

Title: Current understanding of pathogenic mechanisms and disease models of citrin deficiency

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Denis Lacabanne, Alice P. Sowton, Bosco Jose, Edmund R. S. Kunji and Sotiria Tavoulari declare no conflict of interest.

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This article does not contain any studies with human or animal subjects performed by the any of the authors.

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Abstract

Citrin deficiency (CD) is a complex mitochondrial disease with three different age-related stages: neonatal intrahepatic cholestasis caused by CD (NICCD), failure to thrive and dyslipidemia caused by CD (FTTDCD), and type II citrullinemia (CTLN2), recently renamed to adolescent and adult CD (AACD). Whilst highly prevalent in the Asian population, CD is pan-ethnic and remains severely underdiagnosed. The disease is caused by the dysfunction or absence of the mitochondrial aspartate/glutamate carrier 2 (AGC2/SLC25A13), also known as citrin. Citrin deficiency results in a direct impairment of the malate-aspartate shuttle and the urea cycle, with expected knock-on effects on a multitude of other metabolic pathways, leading to a complicated pathophysiology. Here, we discuss our current knowledge of the molecular mechanism of substrate transport by citrin, including recent advances against its calcium regulation. We also discuss the different types of pathogenic variants found in CD patients and new insights into their pathogenic mechanisms. Additionally, we provide a summary and assessment of the efforts to develop preclinical models as well as treatments for the disease.

1. Introduction

Citrin deficiency (CD) is an inborn error of metabolism and a secondary urea cycle disorder caused by pathogenic variants of the *SLC25A13* gene (OMIM #603859), encoding the mitochondrial aspartate/glutamate carrier 2 (AGC2), also called citrin (Kobayashi et al. 1999; Palmieri et al. 2001). This carrier protein, located in the mitochondrial inner membrane, is responsible for the symport of glutamate with a proton into the mitochondrial matrix and the export of aspartate from the matrix to the cytoplasm (Azzi et al. 1967; LaNoue et al. 1974a, b; Dierks and Krämer 1988; Dierks et al. 1988; Palmieri et al. 2001) (**Figure 1**). To date, more than 120 different pathogenic variants of citrin have been reported in the literature worldwide (reviewed in Tavoulari et al. 2022, with additional ones in **Table 1**) and 168 in Human Gene Mutation Database (HGMD), but the number of identified variants continues to grow with an increasing number of diagnoses each year.

Citrin is expressed variably across tissues, with the liver, kidney, heart, pancreas and small intestine exhibiting the highest levels of AGC2 (Iijima et al. 2001; Begum et al. 2002; Del Arco et al. 2002), whereas aralar (AGC1), a paralog of citrin encoded by *SLC25A12*, is primarily expressed in the brain, heart, and skeletal muscle (Del Arco and Satrústegui 1998). Despite their common function, the different tissue distribution of citrin and aralar results in distinct clinical phenotypes associated with their pathogenic variants. Whilst variants of *SLC25A13* cause CD, variants of *SLC25A12* lead to AGC1 deficiency, an infantile-onset encephalopathy characterized by defective myelin synthesis and associated with hypotonia, arrested psychomotor development, and epilepsy (Wibom et al. 2009; Falk et al. 2014). CD is considered to affect the liver primarily, whereas variants of *SLC25A12* do not result in hepatic phenotypes, likely due to the low aralar content in human livers, estimated in different studies to be either absent (González-Moreno et al. 2023) or 13 times lower than citrin (Wang et al. 2019).

CD was first described six decades ago (Morrow et al. 1967) and was originally characterized as both a rare and East-Asia-specific disease. It was first studied in Japan, where it is highly prevalent (Saheki and Kobayashi 2002; Kido et al. 2022), but is now recognized as being pan-ethnic with increasing numbers of patients of non-Asian descent being diagnosed worldwide (Ben-Shalom et al. 2002; Dimmock et al. 2009; Hutchin et al. 2009; Fiermonte et al. 2011; Vitoria et al. 2013; Avdjieva-Tzavella et al. 2014; Pinto et al. 2020; Bölsterli et al. 2022; Sachs et al. 2023; Häberle 2024; Kido et al. 2024). Additionally, CD is not as rare as previously thought, with an incidence of 1 in 17,000 births in Japan (Lu et al. 2005) and carrier rates measured at 1:41 in Singapore (Bylstra et al. 2019), 1:31 in Vietnam (Tran et al. 2021) and as high as 1:28 in the Southern regions of China (Lin et al. 2020).

Clinically, CD manifestations are diverse and show vast inter-patient variability, as reviewed comprehensively elsewhere (Saheki et al. 2002, 2020; Song et al. 2017; Kido et al. 2024). Briefly, from birth, CD patients can present with neonatal intrahepatic cholestasis associated with CD (NICCD) (OMIM #605814), which is characterized by jaundice, cholestasis, hypoproteinemia, hypoglycemia, galactosemia, and multiple aminoacidemias, including citrullinemia (Kido et al. 2024). Following NICCD, patients enter an adaptation stage, where they may experience a variety of milder clinical symptoms, typically accompanied by an aversion to carbohydrate-rich food (Okamoto et al. 2021). However, many patients at this stage suffer from failure to thrive and dyslipidemia caused by CD (FTTD CD), where they may suffer from growth impairment, fatigue, dyslipidemia, pancreatitis, and gastroenteropathy (Song et al. 2009; He et al. 2019; Pinto et al. 2020). If undiagnosed or poorly managed, CD can escalate in a subset of patients, leading to development of citrullinemia type II (CTLN2) (OMIM #603471) (Saheki and Kobayashi 2002; Saheki et al. 2002), recently renamed as adolescent and adult CD (AACD; Kido et al. 2024).

Often, this stage is characterized by frequent attacks of hyperammonemia, neuropsychiatric symptoms, and even brain edema, alongside a steatotic hepatic phenotype (Komatsu et al. 2008; Kido et al. 2024). However, it is unknown why AACD manifests only in a subset of patients and what triggers it. The precise molecular mechanisms driving these diverse phenotypes remain poorly understood, highlighting the need for more focused molecular research to unravel the links between genotype and clinical presentation.

Here, we review new insights into the molecular function of citrin, including how distinct pathogenic variants may be associated with discrete mechanisms of citrin dysfunction, before considering current efforts in the development of preclinical models of CD in the hunt for effective treatments of the condition.

2. Citrin sits at a nexus of metabolic pathways

Citrin connects multiple metabolic pathways (**Figure 1**) and its pathogenic variants, as seen in CD, disrupt these pathways, leading to diverse phenotypes observed in patients. Citrin is an essential carrier protein of the malate-aspartate shuttle (MAS) (Chappell 1968), working alongside the mitochondrial oxoglutarate carrier (OGC, *SLC25A11*) (**Figure 1**). Whilst MAS involves a series of interconnected enzymatic reactions and transport steps, its net effect is redox shuttling, leading to NADH in the cytosol being oxidized and NAD⁺ in the mitochondrial matrix being reduced (Borst 2020). MAS therefore serves to regenerate cytosolic NAD⁺ for glycolysis, whilst translocating electrons produced during glycolysis into the mitochondrial matrix to feed into the electron transport chain and generate ATP. However, cytosolic NAD⁺ regeneration is vital beyond transferring energy equivalents between the cytosol and mitochondria, with many cytoplasmic reactions also being redox dependent, including serine biosynthesis, alcohol metabolism and post-translational modification of proteins (Isselbacher and Greenberger 1964; Diehl et al. 2019; Kulkarni and Brookes 2019). It is commonly

assumed that many of the phenotypic manifestations of CD, such as fatigue, ultimately arise from elevations in the cytosolic NADH/NAD⁺ ratio as a result of MAS dysfunction (Saheki and Kobayashi 2002). However, this assumption has not been confirmed in patients, and the only evidence of altered NADH/NAD⁺ ratios in CD is derived from cultured cell models (Yau et al. 2023; González-Moreno et al. 2023) or inferred indirectly from lactate/pyruvate ratios in animal models (Sinasc et al. 2004; Moriyama et al. 2006, 2015; Saheki et al. 2007, 2019).

In addition to maintaining redox balance, citrin contributes to detoxification of ammonia, derived from deamination of amino acids, via the urea cycle. The export of aspartate from the mitochondrial matrix into the cytosol by citrin is a key transport step required for the urea cycle in the liver (**Figure 1**). These reactions convert waste ammonia from protein breakdown into urea for excretion. Aspartate is required in the cycle in the intermediate step, combining with citrulline, itself exported from the mitochondria in exchange for ornithine by the mitochondrial ornithine carrier (ORC), to form argininosuccinate (Shambaugh 1977). Citrin is critical for ensuring sufficient aspartate is available in the cytosol for continuous urea cycle flux. In CD, insufficient aspartate export is hypothesized to contribute to the accumulation of ammonia to toxic levels. Patients who progress to the most severe form of CD, AACD (previously CTLN2), display clinical symptoms associated with hyperammonemia, namely neuropsychiatric symptoms, which can progress to coma and brain edema (Song et al. 2017).

