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CPS1: Looking at an Ancient Enzyme in a Modern Light

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Abstract

The mammalian urea cycle (UC) is responsible for siphoning catabolic waste nitrogen into urea for excretion. Disruptions of the functions of any of the enzymes or transporters lead to elevated ammonia and neurological injury. Carbamoyl phosphate synthetase 1 (CPS1) is the first and rate-limiting UC enzyme responsible for the direct incorporation of ammonia into UC intermediates. Symptoms in CPS1 deficiency are typically the most severe of all UC disorders, and current clinical management is insufficient to prevent the associated morbidities and high mortality. With recent advances in basic and translational studies of CPS1, appreciation for this enzyme's essential role in the UC has been broadened to include systemic metabolic regulation during homeostasis and disease. Here, we review recent advances in CPS1 biology and contextualize them around the role of CPS1 in health and disease.

Introduction

In ureotelic animals, nitrogen produced as a byproduct of amino acid catabolism is detoxified in the liver by the urea cycle, a system of 5 (6 including n-acetylglutamate synthase [NAGS]) enzymes and two transporter proteins (Figure 1). The first enzyme in the cycle is carbamoyl phosphate synthetase 1 (CPS1; E.C. 6.3.4.16), catalyzing the ATP-dependent condensation of bicarbonate and ammonia to form carbamoyl phosphate[1].

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Carbamoyl phosphate undergoes subsequent stepwise catalysis to form citrulline, argininosuccinate, arginine, and finally ornithine and urea, through the actions of ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase, respectively, which is released into the blood for excretion by the kidneys[2]. Partial or total loss of function in any of the urea cycle proteins results in urea cycle disorders (UCDs), which occur in aggregate between 1:35,000[3,4] and 1:50,000[5,6] live births. CPS1 deficiency (OMIM #237300) is an autosomal recessive disorder[7] with a prevalence of 1 in 300,000[4] to 1 in 1.3 million births[3]; estimates vary widely due to difficulty in diagnosis. Patients typically present with lethargy, vomiting, encephalopathy, and coma; subsequent blood analysis typically demonstrates elevated ammonia and glutamine along with reduced citrulline and arginine[8]. While the majority of cases appear in the neonatal period, others present less frequently occurring any time from childhood to adulthood depending on the extent of CPS1 functional loss. In neonates, up to 50% of symptomatic patients perish despite treatment[4], many with the first episode. Late-onset patients have survival of over 90%[9]. Long-term neurological deficits are found in the vast majority of neonatal onset cases, with a smaller proportion occurring in those diagnosed as late-onset[6]. CPS1-deficient patients tend to have the most severe symptoms of all UCDs[4], contributing to the need for new treatment options for patients.

Clinical management of CPS1 deficiency is largely dependent on dietary protein restriction, administration of the nitrogen scavenging compounds phenylbutyrate and benzoate[10,11], and L-arginine supplementation[11]. Keto analogues of amino acids (e.g. valine or phenylalanine) have also been used successfully[12]. However, these therapies are unable to prevent progressive neurological decline due to recurrent hyperammonemia, which often requires emergent hemodialysis to effectively clear[13]. Dialysis is effective in preventing mortality in severe cases but is unable to change the ultimate neurocognitive outcomes of the disorder. Early diagnosis and aggressive treatment are therefore essential for improving prognoses; however, initial diagnosis is hindered by the lack of a specific metabolite that distinguishes it from NAGS deficiency[14], necessitating genome sequencing or functional testing. Prenatal and newborn screening could have a meaningful impact in speeding up diagnosis, though it is not routinely performed for CPS1[15]. Orthotopic liver transplantation is effective in treating deficiency of CPS1[16,17], though there are potential associated complications including long-term risk of allograft rejection and post-operative infections[18]. Despite the high level of survival post-surgery[19], CPS1 patients require long-term immunosuppression and may still require citrulline supplementation due to loss of CPS1 activity in enterocytes, the primary source of plasma citrulline[8,17].

CPS1 deficiency has been at the mercy of an unchanging therapeutic field for the better part of 40 years. The recent advancement in gene therapy successes in a variety of metabolic diseases, including ornithine transcarbamylase deficiency[20], and the development of several model systems to study CPS1 biology in the past 10 years indicate that the time is appropriate for a fresh look at the state of the field for CPS1 deficiency. In addition, CPS1 has been implicated in diseases beyond its classical deficiency[21]. The purpose of this review is to provide an overview of what is known about the biochemistry and regulation of CPS1 and discuss how a variety of recently described models may be used to answer outstanding questions and address the development of much needed novel therapeutics.

CPS1 In Homeostasis:

Gene Structure and Regulation—*CPS1* is located on chromosome 2q34, spanning over 120kb, and contains 38 exons with 37 introns[22–24], the result of an ancient duplication of the CP synthesis domain (large subunit, *carB* in *E. coli*) and subsequent fusion with the glutaminase domain (small subunit, *carA* in *E. coli*) to form the mammalian holoenzyme[25,26]. A core promoter[27] and both a −150bp proximal[28] and −6kb distal[29] enhancer have been identified, which together produce a 5761bp mRNA[24] with well-conserved sequence homology between species[25,30]. There is one mammalian homolog of CPS1: CPS2, which is expressed as part of the ubiquitously expressed CAD (**C**PS2, **a**spartate transcarbamylase, and **d**ihydrooritase) protein complex that regulates pyrimidine synthesis[31]; several orthologs exist across kingdoms, including *E. coli* CPS (eCPS) and CPS3, which is found in some fishes[32]. In addition to the protein-coding mRNA, a 2.3kb long noncoding RNA (lncRNA) is also produced from the *CPS1* locus, specifically intron 21, termed CPS1 intronic transcript 1 (CPS1-IT1)[33]. CPS1-IT1 has recently been the subject of increased investigation due to its putative tumor suppression functions (discussed below).

