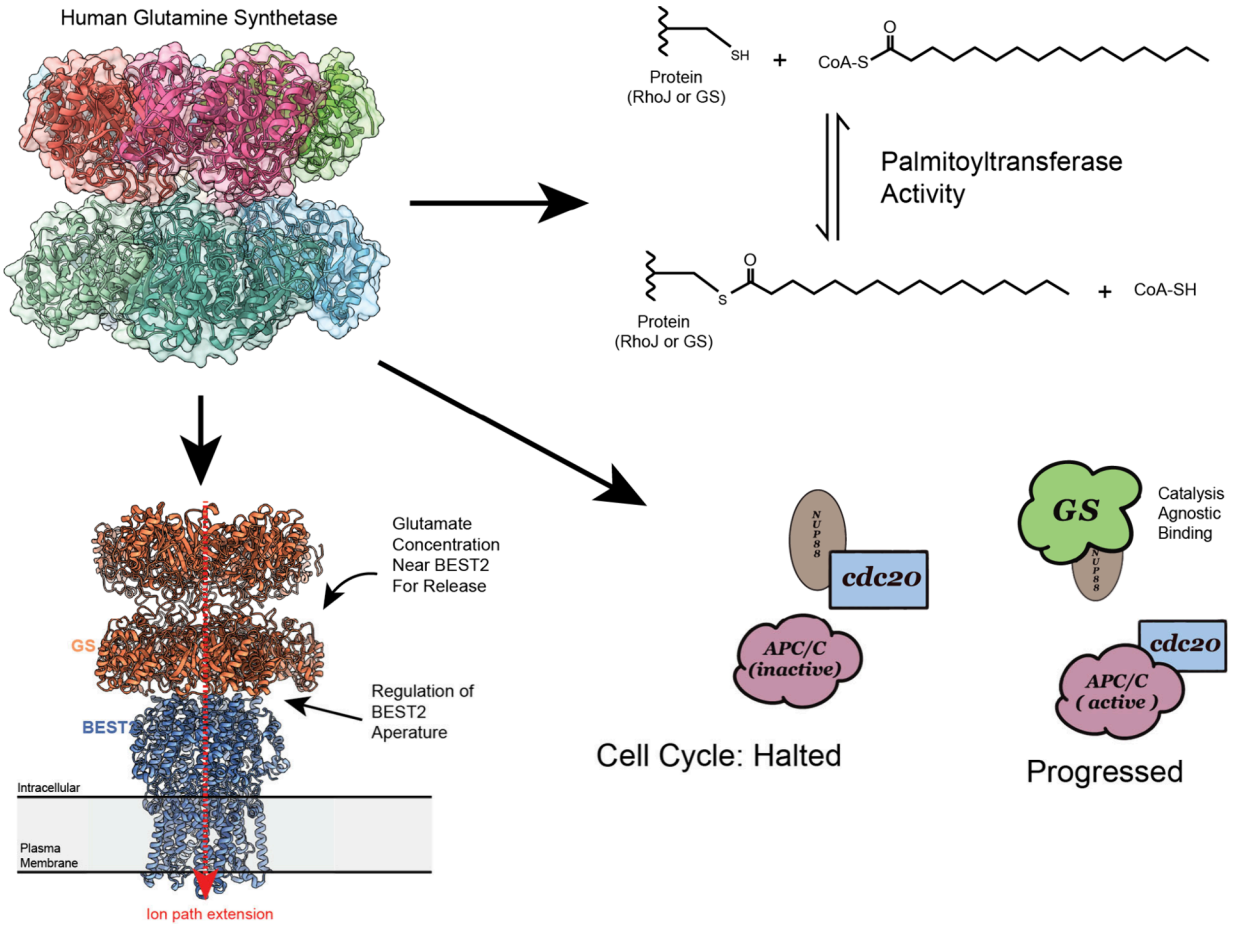

Figure 3. Moonlighting functions of human glutamine synthetase. Top left: PDB 2QC8 colored by subunit and fit into surface representation. Top right: chemical reaction of palmitoylation activity of human GS.[56] Bottom left: GS binding BEST2 ion channel conferring presumed ion concentration and activity modulation.[57] Bottom right: GS interaction with NUP88 can outcompete NUP88 binding to CDC20 thus allowing CDC20 binding to the anaphase promoting complex (APC/C) to promote progression through mitosis.[58]

pentamers.[49] Such an arrangement would support further filament formation. *S. cerevisiae* GS was then subsequently observed to form reversible inclusions upon nutrient stress.[42,45,46,48] Mutational analysis of *S. cerevisiae* GS found mutations at the "head-to-head" interface that could promote or hinder filament formation.[45] Of note, residues important to filament formation are ionizable which supports the hypothesis that stress-induced lowering of intracellular pH drives filament formation. However, structures of the *S. cerevisiae* GS filament form are elusive.