Also in the liver, aspartate is a key precursor for gluconeogenesis. As part of MAS, cytosolic aspartate aminotransferase converts aspartate to oxaloacetate. Whilst oxaloacetate can be converted to malate, which is imported into the mitochondria through OGC as part of MAS, in the cytosol of hepatocytes, oxaloacetate can also be decarboxylated and phosphorylated by phosphoenolpyruvate carboxykinase. This reaction forms phosphoenolpyruvate (PEP) for gluconeogenesis (Marco et al. 1974). Mitochondrial

oxaloacetate may also be exported as malate or phosphoenolpyruvate via other mitochondrial carriers. Whilst other gluconeogenic substrates can be used for gluconeogenesis (alongside pyruvate itself), aspartate is considered to be one of the principal sources of cytosolic oxaloacetate for gluconeogenesis in the liver (Holeček 2023), highlighting the importance of citrin in this pathway. Therefore, CD might be associated with impaired hepatic gluconeogenesis. Alternatively, the hypothesized disturbances in cytosolic NADH/NAD⁺ ratio (caused by CD) have been proposed to impair gluconeogenesis by impairing the conversion of lactate to pyruvate and glycerol-3-phosphate to dihydroxyacetone phosphate. Impaired gluconeogenesis may contribute to hypoglycemia that some CD patients experience (Okano et al. 2019).

Citrin is essential for cellular homeostasis, supporting redox balance and energy metabolism via MAS, ammonia detoxification through the urea cycle, and glucose production by gluconeogenesis. Given these critical functions, disruption of citrin activity due to pathogenic variants can lead to the wide-ranging metabolic disturbances observed in CD, underscoring the importance of the protein in both normal physiology and disease.

3. The structure and function of citrin

3.1 Structural model

Citrin and aralar are unique members of the SLC25 family of mitochondrial carriers due to their structural complexity. They consist of three domains: an N-terminal domain with eight EF-hand motifs (Palmieri et al. 2001; Contreras et al. 2007), a mitochondrial carrier domain responsible for substrate transport (Palmieri et al. 2001), and a C-terminal amphipathic α -helix bound in a hydrophobic cleft within the N-terminal domain (Thangaratnarajah et al. 2014) (**Figure 2**).

Unlike other SLC25 family members (Kunji and Crichton 2010; Ruprecht and Kunji 2020, 2021; Cimadamore-Werthein et al. 2024), citrin and aralar form homodimers mediated by the N-terminal domain (Thangaratnarajah et al. 2014). EF-hands 4-8 do not bind calcium but instead form a large dimerization interface of about 2,000 Å² (Thangaratnarajah et al. 2014). It is likely that the two carrier domains in the dimer do not interact and function independently of each other (Thangaratnarajah et al. 2014; Cimadamore-Werthein et al. 2024; Tavoulari et al. 2024).

The carrier domain of citrin and aralar resembles canonical SLC25 family members, such as the ADP/ATP carrier or uncoupling protein 1 (Ruprecht et al. 2014, 2019; Jones et al. 2023). It has a threefold pseudo-symmetrical structure (Kunji and Harding 2003) formed by three sequence repeats (Saraste and Walker 1982). Each domain consists of two transmembrane helices linked by a loop containing a matrix helix (Kunji and Harding 2003; Pebay-Peyroula et al. 2003), folding into a six-helical bundle with a central water-filled cavity (Pebay-Peyroula et al. 2003; Ruprecht et al. 2014, 2019) (**Figure 2**).

Whilst the atomic structure of full-length citrin has not been resolved, a model has been proposed (Tavoulari et al. 2022) (**Figure 2**) based on the structures of the N- and C-terminal domains of citrin (Thangaratnarajah et al. 2014) and homology models of the carrier domain based on the ADP/ATP carrier (Ruprecht et al. 2014, 2019).

3.2 Transport mechanism

Like other members of the SLC25 family, citrin operates via a ping-pong transport mechanism (Cimadamore-Werthein et al. 2024), where glutamate and a proton have to be imported into the matrix before aspartate can be exported. This mechanism involves cycling between two conformational states, the “matrix-open” state or “m-state”, in which the substrate binding site is open to the mitochondrial matrix and the “cytoplasmic-open” state or “c-state”, where it is

open to the intermembrane space (Erdelt et al. 1972; Klingenberg and Buchholz 1973; Ruprecht et al. 2014, 2019) (**Figure 2c**). Glutamate and a proton bind to their binding sites in the c-state (Robinson and Kunji 2006; Kunji and Robinson 2006, 2010), after which the carrier converts to the m-state, via an occluded state, which is closed to both sides of the membrane. After the substrates are released into the matrix, mitochondrial aspartate binds to the m-state and triggers conformational changes, shifting the protein to the c-state via an occluded state. Subsequently, aspartate can leave the binding site and diffuse into the intermembrane space, completing the exchange of substrates across the inner membrane of mitochondria. Although atomic details of this translocation mechanism of citrin are unavailable, the carrier domain of citrin shares all of the structural features previously proposed for the best studied example of this protein family, the ADP/ATP carrier (Ruprecht et al. 2014, 2019; Ruprecht and Kunji 2020; Kunji et al. 2020). Recently, several missense pathogenic variants in residues involving the substrate binding site and key networks for state interconversions have been studied, showing detrimental effects on the transport activity and confirming the critical functional role of these residues (See **Section 4**; Tavoulari et al. 2024). The roles of the N-terminal domain and C-terminal α -helix remain unclear, though recent evidence shows that pathogenic variants at different positions of the N-terminus interfere with protein biogenesis (Tavoulari et al. 2024), also discussed in **Section 4**.

3.3 The ambiguous role of calcium

Several studies have previously shown that the malate-aspartate shuttle is activated by calcium, a property attributed to the mitochondrial aspartate/glutamate carriers (Sugano et al. 1988; Palmieri et al. 2001; Pardo et al. 2006; Contreras et al. 2007; Mármol et al. 2009; Koshenov et al. 2022), which are known to bind calcium (Del Arco et al. 2000) at a single site, the preformed EF-hand 2 (Thangaratnarajah et al. 2014). One caveat of these

investigations is that the transport activity of the AGCs cannot be measured directly in a cellular or mitochondrial context. Instead, AGC activity is inferred from NADH oxidation measurements of MAS activity reconstituted from isolated mitochondria (Pardo et al. 2006; Contreras et al. 2007; Mármol et al. 2009) or from metabolic changes that could be resulting from changes in MAS activity (Sugano et al. 1988; Palmieri et al. 2001; Koshenov et al. 2022). Some studies have also employed knock-down of AGC to establish its direct involvement in the calcium effect on MAS (Mármol et al. 2009; Koshenov et al. 2022). However, MAS is part of a complex metabolic network, involving the conversion of malate to oxaloacetate. This step is also a critical step in the tricarboxylic acid (TCA) cycle, which itself is upregulated by calcium (McCormack et al. 1990; Glancy and Balaban 2012). Thus, MAS and TCA cycle activities are critically linked, meaning an upregulation in the TCA cycle by calcium could lead to an increase in MAS activity, unrelated to changes in citrin activity.

In vitro studies with reconstituted purified citrin have also been employed to evaluate the above findings, but no consensus has been achieved so far. First, it was reported that reconstituted aralar1 from *Drosophila melanogaster* is activated by calcium *in vitro* (Lunetti et al. 2021). In contrast, we have recently found that wild-type human citrin activity, measured in proteoliposomes, was unaffected after calcium had been removed and replaced by magnesium (Tavoulari et al. 2024; **Figure 3D**). The calcium replacement by another divalent cation had not been considered in previous *in vitro* activity measurements. However, this step is necessary, as cation-free conditions can destabilize proteoliposomes, preventing the formation of concentration gradients required for transport activity and can be mistaken for protein inactivation in the absence of calcium (Tavoulari et al. 2024).

As achieving calcium-free conditions is generally challenging and can create artifacts in proteoliposomes, another approach was employed to eliminate the calcium binding site by mutagenesis. We have shown that a mutant of human citrin (D66A/T68A/D70A/E77A), where

the critical residues of the calcium binding site in EF-hand 2 are mutated to alanine, rendering the protein unable to bind calcium, had transport activity comparable to wild-type citrin (Tavoulari et al. 2024; **Figure 3A-D**). These results contrast with previous studies where EF-hand 1 and EF-hand 2 mutagenesis in aralar resulted in decreased MAS activity, as measured by changes in NADH oxidation rates (Mármol et al. 2009). For human citrin, elimination of the calcium binding site did not affect the stability or oligomeric state of citrin (**Figure 3E-F**). Furthermore, the quadruple alanine mutant localized to the inner mitochondrial membrane, showing that calcium binding was not critical for biogenesis either (**Figure G-I**).