Transcriptional regulation is complex, largely having been studied in rodents historically, and occurs at the core promoter in addition to the two enhancer regions. The promoter contains a CAAT/enhancer binding protein (C/EBP) binding site necessary for transcriptional activation[34,35], in addition to an activator protein 1 (AP1) binding site, which is an important negative transcriptional regulator[36,37]. AP1 binding to the promoter is mediated by the distal enhancer[38], which is also responsible for the complex, liver-specific response to stimuli[39]. Cyclic AMP (cAMP) response binding protein (CREB) and glucocorticoid receptor (GR; via glucagon/cAMP and glucocorticoid signaling, respectively) bind the distal enhancer to promote transcriptional activation[40]. Moreover, AP1, C/EBP, GR, and hepatocyte nuclear factor 3 (HNF3) family protein binding sites are all found within the distal enhancer and mediate its function[41–43]. In addition to the distal enhancer, the proximal enhancer also contains C/EBP binding sites and a GAGA box, integrating the transcriptional effects of the distal enhancer[28].

CPS1 expression, in addition to the other urea cycle enzymes, is indirectly regulated by various factors. miR-10a-3p negatively regulates the glucocorticoid receptor subunit NR3C1, which in turn reduces *CPS1* expression[44]. Chromatin remodeling by Baf60a, a subunit of the Switch/Sucrose Non-Fermentable (SWI/SNF) complex, also reduces transcription when bound to the cofactor YB1. Competitive binding of YB1 and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) to Baf60a clarifies the direct role of PGC1α in the induction of *CPS1* expression[45,46]. AMP kinase (AMPK) activation suppresses *CPS1* expression[47,48], though the exact mechanism is unclear; however, it has recently also been shown to increase transcription of urea cycle enzymes in general including *CPS1*[49], possibly through PGC1α signaling[50].

Protein Structure and Function—First identified in the 1950s[1] as the enzyme responsible for generating the citrulline precursor carbamoyl phosphate[51], CPS1 has been the subject of extensive structural and biochemical characterization over the decades since. CPS1 is a 165kD protein that makes up 15–20% of total hepatic mitochondrial protein[52,53].

After translation, the proenzyme is shuttled from the cytoplasm into the mitochondrial matrix, where it is cleaved to form the mature protein[54]. CPS1 is also found in the enterocytes of the small intestine, where it contributes to pyrimidine biosynthesis[55,56] and circulating citrulline/arginine via the gut-renal axis[57].

CPS1 catalyzes the overall formation of carbamoyl phosphate in three distinct steps (Figure 2A): i) bicarbonate phosphorylation to form carboxyphosphate; ii) condensation of carboxyphosphate with ammonia to form carbamate; and iii) carbamate phosphorylation to form carbamoylphosphate[58]. Steps i and iii consume 1 ATP each[59,60], with ammonia being consumed from the coupled Glutaminase (encoded by the *GLS* gene) reaction that releases glutamate and ammonia[61]. Exogenously supplied ammonia is necessary for CPS1, in contrast to the other homologs (eCPS, CPS2, and CPS3), due to loss of glutaminase activity in the small subunit from a conversion of the catalytic cysteine residue to serine[25]. CPS1 is organized into multiple domains, individually responsible for the partial reactions[62–64], enzyme stability[65,66], and allosteric regulation by n-acetylglutamate (NAG)[67] (Figure 2B). X-ray crystallography studies of eCPS demonstrated that a 96Å internal tunnel is responsible for shuttling reaction intermediates between domains[58,68–70], which was subsequently confirmed with the crystal structure of the human enzyme[71]. This crystal structure was also used to confirm the binding site of NAG and the impact of clinical mutations in that region[72,73].

CPS1 enzyme function is coordinately regulated with the rest of the urea cycle in response to various stimuli. Upon feeding, the urea cycle, including CPS1, is upregulated to metabolize the digested proteins. During fasting, rising nicotinamide adenine dinucleotide (NAD) levels activate Sirtuin proteins; specifically, Sirtuin 5 (Sirt5) deacetylates[74,75] and deglutarylates[76] CPS1, removing the inhibitory protein modifications from the critical cysteines in ATP binding sites. Glucagon administration rapidly increases the intramitochondrial concentration of NAG (the essential allosteric activator of CPS1), binding more CPS1 molecules and boosting activity[77]. Glucagon signaling via cAMP also increases the expression of PGC1α, which rapidly induces increased Sirt5 expression to deacetylate, and thus activate, CPS1[45]. In contrast, Sirt5 is negatively regulated by miR-19b during low protein intake, ultimately reducing CPS1 activity to inhibit catabolism of critical amino acids[44]. Sirt5 is also inhibited by AMPK[78], reducing CPS1 activity, potentially as a result of urea cycle flow and AMP generation by argininosuccinate synthase[79]. Altogether, CPS1 regulation in response to fasting and feeding, mediated principally by glucagon and glucocorticoid, is diurnal and an important aspect of liver-wide circadian regulation[80]. A schematic diagram of CPS1 gene and protein regulation is depicted in Figure 3.