Taken together, these studies offer evidence to support that oligomeric state remodeling to tune activity is common among GS homologues and can present unique modes of regulation. The large diversity of GS sequences, including some organisms with multiple GS genes, suggests functional diversity that is largely unexplored. GS catalytic activity serves different functions based on the metabolic needs of the organisms providing a wealth of potential future studies exploring these regulatory mechanisms required by these metabolic niches. Finally, the apparent neofunctionalization of Type I-γ GS suggests further diversity of catalytic activity in this enzyme class to be discovered.

Moonlighting Functions. Metabolic enzymes have been increasingly implicated in supporting functions outside of their normal catalytic mechanisms which are collectively named "moonlighting" functions.[50−55] Human glutamine synthetase has now been identified to perform multiple noncanonical moonlighting functions that are key to cellular processes and signaling (Figure 3).

While studying the molecular signaling pathways underlying angiogenesis, Eelen and colleagues (2018) identified GS expression as indispensable and revealed an additional role for human GS as a palmitoyltransferase.[56] The authors provided evidence that human GS could autopalmitoylate and palmitoylate RHOJ, a protein also identified as crucial for angiogenic signaling. Notably, this activity was independent of GS's canonical catalytic site, as palmitoylation activity was not impacted by common active site disease variants, suggesting a distinct mechanism. Although CoA may likely bind the nucleotide pocket of GS, the remaining fatty acid tail's binding site is unclear. Further research is needed to determine the structural determinants of this alternative catalytic function.

Human GS was also identified as a regulator of the Anaphase Promoting Complex (APC). Zhao and colleagues (2022) discovered that GS directly interacts with the nuclear pore protein NUP88, preventing NUP88 from binding to CDC20.[58] This interaction was found important for the activation of the CDC20-mediated anaphase-promoting complex/cyclosome (APC/C), ensuring proper mitotic progression. The study shows that GS depletion, independent of its enzymatic activity, leads to mitotic arrest and multinucleation, which inhibits tumor growth in various cancer models. Additionally, the

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authors demonstrate that this function is independent of catalytic activity as this function could be faithfully fulfilled by low-activity variants of GS. Furthermore, the authors found the interaction site of NUP88 with GS through mutagenesis to be at an "edge" of a pentameric ring and far from the active site.

Glutamine synthetase (GS) exhibits another moonlighting function through its interaction with Bestrophin-2 (BEST2), a calcium-activated anion channel. Owji and colleagues (2022) solved a GS-BEST2 complex structure by cryoEM and demonstrated direct modulation of BEST2 activity.[57] Not only did GS alter BEST2 activity, but it was further found that ions could be conducted through the GS central pore and to BEST2 for transport. Interestingly, binding to BEST2 did not appear to impact GS activity in this study. A presumed functional rationale of GS-BEST2 binding was to concentrate glutamate, which is a substrate for both GS and BEST2, at the ion channel aperture for transport.

Taken together, these studies offer insight into the diversity of functions that can occur outside of the active site in metabolic enzymes and build on recent discoveries of nonenzymatic "moonlighting" roles of metabolic enzymes.[50−55]

CONCLUSION AND OUTLOOK

Glutamine synthetases are nearly ubiquitous in nature and homologues display a diverse array of structures and modes of regulation. While the mechanism of the canonical reaction was uncovered decades ago, new studies are highlighting the myriad of regulatory mechanisms presented to this enzyme. Many of these mechanisms involve major shifts in the enzyme's quaternary structure, underscoring the sensitivity of GS' composite active site architecture to structural remodeling.

Furthermore, the newly discovered moonlighting roles of this enzyme present an exciting area of biological discovery. Given that many organisms harbor multiple GS isoforms it is possible, if not likely, that these isoforms will display different functions and functional regulation. Indeed, GS expression is increasingly linked to parasite proliferation (tuberculosis,[59−61] malaria[29,62]), cancer,[18,19,30,63] preventing hyperammonemia, and neurological disorders[64] which underlies the calls for specific drugs to inhibit GS activity. Thus, these newly discovered regulatory mechanisms reveal vulnerabilities that may inform the next generation of antibiotics, anticancer therapeutics, and herbicides.

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Notes

The authors declare no competing financial interest.

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