In structural studies, conformational changes of the N-terminal domain of aralar alone have been observed in a calcium-dependent manner (Thangaratnarajah et al. 2014), which could be consistent with a mechanism of calcium activation. However, these conformational changes were only observed in the absence of the amphipathic helix of the C-terminal domain, which is not relevant in the context of the full-length protein (Thangaratnarajah et al. 2014). A structure of an N- and C-terminal domain fusion of citrin, mimicking the correct structural context, could be obtained in a calcium-bound state, but not in a calcium-free state, despite the use of excess chelators (Thangaratnarajah et al. 2014), suggesting that the conformational changes might not occur in the presence of the amphipathic helix, independent of calcium. Interestingly, the structure of the EF-hand 2 in the N-terminal domain structure of aralar is the same whether calcium is bound or not (Thangaratnarajah et al. 2014), suggesting that calcium binding or release cannot trigger conformational changes. Furthermore, EF-hand structural mechanisms usually involve EF-hand pairs, both capable of binding or releasing calcium, however this is not the case in citrin. Thus, based on our functional and structural analyses of citrin, no functional role can be assigned to the bound

calcium ion in EF-hand 2. This suggests that calcium may not be playing a role in pathogenic mechanisms in citrin deficiency.

4. Pathogenic variants of citrin: More than one mechanism at work

Although CD is a monogenic disease, it involves a wide range of pathogenic variants of *SLC25A13*. Splice site variants, deletions, insertions, nonsense and missense variants have all been reported (**Figure 4, 5 and Table 1**) (also reviewed in Tavoulari et al. 2022), with more pathogenic variants in citrin reported than in all other mitochondrial carrier proteins combined (Kunji et al. 2020). Compound heterozygote patients are more common, representing 53%, compared with patients carrying two copies of the same pathogenic allele (Tavoulari et al. 2022).

The most prevalent pathogenic allele is c.851_854delGTAT, a deletion variant, found in more than 50% of patients in China and in approximately 30% of patients in Japan and Korea (Tabata et al. 2008; Oh et al. 2016). Other highly prevalent pathogenic alleles contain splice site variants (Tabata et al. 2008; Oh et al. 2016; Lin et al. 2020), with prevalence ranging from 48% of all pathogenic alleles in Japan to 24% in China. Approximately one-third of the known splice site variants in the *SLC25A13* gene occur in introns 6 and 7 (1.8 kb and 1.4 kb, respectively). These variants lead to aberrant splicing, introducing premature stop codons and resulting in truncated proteins. Nonsense variants are less prevalent, representing only around 4% of Chinese patients (Tavoulari et al. 2022).

Most splice site, deletion, insertion, or nonsense variants disrupt the citrin carrier domain, which is incompatible with these variants being functional transporters. However, in many variants, the mRNA may undergo nonsense-mediated decay (Broгна and Wen 2009), potentially abolishing protein expression altogether, although direct experimental or clinical

data have not been available to support this hypothesis in the majority of cases (Yasuda et al. 2000).

Missense variants represent 20% of Chinese CD cases. To date, more than 50 missense variants have been reported, of which about 70% are located in the carrier domain of citrin, with the remaining ones located in the N-terminal domain (**Figure 5A, B**). It is expected that these variants produce full-length protein products, and whilst limited data is available, normal expression levels have been reported for G437E in fibroblasts of a heterozygous patient (Fiermonte et al. 2011) and for A25E in a homozygous patient (Dimmock et al. 2009).

Recently, we have proposed that there are distinct mechanisms of dysfunction elicited by different missense variants in citrin (**Figure 5C-F**) (Tavoulari et al. 2024), which may be relevant to the broad phenotypic landscape observed in CD patients. In *in silico* and *in vitro* tests of 33 missense variants, most of the investigated ones affecting the carrier domain led to severely impaired substrate transport activity. Complete or near-complete loss of transport activity was measured when variants occurred in functional elements of the carrier, known to be crucial for substrate translocation in other members of the SLC25 family, such as the putative substrate binding site (K405N, D493G and R588Q) and the cytoplasmic or matrix networks (D350N, C489R, T456R). As an exception, conservative substitutions, like K453R in the matrix network, reduced activity, but did not inactivate the protein completely. Severe effects on transport activity were reported for several additional substitutions in the carrier domain at structurally important positions or residues important for dynamic changes. Whilst activity was severely affected for most variants in the carrier domain, the expression and mitochondrial localization of most of these variants were unaffected (Tavoulari et al. 2024).

Conversely, most N-terminal missense variants tested retained high levels of transport activity, and some, as for example L95D, were comparable to wild-type citrin. However, the majority of the tested N-terminal domain variants interfered with the ability of the protein to

localize to the mitochondria, despite being positioned in different regions of the N-terminal domain (Tavoulari et al. 2024). Therefore, it appears that pathogenic variants in the N-terminal domain interfere with mitochondrial localization, suggesting that it plays a critical role in the biogenesis of citrin, although further evidence is necessary to support this hypothesis.

5. Therapies and models of citrin deficiency

The dysfunction of citrin manifests in a complex clinical phenotype that includes hyperammonemia, dyslipidemia, and hypoglycemia across different life stages. Therapeutic options remain limited, and no current treatment addresses the underlying metabolic dysregulation comprehensively. To bridge this gap, effective preclinical models have become essential for evaluating therapeutic strategies aimed at restoring metabolic homeostasis. Here, we discuss published efforts to develop preclinical models of the disease, as well as therapies used or under investigation. Future directions on developing treatments and further understanding of the pathology have been reviewed elsewhere (Walker 2025).

5.1 Cell models

The first cellular models of CD were developed with human induced pluripotent stem cells (hiPSCs) derived from dermal fibroblasts of a CD patient carrying the c.851_854delGTAT and c.1750+72_1751-4dup17ins (IVS16ins3kb) variants (Kim et al. 2016) (**Figure 6**). Subsequently, these hiPSCs were differentiated into hepatocyte-like cells and demonstrated lower rates of ureagenesis from NH₄Cl compared with hepatocyte-like cells generated from hiPSCs of non-CD patients, indicating that they recapitulate the urea cycle defects observed in CD. This finding has recently been recapitulated in hepatocyte-like cells derived from hiPSCs developed from peripheral blood mononuclear cells of two CD patients (patient 1: c.1177+1G>A and c.1801G>T, patient 2: c.1177+1G>A and c.851_854delGTAT) (Okano et

al. 2024). The CD-hiPSCs developed by Kim *et al.* also showed increased triacylglycerol accumulation under high-glucose conditions, linked to reduced expression of PPAR α and its target genes (previously indicated in CD (Komatsu *et al.* 2015), as well as downregulation of mitochondrial marker genes, such as TOM20 and Parkin (Kim *et al.* 2016). This patient-derived model system therefore provides evidence for aberrant regulation of fatty acid metabolism *via* PPAR α and mitochondrial abnormalities as a central mechanism in the fatty liver phenotype of CD. However, the cells in the study of Kim *et al.* are derived from only one patient, carrying two indel variants. Whether these pathophysiological mechanisms are applicable to CD as a whole, given the numerous distinct variants associated with it, remains to be investigated.

More recent work has used HepG2, a human hepatoblastoma cell line (Arzumanian *et al.* 2021), and CRISPR/Cas9 gene editing to create an *SLC25A13* knockout via targeted deletion of a ~43 kb region spanning exons 3 and 4 in one allele, with a serendipitous 10 bp deletion in the second allele (Yau *et al.* 2023) (Figure 6). Yau *et al.* proposed that glycolytic capacity, fatty acid oxidation and mitochondrial respiratory activity are all impaired in the citrin-deficient cells, whilst proteins and genes involved in lipogenesis, cholesterol and bile acid metabolism are upregulated. Supplementing the cells with nicotinamide riboside, an NAD⁺ precursor (Bieganowski and Brenner 2004), reversed several phenotypic abnormalities, suggesting its potential as a treatment for CD.

However, this system requires further validation as some fundamental issues have not been addressed. First, HepG2 cells carry chromosomal abnormalities (Simon *et al.* 1982; Wong *et al.* 2000; Zhou *et al.* 2019), including, importantly for CD research, trisomy of chromosome 2, on which aralar and mitochondrial glycerol-3-phosphate dehydrogenase (mGPD) are located. Whether HepG2 cells show elevated levels of aralar expression and function, as has been suggested previously (Infantino *et al.* 2019), needs to be evaluated

carefully as it will influence whether citrin models need to be initiated on a wild-type or aralar-suppressed background. Aralar upregulation in HepG2 cells has also been suggested to result from epigenetic upregulation in culture (Infantino et al. 2019) and thus the impact of epigenetic regulation also needs further investigation. mGPD is a key enzyme for the glycerol phosphate shuttle, which provides an alternative route of transferring energy equivalents between the mitochondria and cytosol. Trisomy of chromosome 2 may therefore not only contribute elevated aralar, but elevation of mGPD, and possibly reduce reductive stress in CD models.