CPS1 In Disease:

Classical CPS1 deficiency has long been recognized as being the result of loss of CPS1 enzymatic function[81] resulting in hyperammonemia and neonatal mortality if not rapidly recognized and controlled. However, CPS1 expression has emerged as playing a role in other diseases, including cancer, which has been known since at least the 1980s but has not been used as more than a diagnostic[82], cardiovascular disease, and obesity.

Cancer—Due to the essential role of CPS1 in detoxifying ammonia and providing the precursor molecules for pyrimidines and arginine, its role in promoting cancer growth may not seem surprising. CPS1 has been found to be upregulated in a wide variety of cancer types, including gastric[83] and lung[47], though not in others (e.g. breast cancer)[84]. Counterintuitively, CPS1 is often found to be decreased or absent in small intestinal cancers[85] and hepatocellular carcinomas[82]. The reason for this downregulation is unclear, though aberrant global methylation may play a role[86]. Several studies have confirmed the role of CPS1 in supplying pyrimidines to the rapidly growing tumor, its loss causing deleterious effects on tumor cell viability and proliferation[47,87]. CPS1 upregulation may be multi-faceted depending on the tissue of origin: in non-small-cell lung carcinomas, liver kinase B1/AMPK-mediated CPS1 transcriptional repression is lost, allowing the gene to be transcribed[48]. In bladder cancer, caspase recruitment domain family member 10 (CARD10), via nuclear factor kappa B (NF-κB), activates CPS1 expression[88].

Though these mechanisms are becoming clearer, two major questions remain. First, what factors are promoting CPS1 activity? Regardless of its upregulated expression, CPS1 is completely dependent on NAG to function; there is no activity in its absence. Therefore, the co-upregulation of CPS1 and NAGS is important to address in future studies to more fully understand if co-induction of the two is happening, whether some other external small molecule is promoting CPS1 activity (such as with glycerol, described below), or if there are gain-of-function mutations that are yet to be identified. Second, what is the precise mechanism of CPS1-mediated pyrimidine synthesis? CPS1 is principally a mitochondrial enzyme, shuttled into the matrix immediately after translation[54], sequestering it away from the pyrimidine-synthesizing CAD enzyme, which contains CPS2 that catalyzes the same reaction as CPS1 in the cytoplasm. CAD-mediated pyrimidine synthesis is distinct from any CPS1 activity, and CPS1 silencing in cancer cells did not demonstrate any impact on CAD abundance[48], ruling out the possibility that the effects of CPS1 loss in these cells was impacted by reduced CPS2. In addition, carbamoyl phosphate is a labile molecule that does not typically traffic to the cytoplasm from the mitochondria, though it may spillover after excessive accumulation[89], suggesting that aberrant CPS1 activity may overwhelm the mitochondrial matrix with carbamoyl phosphate that then interacts with CAD. Some small amount of CPS1 protein may be present in cytoplasm normally[55], and oncogenic overexpression may result in a level of cytoplasmic protein that significantly contributes to pyrimidine homeostasis. Whether overexpression alone is sufficient to drive tumorigenesis, or if it is a byproduct of global aberrant gene expression, remains to be investigated.

By investigating outstanding questions about CPS1 expression in cancer, CPS1 may become an attractive target for therapeutic development. Indeed, N-carglumic acid (NCA), the structural analog of NAG and active ingredient in Carbaglu©, was shown to inhibit cancer cell growth and induce apoptosis in several different types of cancers[90]. This is especially intriguing as NCA *increases* CPS1 activity, which intuitively might promote cancer growth, and warrants more in-depth investigation to fully elucidate the mechanisms involved. Additionally, a recently performed drug screening study identified two small molecules, termed H3B-120[91] and H3B-616[92], that selectively and potently inhibit CPS1 function by

blocking ATP hydrolysis, paving the way for new, more targeted small molecule-based anti-cancer drugs.

Beyond the protein itself, the discovery that the *CPS1*-derived lncRNA CPS1-IT1 impacts tumor proliferation[87,93–95] has also renewed focus on CPS1 in the context of cancer. CPS1-IT1 expression is correlated with better prognoses in hepatocellular carcinoma[93], ovarian cancer[96], lung cancer[97], and colorectal cancer[98]. Its overexpression reduces tumor cell proliferation and induces apoptosis[96,97] by inhibiting hypoxia inducible factor 1α signaling[98] and Cyr6-mediated angiogenesis[95] in colorectal and melanoma cells, respectively. These anti-tumor proliferation properties make CPS1-IT1 an appealing gene therapy candidate for use in a wide variety of tumors, either by delivering the full sequence directly to cancer cells or by promoting its expression from the endogenous locus.

Cardiovascular Disease—Appreciation for the role of CPS1 in cardiovascular disease has recently increased due to the identification of single nucleotide polymorphisms (SNPs) associated with it. The SNP rs1047891 encodes a C>A transversion, a missense mutation in exon 36 leading to the incorporation of asparagine instead of threonine at amino acid residue 1405 (T1405N). T1405N was initially associated with protection against neonatal hypertension[99], as well as post-cardiac surgery and post-bone marrow transplant complications[100]. However, T1405N has subsequently been shown to functionally impair CPS1 activity by 30–40%[101], contradicting the hypothesis that T1405N promotes increased circulating arginine and endothelial nitric oxide signaling (eNOS)[100]. Further studies aimed at determining the impact of this SNP have shown varying results that prevent it from being clearly shown as pathogenic[102,103]. Specifically, the hypothesis that reduced CPS1 activity leads to restricted nitric oxide signaling substrates, thereby contributing to hypertension, has not been demonstrated in adults[102], despite being found in neonates[100]; the seemingly contradictory results have led to the concept of environmentally determined gene expression (EDGE), in which protein variants only show a measurable phenotype under some environmental stress condition[100]. As newborns have lower CPS1 expression than adults[104] and a higher metabolic demand, diminished CPS1 function at this time may exhibit pathological effects until it is masked by increased total protein to accommodate the disparity. In support of the EDGE hypothesis, T1405N has also been correlated with hyperammonemia in epilepsy patients receiving valproic acid[105], demonstrating that an environmental stressor for an unrelated disorder may exacerbate underlying genetic deficits that would otherwise show no phenotype. T1405N is found in 30% of the population[106] and may therefore present an important marker to consider in personalized medicine when determining other disease susceptibilities as well as treatment strategies.