Second, some other known genomic abnormalities may have functional implications for evaluating treatments for CD in an *in vitro* CD model. HepG2 cells have been shown to lack two key enzymes of the urea cycle (namely arginase 1 and ornithine transcarbamylase), diminishing the capacity of wild-type HepG2 to produce urea from NH_4Cl (Mavri-Damelin et al. 2007). Therefore, evaluation of treatments to ameliorate the secondary urea cycle disorder requires re-establishment of a functional urea cycle in HepG2. Beyond differences in the urea cycle, HepG2 cells exhibit known metabolic differences compared with primary hepatocytes, including altered insulin-sensitive glucose metabolism (Molinaro et al. 2020).

Finally, the work by Kim *et al.* highlighted that there may be differences in mitochondrial content between CD patient cells and healthy controls, alongside differences in mitochondrial morphology (Kim et al. 2016). Therefore, any measurement of mitochondrial respiratory activity or metabolite level needs to be carefully normalized to the number of cells actually respiring in any particular assay. Growth rates between citrin knockout cells and controls are likely to be different. Ideally, normalization should also include a marker of mitochondrial content (Larsen et al. 2012) to account for any differences in mitochondrial density as a result of the CD phenotype. Thus, it is still unknown whether there is an underlying deficit in mitochondrial respiratory function in these HepG2 citrin-deficient cells, or whether the

observed phenotype is merely a result of reduced cell number and mitochondrial density related to the citrin deficiency.

5.2 Animal models

Efforts to develop an animal model of CD were initiated decades ago, focusing on mice (**Figure 6**). As with many monogenic diseases, a knockout mouse was first generated. However, despite showing reduction in hepatic MAS activity through loss of the primary hepatic isoform of AGC, the citrin-KO mouse showed no phenotypic similarities to either AACD (previously CTLN2) or NICCD, even when mice were aged up to 12 months (Sinascac et al. 2004). The lack of a CD phenotype was attributed to the high level of activity of the glycerol phosphate shuttle in murine livers compared with humans, which allows reducing equivalents to be shuttled into the mitochondria, circumventing the disruption of the MAS (Williams et al. 1986; Macdonald et al. 2021). Thus, a double KO mouse was generated, with genes for both citrin and mGPD disrupted, to circumvent the limitations of the citrin-KO model (Saheki et al. 2007). This citrin-mGPD-double KO mouse presented growth retardation, hyperammonemia, hepatic triacylglycerol accumulation, as well as a similar aversion to carbohydrate-rich foods and alcohol seen in CD patients and reduced food intake when fed a carbohydrate-rich, protein-poor rodent chow (Saheki et al. 2012; 2017). However, there are several caveats to using this double KO mouse as an animal model of CD. Firstly, mGPD remains active in the human pathology, and, whilst the activity of the glycerol-phosphate shuttle is reportedly low in the human liver (Macdonald et al. 2021), it remains a mechanism of transport for reducing equivalents in and out of the mitochondria. Further, whether the shuttle activity compensates for the disruption of the MAS in CD is unknown. Additionally, single mGPD KO mice show phenotypic alterations that may confound the results of interpreting that of a double KO mouse, including impaired weight gain, lower plasma glucose

and altered urea cycle enzyme activity (Eto et al. 1999; Saheki et al. 2007; Madiraju et al. 2014). An additional problem with the double KO mouse was that it was generated on an inbred mouse strain background (C57Bl/6J), unlike the citrin-KO mouse, which was generated on a mixed genetic background. Saheki *et al.* generated the double-KO mouse in the C57Bl/6J background, due to the presence of a mild phenotype with the citrin-KO mouse (characterized by mild hypoglycemia and fatty liver) when it was congenic on the C57Bl/6J background (Saheki et al. 2007). The C57Bl/6J mice, however, carry known genetic polymorphisms and mutations affecting metabolism and glycemic tolerance compared to other mouse strains (Toye et al. 2005). This includes a 17,814 bp deletion in the *Nnt* gene, encoding nicotinamide nucleotide transhydrogenase. This gene is crucial for mitochondrial NADPH regeneration, and its loss disrupts glucose homeostasis (Toye et al. 2005). These disruptions are likely to exacerbate mitochondrial redox potential issues caused by citrin-mGPD knockouts (Sheeran et al. 2010).

5.3 Treatments and biomarkers proposed through CD models

Despite the caveats, both mouse models have provided insights into CD and potential treatments and biomarkers. Elevated hepatic glycerol-3-phosphate has been identified as a key metabolic signature in the double-KO mouse, which shows further hepatic accumulation following an oral sucrose challenge (Saheki et al. 2011, 2017). Oral sucrose administration has also been shown to result in increased hepatic citrulline content, with decreased citrate and malate concentration, both attributed to inhibition of ureagenesis and the TCA cycle, respectively (Saheki et al. 2011). Glycerol-3-phosphate has also been proposed to be a potential diagnostic marker of CD, alongside glycerol, based on the accumulation of these metabolites in the urine of the double KO mouse (Moriyama et al. 2015). Urine glycerol and glycerol-3-phosphate were also found to accumulate in CD patients aged 1-9 years

(Moriyama et al. 2015), highlighting that these metabolites may indeed be useful diagnostic markers for CD, especially in the silent period where few other clinical symptoms are present (Okano et al. 2013).

Both metabolic and genetic therapies have been proposed for the treatment of CD and have been investigated using both the citrin-deficient (Moriyama et al. 2006; González-Moreno et al. 2023) and double-KO mouse (Saheki et al. 2012, 2019; Cao et al. 2019). Using *in situ* liver perfusion, Moriyama *et al.*, tested the individual effects of pyruvate, aspartate, and citrate to improve ureagenesis from ammonia in citrin-deficient mice. This analysis showed that a hepatic perfusion of pyruvate at millimolar concentrations led to an increase in the rates of ureagenesis from NH_4Cl in the citrin-deficient liver to levels seen in the wild-type, whilst aspartate and citrate were unable to bring about this effect (Moriyama et al. 2006). However, the inability to achieve plasma concentrations of pyruvate to those shown to be necessary by Moriyama *et al.*, makes pyruvate therapy an unlikely target for CD treatment. Through addition of various supplements to a purified rodent diet lower in protein and higher in carbohydrate to standard laboratory chow, Saheki *et al.* demonstrated that increased dietary protein content improved food intake and weight gain in the double-KO mouse (Saheki et al. 2012). Supplementing the diet with alanine, sodium glutamate, or MCTs improved food intake and weight gain in the double-KO mouse, whilst also suppressing the hepatic increase in glycerol-3-phosphate and citrulline following an enteral sucrose challenge, although to a lesser extent with MCT supplementation (Saheki et al. 2012). Similarly, oral supplementation with ornithine, combined with either alanine or aspartate has been shown to suppress the elevation in blood ammonia and plasma citrulline in the double-KO mouse, following a combined sucrose-glycine challenge (Saheki et al. 2019). Taken together, these studies highlight that the natural avoidance of carbohydrate-rich foodstuffs in preference for diets richer in protein and fat content by patients may have a molecular basis in minimizing the risk

of hyperammonemia and citrullinemia associated with CD. Patients may therefore be “self-supplementing” their diets in a manner to minimize potentially pathological phenotypes associated with CD (Okamoto et al. 2021). Optimizing dietary protocols for the stages of CD remains an active area of research.

Additional treatments, including gene therapies, are being explored in these models (also reviewed in Vuković et al. 2024). González-Moreno *et al.* used the *Ctrn*^{-/-} mouse to show that aralar can functionally replace citrin, and therefore could serve as a potential gene therapy in CD (González-Moreno et al. 2023). Primary hepatocytes from *Ctrn*^{-/-} mice showed normalized cytosolic NADH/NAD⁺ ratios when transfected with exogenous aralar, whilst hepatic mitochondria expressing exogenous aralar under a liver-specific promoter showed a small increase in the activity of the MAS, as measured by NADH decay rate in isolated mitochondria (González-Moreno et al. 2023). This approach provides a potential therapeutic route by which aralar could replace the mutated citrin protein in CD. However, whether aralar can ameliorate any of the pathological symptoms of CD remains undetermined, as does the interplay between exogenous aralar overexpression when residual citrin activity is present, as is the case of some missense variants in CD.

As an alternative approach to treating citrin deficiency, codon-optimized human citrin (hCitrin) mRNA encased in lipid nanoparticles (LNPs) has been shown to achieve a small but significant increase in hepatic citrin expression (2-5% of wild-type) in the *Ctrn*^{-/-} mouse 24 hours following intravenous administration (Cao et al. 2019). Furthermore, double-KO mice receiving three weekly intravenous injections of hCitrin-mRNA-LNPs showed an increased preference for voluntary sucrose consumption, indicative of a reduction of the canonical aversion to carbohydrate-rich foods associated CD (Cao et al. 2019). Following an oral sucrose challenge, hCitrin-mRNA-treated double-KO mice further showed hepatic citrulline and plasma ammonium concentrations lower than those of double-KO mice treated with a

control mRNA, with concentrations of these metabolites maintained at levels similar to those measured in wild-type mice (Cao et al. 2019). Although it is still unclear how such a low expression of citrin can ameliorate the clinical phenotype, these results suggest that mRNA therapy, delivered via LNPs, may be a promising avenue to explore as a treatment strategy for CD. Several questions remain, such as which levels of citrin expression would be required to reverse the disease phenotype at different stages. Another question is whether introducing a full-length protein transcript into patients with severe truncations would result in immunological reactions.

Despite the clear outstanding questions, clinical guidelines for the management and treatment of CD are comprehensively evaluated in several recent reviews (Okano et al. 2019; Hayasaka 2023; Inui et al. 2024).

6. Conclusion

CD represents a complex metabolic disorder, encompassing a wide range of genetic variants that impact key metabolic processes, particularly in the liver. The variable clinical presentations of the disease, spanning from neonatal intrahepatic cholestasis to adult-onset citrullinemia type II, underscore the intricacy of the role of citrin in cellular metabolism and the challenges inherent in devising targeted therapeutic strategies. Whilst progress has been made in elucidating the structural and functional implications of various pathogenic variants, the precise mechanisms by which these variants disrupt metabolic homeostasis remain incompletely understood. Refined preclinical models, that more accurately mimicking the human pathology at different disease stages, are required to elucidate the molecular mechanisms underlying CD. Dietary modifications, gene therapy, and mRNA-based treatments offer promising avenues, but in order to take advantage of these technologies, reliable disease models will also be key. Addressing these gaps will be crucial for transforming

our understanding of CD into effective, patient-centered treatments that can improve quality of life and long-term outcomes significantly for those affected by this challenging disorder.

Table 1. Recently identified novel pathogenic variants.

Exon	Genotype	Protein prediction	Reference
Exon 3	c.173_174delTG	Val58fs81X	(Bölsterli et al. 2022)
Intron 8	c.848+1G>T	Arg284fsX	(Bölsterli et al. 2022)
Exon 11	c.1173T>G	Tyr391X	(Bölsterli et al. 2022)
Exon 13	c.1307_1308delinsAA	Gly436Glu	(Bölsterli et al. 2022)
Exon 16	c.1629dup	Ile544fs24X	(Bölsterli et al. 2022)
Exon 7	c.674C>CA	Ser22X	(Kim and Jung 2022)
Intron 8	c.848 + 6T > C	Arg284fsX	(Sachs et al. 2023)
Exon 11	c.1043C>T	Pro348Leu	(Sun et al. 2023)
Exon 12	c.1216dupG	Ala406fs418X	(Sun et al. 2023)
Exon 3	c.135G>C	Leu45Phe	(Sun et al. 2023)
Intron 2 Exon 3	c.70-63_133del	Try24fs34X	(Nguyen et al. 2023)
Exon 18	c.[1956C>A;1962del]	[Gln652Lys;Phe654Lfs45X]	(Nguyen et al. 2023)
Exon 15	c.1591G > A	Gly531Ser	(Baskar et al. 2023)
Exon 14	c.1402C>T	Val468Leu	(Jiang et al. 2023)
Exon 15	c.1474C>T	Arg492Trp	(Hahn et al. 2024)

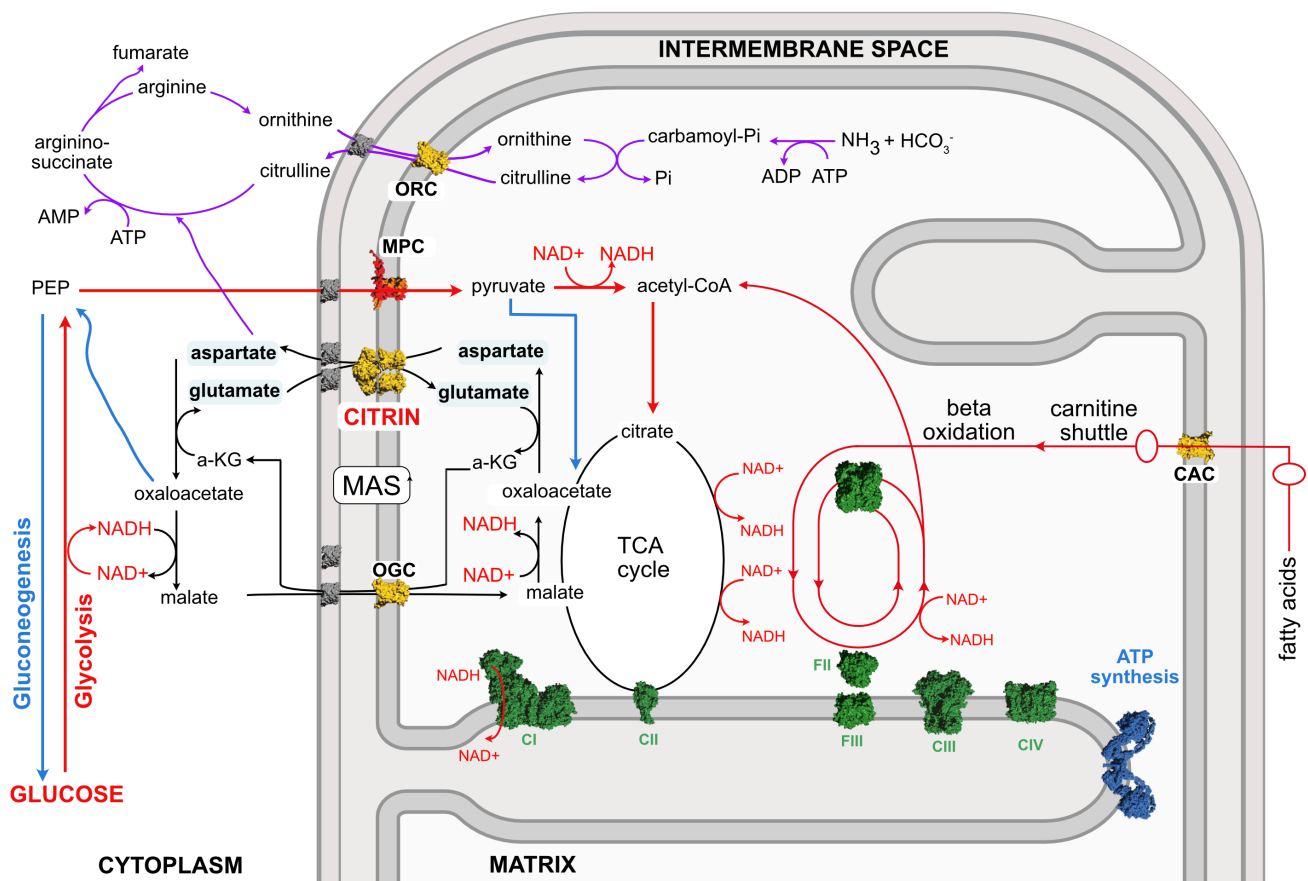


Figure 1. Metabolic pathways related to citrin function

Schematic representation of the malate-aspartate shuttle (black), glycolysis (red), tricarboxylic acid (TCA) cycle (black), gluconeogenesis (blue), ammonia fixation/urea cycle (purple) and fatty acid oxidation (red). Citrin is shown in yellow together with the oxoglutarate carrier (OGC), ornithine carrier (ORC) and the carnitine/acylcarnitine carrier (CAC). The mitochondrial pyruvate carrier heterodimer (MPC) is shown in red/orange. The respiratory chain complexes 1 to 4 (CI–CIV), acyl-CoA dehydrogenases (FI), electron transfer flavoprotein (FII), and ETF-ubiquinone oxidoreductase (FIII) are shown in green and the dimer of ATP synthase in blue. PEP stands for phosphoenolpyruvate and NAD for nicotinamide adenine dinucleotide. NAD⁺ and NADH are highlighted in red.