In addition to the T1405N variant, rs715 has been identified as a SNP associated with disease. rs715 is a T>C transition in the 3’ UTR of *CPS1* and is associated with reduced urea cycle intermediates, increased glycine, and protection against coronary artery disease[107,108]. The exact mechanism of rs715 is uncertain; however, it is part of the same haplotype as T1405N and thus may only be indicative of T1405N presence and subsequent impact. Reduced activity from the T1405N variant is proposed to lead to increased circulating glycine; the relationship between glycine and betaine, which controls blood pressure, may also explain how reduced urea cycle-dependent glycine catabolism leads to decreased blood

pressure and improved cardiovascular health[107,108]. Additional studies are needed to further elucidate the complex interactions of CPS1 and glycine on cardiovascular health, as well as the components of EDGE. Studies of this sort would benefit immensely from a robust cell or animal model, as they are currently largely limited to correlative studies or small studies in healthy adults.

Obesity—The rising incidence of obesity worldwide has led to increased attention on finding novel underlying mechanisms and potential treatment strategies. Urea cycle function is reduced in obesity[109] as elevated lipid availability decreases glucose utilization for energy and inhibits protein catabolism, possibly facilitated in part by SWI/SNF-mediated chromatin remodeling[46]. Increased ammonia has been implicated in the effects of high-fat diet (HFD)[110], making CPS1, as the rate-limiting enzyme, an intriguing target for therapeutic development. In obese patients undergoing dietary intervention and weight maintenance, patients who regained previously lost weight had increased baseline CPS1 expression relative to those that maintained weight loss, in addition to having reduced glycine[21]. Inter-individual differences in CPS1 expression may reflect a genetic predisposition to metabolize proteins for energy more than the general population, reducing patient ability to utilize stored lipids[21]. The connection between obesity and cardiovascular disease has long been known, and these new studies provide a previously unrecognized link between them through CPS1 activity and glycine/betaine metabolism[21,107]. Studies in obesity may also point to possible therapies, even ones as simple as modulating dietary protein intake. For developing pharmaceuticals, the dietary flavonoid nobiletin is found in citrus fruits and influences a broad array of metabolic processes (reviewed in Huang et al. 2016[111]). In mice fed HFD, nobiletin was found to increase *Cps1* transcription via C/EBP[112]. While further work is required to fully elucidate cellular targets and mechanisms, small molecules may provide a robust means to rescue the reduced CPS1 expression and activity associated with obesity. In contrast, the H3B-120/616 small molecule inhibitors of CPS1[91,92] may provide a means of keeping CPS1 expression in check to help maintain weight and prevent relapse. Together, a variety of approaches and small molecules may be used to fine tune CPS1 activity to optimize therapeutic benefits for individual patients.

Other Diseases—In addition to cardiovascular disease, the T1405N variant of CPS1 has been associated with necrotizing enterocolitis in newborns[113]. However, a follow up study from the same group failed to show diminished circulating arginine levels, making the strength of the correlation unclear[114]. Further studies are needed to determine the effects, if any, of T1405N on arginine levels and their subsequent influence on the development of enterocolitis.

Beyond obesity and cardiovascular disease, CPS1 has also recently been implicated in major depressive disorder. Four novel SNPs were reported to be associated with major depressive disorder in patients from the National Institute on Aging – Late Onset Alzheimer's Disease Family Study[115]. In context with other SNPs identified in this study in the glutaminase (*GLS*) and glutamine synthetase (*GLUL*) genes, which are also closely linked to ammonia metabolism, the authors hypothesized that reduced CPS1 enzyme activity leads to increased circulating ammonia and a concomitant increase in neural GLUL-derived glutamine.

Increased glutamine leads to decreased glutamate (the substrate for GLUL), an important neurotransmitter, which is a known cause of neuronal dysfunction[116]. As other urea cycle enzyme deficiencies have been linked to neuronal phenotypes[117], a link between the urea cycle and the brain through CPS1 is distinctly possible and warrants further investigation.

Finally, CPS1 may also play a role in regulating the immune response to injury. During acetaminophen-induced acute liver injury in mice, CPS1 was shown to be released into the circulation and taken up by macrophages. CPS1 uptake led to macrophage polarization and subsequent liver homing, where they mediated an anti-inflammatory protective response to the injury[118]. Strikingly, these effects were shown to be independent of enzyme function, leading to the conclusion that CPS1 acts as an immunomodulatory cytokine. Though the mechanism of action remains unclear, the capacity of CPS1 to promote macrophage polarization and result in reduced inflammation regardless of whether it is administered before or after injury suggests that it may be a novel way to treat liver injury in a variety of contexts, including hepatitis, substance abuse, and autoimmune diseases.