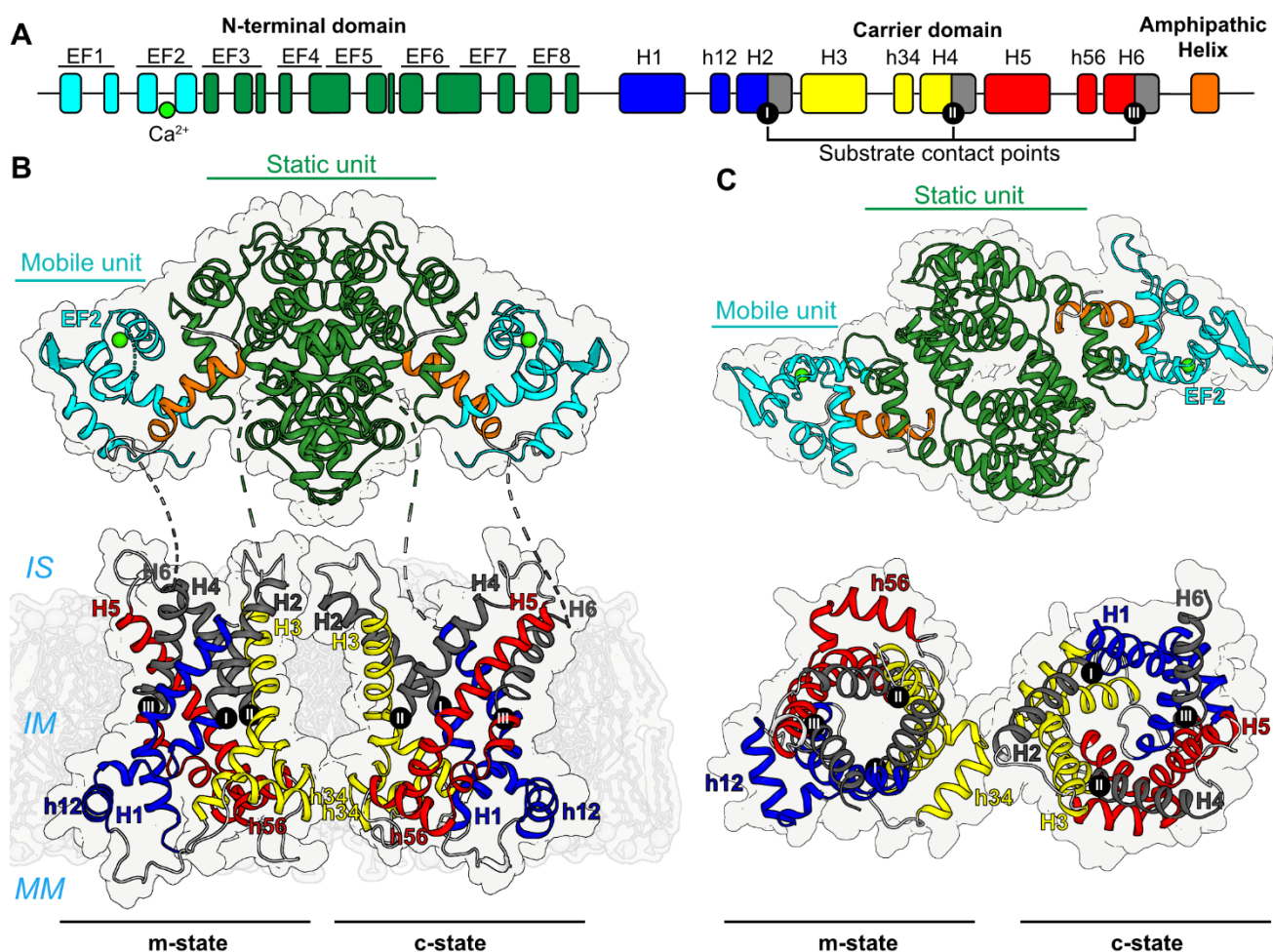


Figure 2. Structural model of citrin

(A) Domain structure of citrin. In the N-terminal domain the EF-hand-containing mobile unit and static unit are shown in cyan and green, respectively. The calcium bound in EF-hand 2 is shown as a green sphere. In the carrier domain, the three core elements are shown in blue, yellow and red and the three gate elements in gray. The C-terminal domain is shown in orange. (B-C) Lateral and cytoplasmic view of the citrin structural model in the same color scheme as in (A).

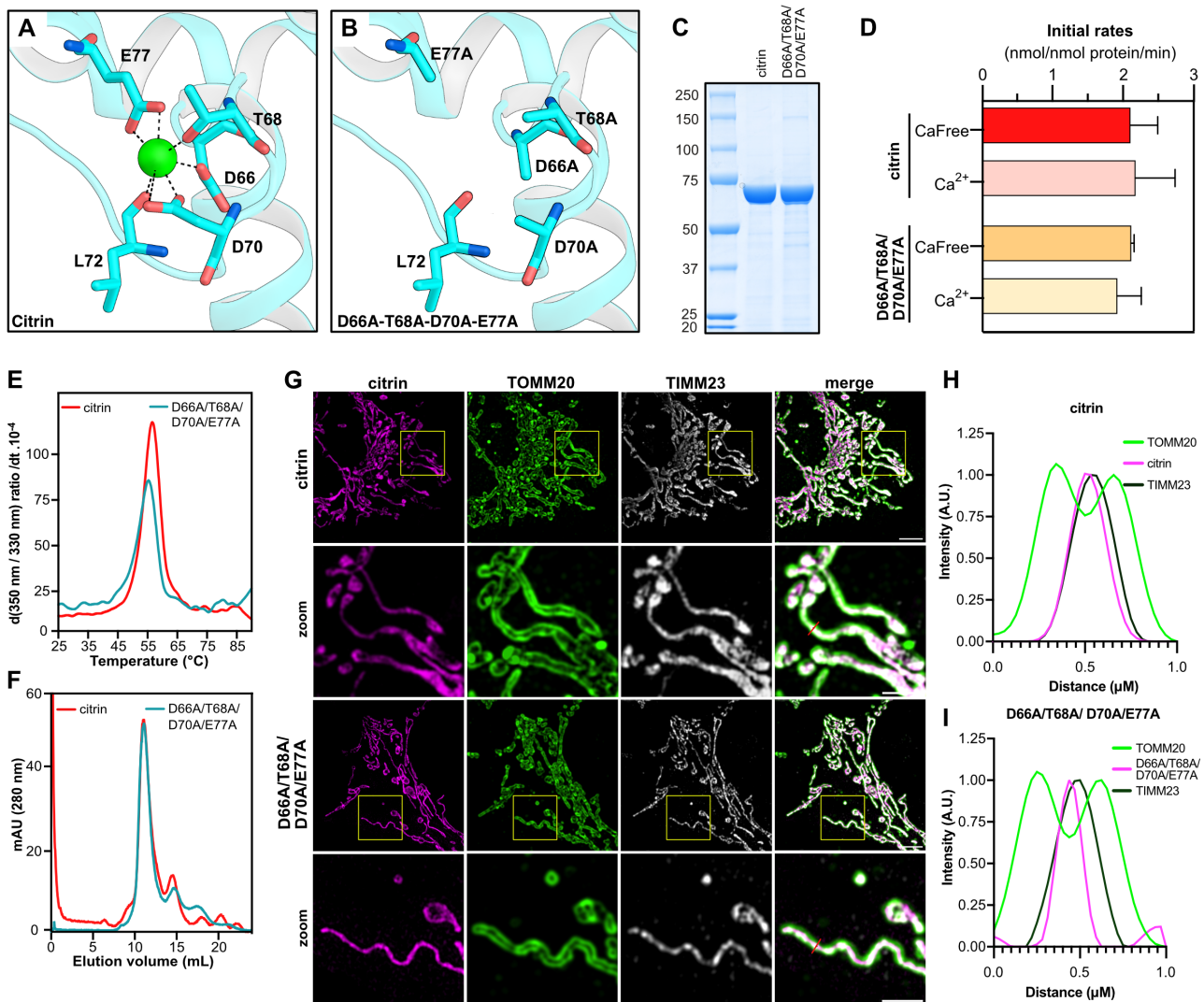
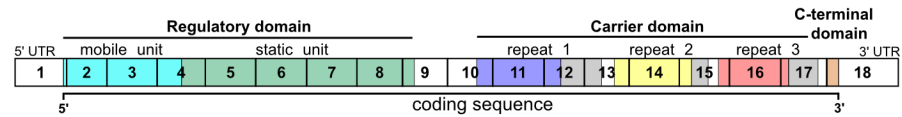


Figure 3. The transport activity of reconstituted citrin is not regulated by calcium

(A) The calcium-binding site of citrin in the presence of calcium (green sphere). (B) The mutated calcium binding site in the D66A/T68A/D70A/E77A mutant. (C) Purification of wild-type citrin and the D66A/T68A/D70A/E77A mutant for *in vitro* assays. (D) Initial rates of substrate transport for wild-type citrin and D66A/T68A/D70A/E77A, in the presence or absence of calcium. (E) Thermostability analysis for wild-type citrin and D66A/T68A/D70A/E77A. (F) Oligomeric state of wild-type citrin and D66A/T68A/D70A/E77A via size exclusion chromatography. (G) Super-resolution mitochondrial imaging of a HAP1 cell line, where both endogenous citrin and aralar are knocked-out, expressing transiently wild-type citrin or D66A/T68A/D70A/E77A. (H) Line-scan analysis of the relative fluorescence

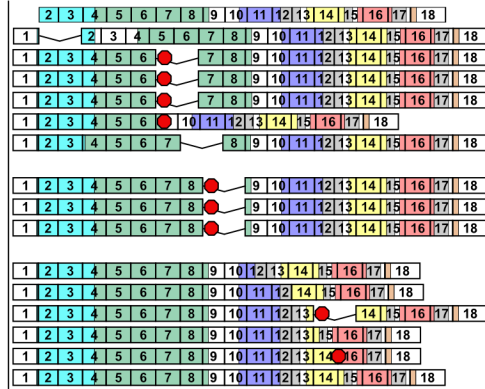
intensity of TOMM20, citrin or D66A/T68A/D70A/E77A and TIMM23 staining for the selected area shown in (F). The Figure has been adapted from Tavoulari et al. 2024.



A Splicing site mutations

c.15G>A (Ex1-1G>A)
g.16-2A>T (IVS1-2A>T)
c.615+1G>C (IVS6+1G>C)
c.615+1G>A (IVS6+1G>A)
c.615+5G>A (IVS6+5G>A)
r.616_848del (Ex7-1G>A)
c.755-2A>G (IVS7-2A>G)
c.754+6T>G (IVS7+6T>G)
c.848+3A>C (IVS8+3A>C)
c.848+1G>T (IVS8+1G>T)
c.848+6T>C (IVS8+6T>C)
c.933G>A (Ex9-1G>A)
c.1019_1177del (IVS11+1G>A)
c.1231_1311del (IVS13+1G>A)
c.1311+2T>G (IVS13+2T>G)
c.1452+1G>A
c.1453_1591del (IVS15+1G>T)
c.1841+3_1841+4delAA

Expected effect on the mRNA



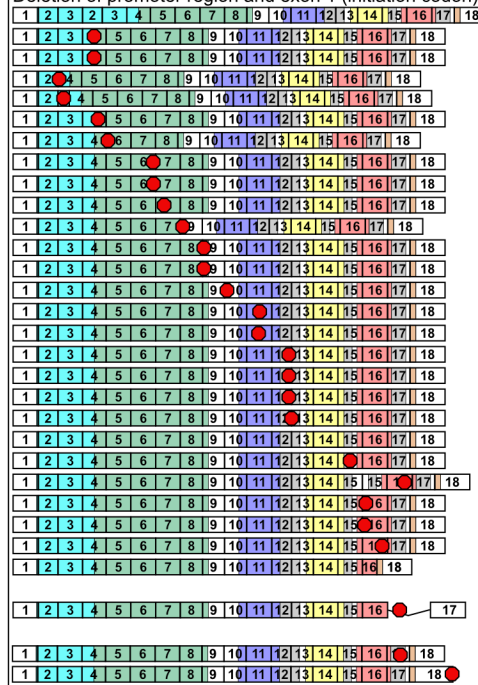
P. mutation

Unclear
Unclear
A206fs212X
A206fs212X¹
A206fs212X¹
A206fs213X
Unclear
K251fs254X¹
R284fs285X¹
R284fs285X¹
R284fs285X¹
Unclear
340_392del
411_437del
A448fs455X¹
438_484del
G485fs491X
Unclear

B Insertion/deletion mutations

c.-3251_c.15+18443del21709bp
r.16_212dup (Ex2_3dup)
c.172_173delGT
c.173_174delTG
c.70_212del
c.70-63_133del
c.265delG
r.329_468del (IVS4ins6kb)
c.478delC
c.495delA
c.674_675insA
c.755-1G>C
c.845_c.848+1delG
c.851_854delGTAT
c.933_c.933+1insGCAG
c.985_986insT
c.1706_1707delTA
c.1092_1095delT
c.1146delA
c.1192_1193delT
c.1216dupG
c.1307_1308delinsAA
c.1374 or 1375delG
c.1453_1591dup
c.1610_1612delTAGinsAT
c.1629dup
c.1638_1660dup
c.1666_1842-31del
(Ex16+74_IVS17-32del516)
c.1750+72_1751-4dup17ins
(IVS16ins3kb)
c.1799-1800insA
c.[1956C>A;1962del]

Deletion of promoter region and exon 1 (initiation codon)



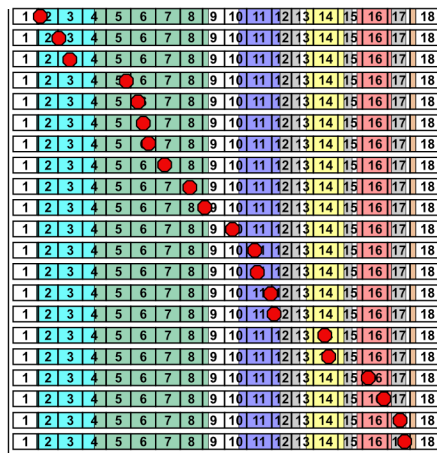
-
Unclear
V58fs81X
V58fs81X
Y24fs34X
Y24fs34X
D89fs94X
E110fs127X
L160fs196X
Q165fs195X
S225X
252fs269X
D283fs285X
R284fs286X
A312fs317X
A329fs372X
S331fs363X
F365fs407X
R383fs407X
L398fs407X
A406fs418X
G436N
A459fs507X
M532fs560X
L537fs538X
I544fs597X
A554fs570X
Q556fs565X

A584fs585X

Y600X
[N652K;
F654Lfs45X]

C Nonsense mutations

c.46G>T
c.72T>A
c.127C>T
c.448G>T
c.475C>T
c.493C>T
c.550C>T
c.674C>A
c.775C>T
c.847G>T
c.955C>T
c.1063C>T
c.1078C>T
c.1173T>G
c.1189C>T
c.1381G>T
c.1399C>T
c.1645C>T
c.1736G>A
c.1813C>T
c.1801G>T



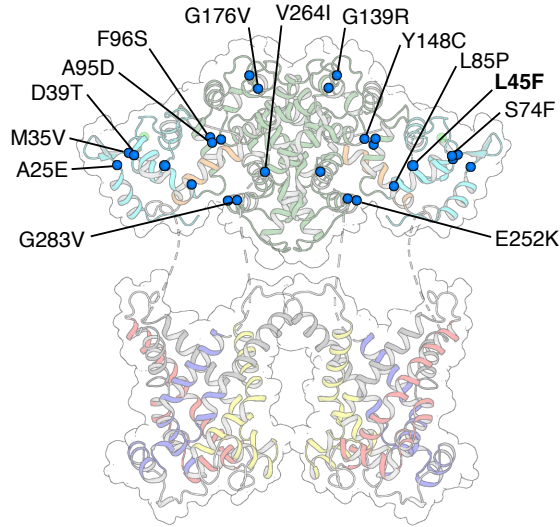
E16X
Y24X
R43X
E150X
Q159X
Q165X
R184X
S225X
Q259X
G283X
R319X
R355X
R360X
Y391X
Q397X
E461X
R467X
Q549X
W579X
E601X
R605X

¹ Splicing effect predicted using Human Splicing Finder (GENOMNIS SAS)

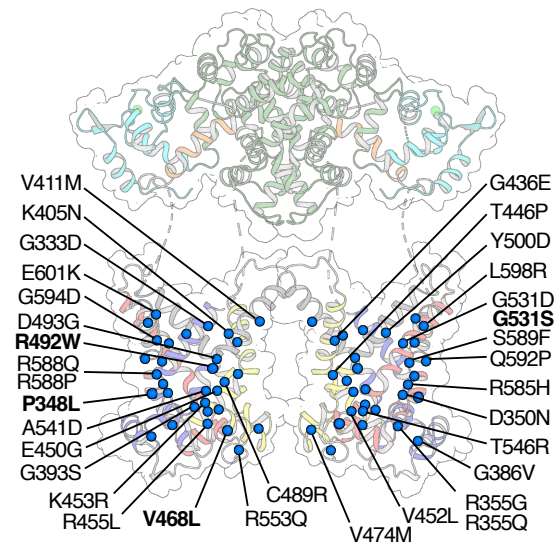
● STOP codon

Figure 4. The wide range of pathogenic mutations of citrin. Positions of splicing, deletion, insertion, and nonsense variants reported in citrin deficiency, shown in the mRNA product (NM_014251.3). The red dots indicate the position of the substitutions. The color coding of the exons corresponds to the protein domains potentially translated, as shown in Figure 2. The potential translated protein outcome is indicated in the last column. The most recent variants (as in Table 1) are shown in bold. The effects of the splicing site variants were predicted using Human Splicing Finder (Desmet et al. 2009).