Emerging Therapeutics

The primary treatment strategy for CPS1 deficiency is protein restriction and the administration of nitrogen scavengers. However, this standard of care is insufficient to prevent recurrent hyperammonemia and neurological insult. Nitrogen scavengers are further limited by toxicity at high doses and unwanted side effects from chronic use[119–121]. Further, these interventions depend on early recognition of the disease, which is not typically diagnosed until after major crises[122], while liver transplantation offers a curative option but is limited by availability and complication risk[18]. The recent development of several model systems has led to the ability to develop and test novel treatments for CPS1 deficiency.

Cell-Based Models—For the majority of the past 60 years, biochemical studies relied on isolating CPS1 from model organisms such as *E. coli* and rats, leaving the clinical impact of various described human mutations uncharacterized. To address this, an expression system overexpressing *E. coli* Cps (eCPS) was developed to test the impact of clinically relevant mutations[123]. Because the structure of eCPS is well-characterized, this system provided the means to test several mutations and determine how they impact enzyme structure and function, which was done initially for 8 variants found in human patients, showing that they correlate well *in vitro* with the clinical presentation[123]. The major limitation to this system is that eCPS is only 40% homologous to human CPS1, with two subunits and an intact glutaminase domain, making some aspects of structure and function difficult or impossible to correlate to human phenotypes.

To address the eCPS system limitations, various other expression systems were generated to study mammalian CPS1. Rat CPS1 shares 95% homology with human CPS1; the cDNA was therefore incorporated into the baculovirus/insect cells overexpression system (using the Sf9 cell line from *Spodoptera frugiperda*) to rapidly generate and purify CPS1 variants to test the effects of mutations[124]. 9 mutations from CPS1 patients were investigated using this system and shown to correlate closely to their clinical phenotypes, aided by the then-recently published crystal structure of the NAG binding domain[72,73]. The same system was

subsequently modified to produce recombinant human CPS1[125], which was used to show that glycerol can activate CPS1 in the absence of NAG. Glycerol-stimulated CPS1 activity had been appreciated previously in rats[67] but had not been directly tested and characterized on human protein. This advancement allows for the development of other potential therapeutics that allosterically activate CPS1 in the context of a damaged NAG binding site. Interestingly, it also suggests how CPS1 may be active without NAG, having implications towards its regulation in cancer (discussed above), as well as suggesting alternative methods of improving CPS1 activity with unrelated small molecules that are NAG binding site-independent. Other human CPS1 cDNA expression systems were generated in the fission yeast *Schizosaccharomyces pombe*[101] and immortalized human cell lines HepG2 (hepatoma) and LO2 (fetal liver)[126]. Testing in the yeast system led to the direct comparison in enzyme kinetics between the threonine and asparagine variants at residue 1405[101] (T1405N, discussed above), offering a potential platform for testing pharmaceutical interventions to increase activity. The human cell lines used were modified with CRISPR/Cas9 to express tdTomato in-frame with CPS1, with fluorescence intensity reflecting CPS1 expression levels. These lines were validated and used in a high-throughput small molecule screen to identify resveratrol as a modulator of CPS1 expression[126] and means to potentially increase ammonia clearance and urea formation in bioartificial livers.

While cDNA-expressing systems have great value, a means to test non-coding mutations is also necessary for a more complete understanding of the CPS1 mutation spectrum. To this end, a modified BAC was generated that contains the entire 120kb *CPS1* gene, including the enhancers and promoter, introns, and untranslated regions, and expressed in the MRC-5V2 human lung fibroblast cell line[127]. This system provides a powerful way to interrogate native human *CPS1* regulation, which would help to confirm the in-depth findings from rodent studies, as well as determine the biological effects of splicing, intronic, and other regulatory mutations that are untestable in the other cell-based systems. In particular, this may be the most impactful way to address the interactions of environmental stressors and SNPs that are essential for driving the EDGE hypothesis postulated to be the cause behind the associated effects of the T1405N variant. The same approach may be used to elucidate the interactions of SNPs identified in major depressive disorder as well as the rs715 SNP that protects against coronary artery disease. Beyond SNPs, this model offers a useful platform for determining the mechanism of AMPK-mediated CPS1 regulation, in addition to how mutations and methylation patterns impact tumorigenesis. Together, the wide variety of cell systems offers a powerful way to investigate the plethora of described clinical mutations in CPS1.

Animal Models—By studying the molecular impacts of various mutations on CPS1 stability and function, we can gain important insights into how and why the deficiency progresses in individuals. However, to design and test potential therapeutics, robust animal models are essential. The severity of disease symptoms and high therapeutic threshold have made the development of animal models technically challenging[30,128], in turn hindering therapeutic development. Other potential treatments have been tried but not validated in controlled clinical studies, such as therapeutic hypothermia[129,130] and hepatocyte transplantation[131], both of which would benefit immensely from testable animal models. To

address the need for new models, our group recently generated and characterized two models of CPS1 deficiency in mice. In the constitutive knock out, neonates homozygous for a deletion in exons 3 and 4, which introduces a premature stop codon, rapidly develop lethal hyperammonemia with milk intake and perish within 24 hours of birth[132]. This model recapitulates the human phenotype as well as a previously published model also describing a constitutive knock out[133]. To our knowledge however, this previous model was never used for any therapeutic development and has since been lost[134].

Though useful for replicating disease phenotypes, the constitutive knock out is technically challenging to use as a platform for proof-of-principle studies due to the rapid death of pups and typical births occurring overnight. To generate a more malleable genetic model, we generated a conditional knock out mouse model that uses the Cre/LoxP system to delete exons 3 and 4 in *Cps1*, introducing a nonsense mutation that leads to gradual decline and death with hyperammonemia and hyperglutaminemia over the course of 3 weeks[135]. With this controlled loss of protein and longer time window for therapy, we demonstrated that murine Cps1 deficiency is treatable using a recombinant helper-dependent adenovirus expressing murine Cps1[135]. We were subsequently able to show using this model that an AAV-based gene therapy approach was feasible and corrected the disorder by expressing human CPS1[136]. Successful gene therapies have been described for the other enzymes in the urea cycle[137–140], and these new studies in CPS1 deficiency lay the groundwork for rescuing the more severe neonatal phenotype in the constitutive knock out and potential clinical translation. With over 250 unique mutations reported, and >90% of them being private mutations[141] (arising in single families only), a successful gene addition approach has the potential to benefit all CPS1 deficiency patients.

A final mouse model was also recently established utilizing human patient hepatocytes and a mouse model of liver repopulation[142]. The triple transgenic $Fah^{-/-}/Rag2^{-/-}/Il2\gamma^{-/-}$ (FRG) mouse is a long-standing liver repopulation model in which a defect of tyrosine metabolism (*Fah* loss) causes endogenous hepatocytes to accrue toxic metabolites and die in the absence of the drug Nitisinone (NTBC)[143]. By withdrawing NTBC after transplantation of human CPS1 patient hepatocytes, the engrafted human cells repopulate the liver with FAH-expressing cells without rejection (*Rag2* and *Il2γ* loss). By controlling the level of repopulation, transplanted FRG mouse livers can be repopulated with human hepatocytes until symptoms of CPS1 deficiency appear, allowing the development of therapeutics in an environment that more closely resembles the native human milieu. One major limitation is the access to primary human hepatocytes from patients, though ever-improving gene editing- and pluripotent cell-based tools may eliminate this issue altogether in the near future. Indeed, this system holds great promise for directly testing the ability of small molecules and gene-based approaches to treat CPS1 deficiency arising from different structural and functional mutations. Glycerol holds promise for activating CPS1 in cases where the NAG binding site is mutated[125]; this therapeutic niche may also be occupied by n-carglumic acid (NCA), which is used to treat NAG synthase deficiency. As CPS1 is not fully saturated with NAG at physiological concentrations[144], increasing CPS1 activity with exogenous NCA may offer an expedient treatment option for patients, provided their particular mutation is amenable as NCA can actually decrease ureagenesis in some CPS1 deficiency contexts[145–147]. NCA may also promote enzyme stability in variants with mutations outside

of active sites that cause protein misfolding[148], highlighting its versatility and the need for further studies.

Additional therapeutics may arise from work in other diseases. Most closely related is work done in the urea cycle deficiencies of Arginase 1[149] and Citrin[150], both of which demonstrated replacement of the missing proteins using lipid nanoparticles (LNPs) delivering mRNA. LNPs as gene therapy vehicles have gained significant traction recently due to their low immunogenicity and high scalability, as well as their amenability to chronic administrations, making them a valuable tool in testing alternative options to classical viral gene therapies. One major limitation to this approach for CPS1 deficiency is the requirement for high levels of protein pan-hepatically[128], which may not be reachable due to general restriction of LNP-mediated expression to the perivasculature and toxicity at high doses; further studies are crucial to determine if this approach is feasible. Similarly, other non-viral approaches, such as plasmid/mini circle delivery and pluripotent stem cell-derived hepatocyte transplantation, may offer safer and more effective strategies as the technologies continue to improve. Obesity studies may also be unexpected sources for discovering means of controlling CPS1 expression and developing therapies. The small molecule nobiletin (discussed earlier) increases CPS1 expression in mice fed HFD. However, it was not able to further increase CPS1 expression in mice fed high protein, potentially limiting its usage to smaller gains in expression not necessarily suitable in CPS1 deficiency. Further studies to determine the extent of transcriptional increase and the possibility of treating more moderate CPS1 deficiency are needed, including other diseases in which CPS1 is downregulated secondarily or cases of acute hyperammonemia.

Final Remarks

CPS1 deficiency is a devastating urea cycle disorder with limited treatment options. Extensive biochemical and molecular characterization make this enzyme a clear target for therapeutic development not only in the classical disorder but also in newly associated diseases including hypertension and obesity. Follow-up studies to proof-of-concept gene therapies are essential to bringing this treatment to the clinic, as well as more detailed investigations of the interactions of small molecules on CPS1 to stabilize and promote its function. New cell and animal models offer a wealth of potential for gaining a deeper understanding of how this urea cycle enzyme may be linked to systemic metabolism and homeostasis.

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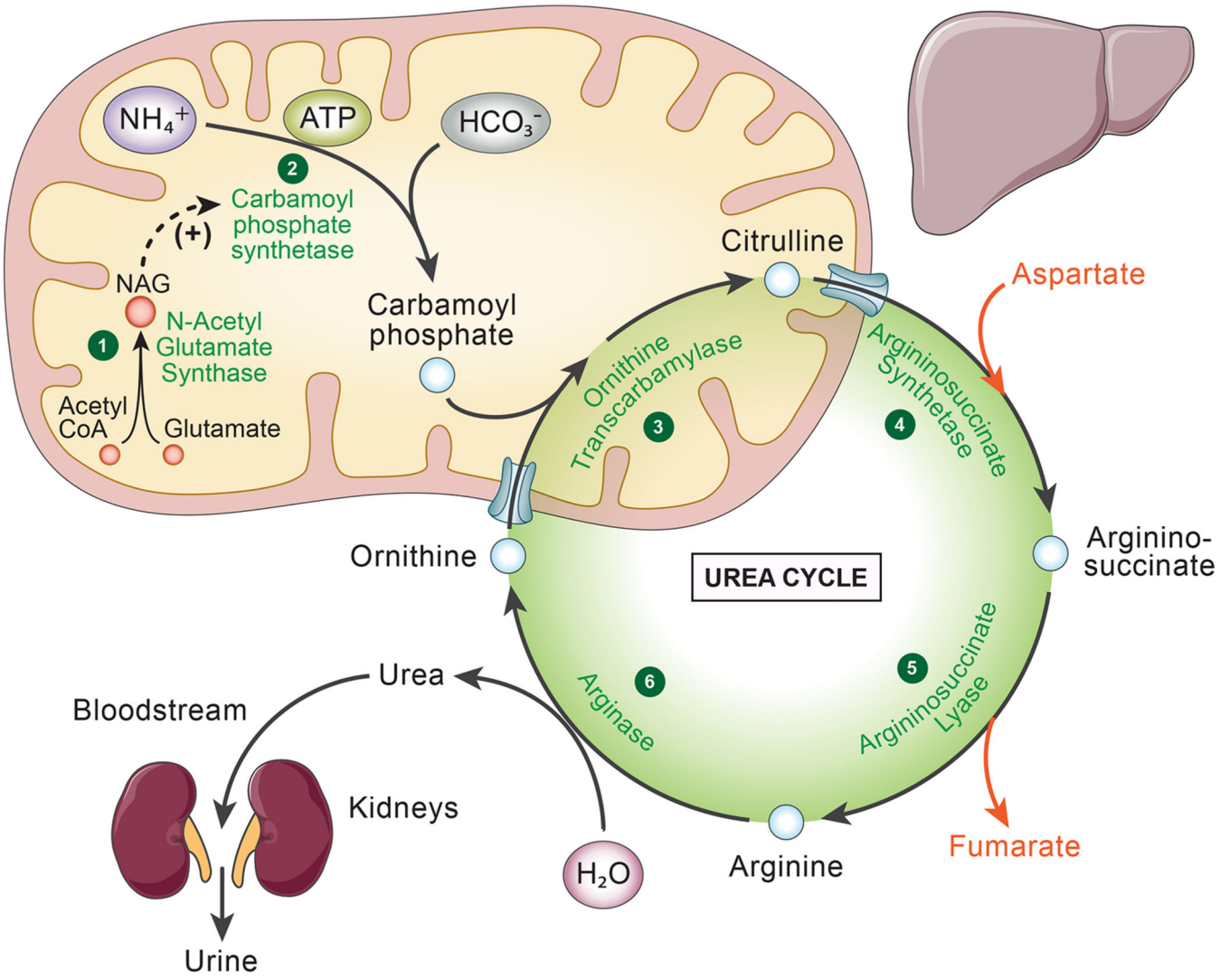

Figure 1.

Diagram of the mammalian urea cycle. CPS1 catalyzes the first step of the urea cycle by condensing ammonia with bicarbonate, generating carbamoyl phosphate that is eventually incorporated into urea and excreted by the kidneys.

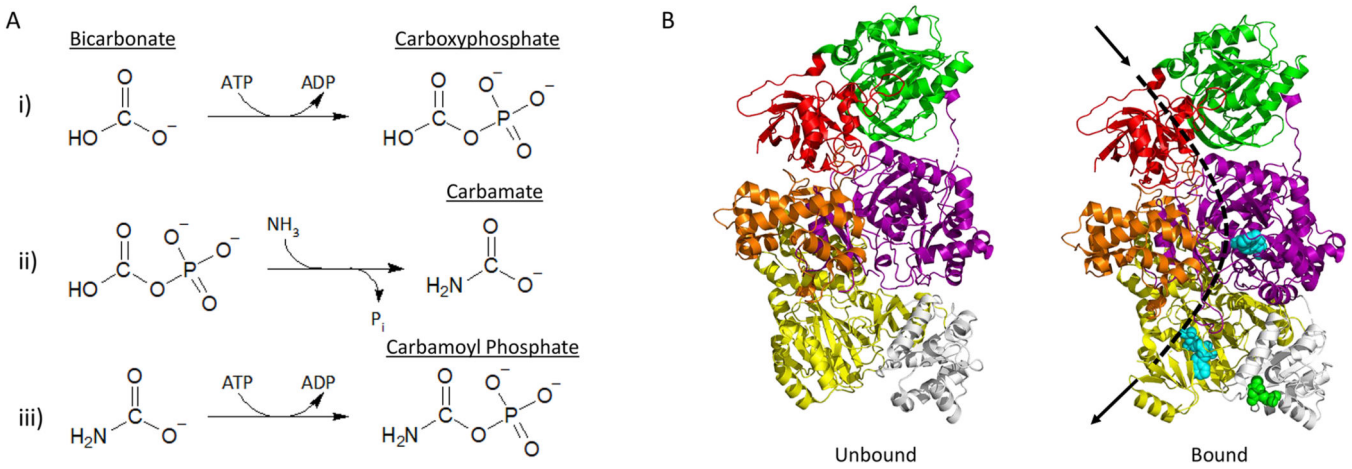

Figure 2.

Structure and biochemistry of CPS1. **A**) CPS1 generates carbamoyl phosphate in 3 steps by phosphorylating bicarbonate (i), condensing carboxyphosphate with nitrogen (ii), and phosphorylating carbamate (iii). **B**) The structure of human CPS1 has two major conformations, not bound to NAG (unbound, left panel) or bound to NAG and ATP (bound, right panel). Conformational changes throughout the enzyme, responsible for the formation of a stable tunnel to shuttle reaction intermediates (dashed line), perpetuate from the NAG-binding domain (white) into the carbamate phosphorylation (yellow), integrating (orange), bicarbonate phosphorylation (purple), glutaminase-like (green), and N-terminal (red) domains. NAG is shown as green spheres in the NAG-binding domain, while ATP molecules are cyan spheres the kinase domains. Based on structures from de Cima et al. 2015.

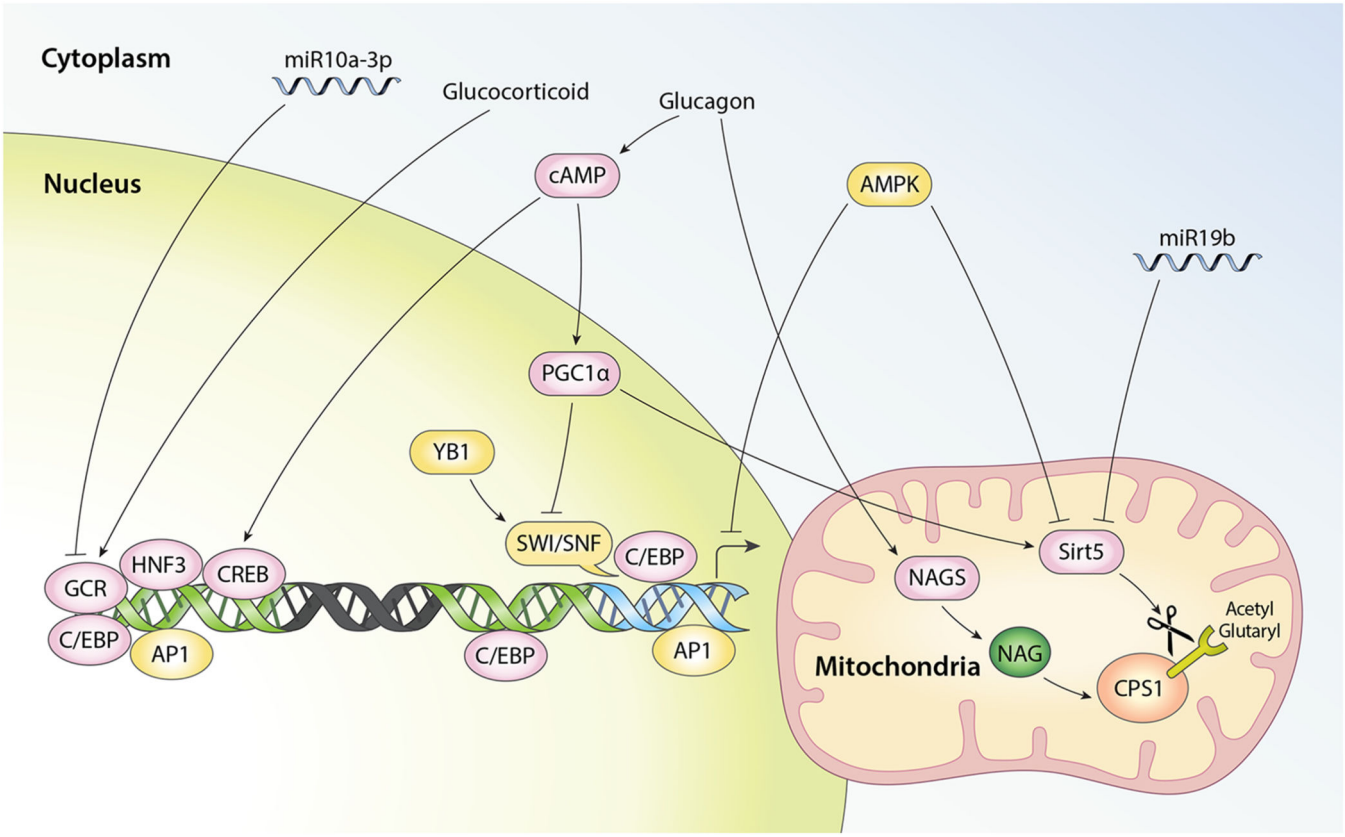

Figure 3.

Interactions regulating the gene and protein expression of CPS1. The promoter (blue DNA) and enhancer (green DNA) elements are responsible for integrating a diverse array of signals to precisely regulate CPS1 expression according to the needs of the cell. Protein level regulation is responsible for rapid responses to metabolic flux. CPS1 activators are shown in pink, with suppressors in yellow. Abbreviations: AMPK, AMP kinase; cAMP, cyclic AMP; PGC1α, peroxisome proliferator-activated receptor-gamma coactivator 1α; C/EBP, CAAT/enhancer binding protein; CREB, cAMP response element binding protein; AP1, activator protein 1; HNF3, hepatocyte nuclear factor 3; GCR, glucocorticoid receptor; YB1, Y-box binding protein 1; SWI/SNF, Switch/Sucrose Non-Fermentable; NAGS, n-acetylglutamate synthase.