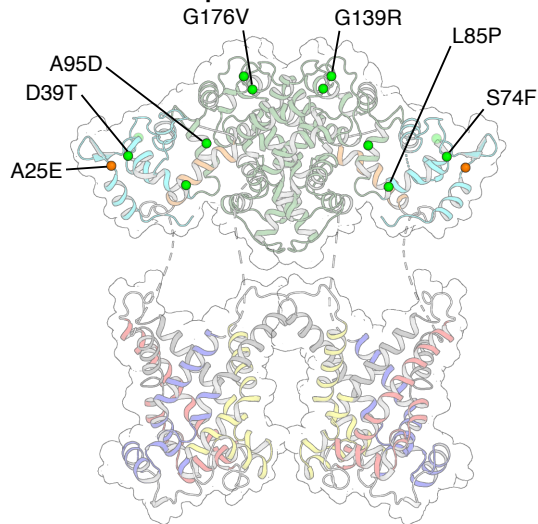
A Missense mutations: N-terminal domain



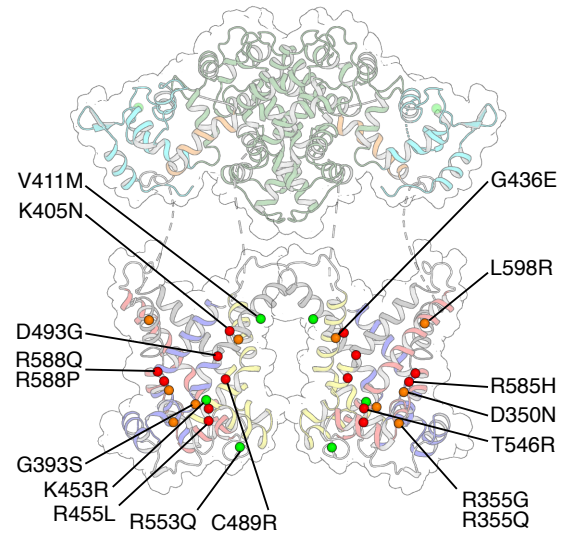
B Missense mutations: carrier domain



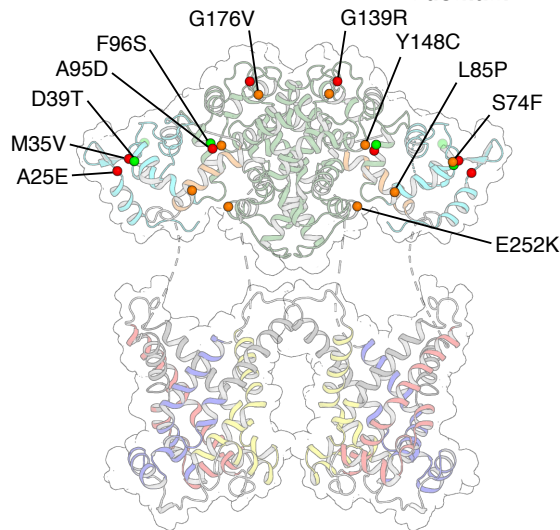
C Effect on transport: N-terminal domain



D Effect on transport: carrier domain



E Effect on localisation: N-terminal domain



F Effect on localisation: carrier domain

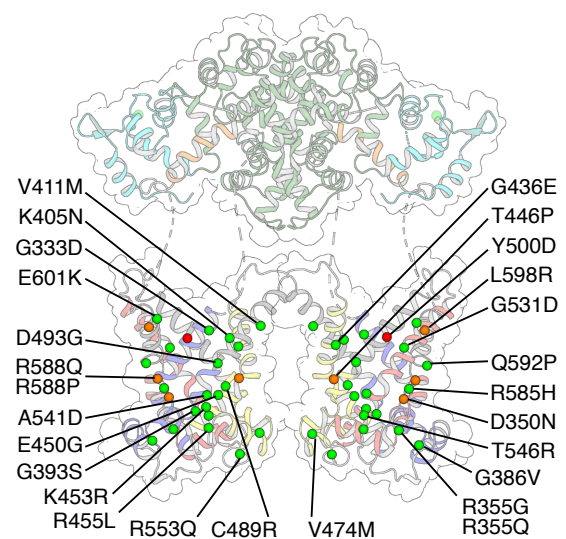
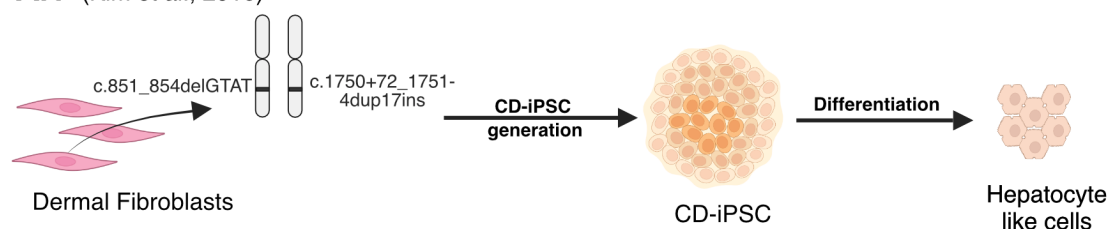


Figure 5. Effects of citrin missense variants on activity and mitochondrial localization.

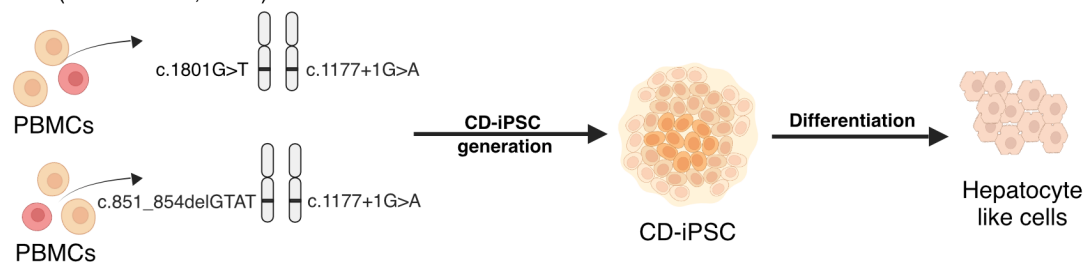
Positions of citrin missense variants in **(A)** the N-terminal domain and **(B)** the carrier domain are highlighted in blue spheres. Novel variants most recently reported are shown in bold (also see **Table 1**). **(C)** Effect of citrin N-terminal pathogenic variants on transport activity. **(D)** Effect of citrin carrier domain pathogenic variants on transport activity. For **(C)** and **(D)**, residues with more than 50% activity are shown in green spheres, with 5–50% activity with orange and with less than 5% activity in red. **(E)** Effect of citrin N-terminal pathogenic variants on mitochondrial localization. **(F)** Effect of citrin carrier domain pathogenic variants on mitochondrial localization. In **(E)** and **(F)**, mutants are hierarchically clustered for having primarily mitochondrial distribution (green spheres), mitochondrial distribution mixed with high levels of non-mitochondrial (orange spheres) or primarily non-mitochondrial distribution (red spheres).

A Patient derived iPSCs

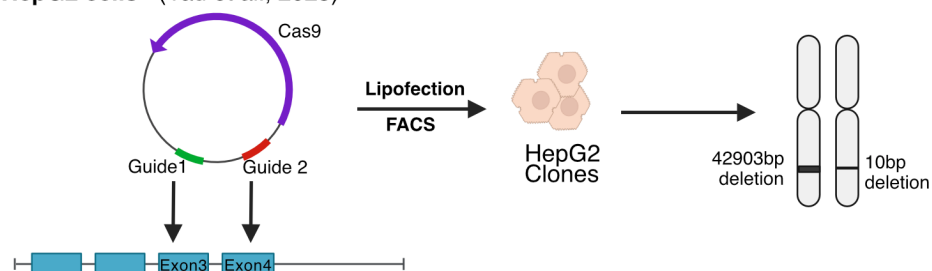
A.1 (Kim *et al.*, 2016)



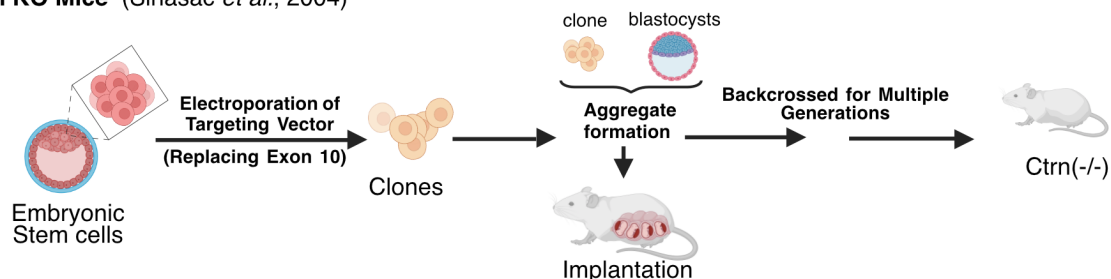
A.2 (Okano *et al.*, 2024)



B Citrin KO HepG2 cells (Yau *et al.*, 2023)



C Citrin KO Mice (Sinascac *et al.*, 2004)



D Citrin/mGPD double KO Mouse (Saheki *et al.*, 2007)

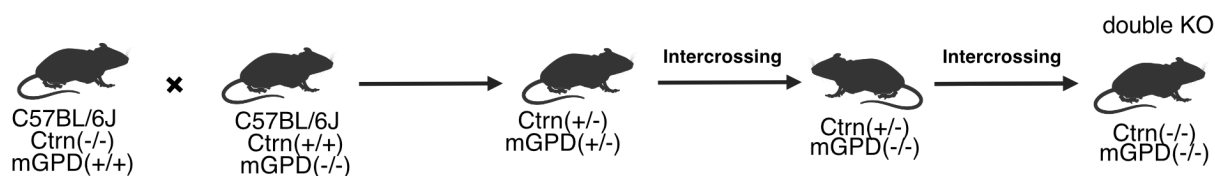


Figure 6. Generation procedures of cellular and mice models of citrin deficiency. With the exception of patient-derived induced Pluripotent Stem Cells (iPSCs), model design efforts have focused on knocking-out the citrin gene. PBMCs: Peripheral Blood Mononuclear Cells, KO: Knock Out, FACS:

Fluorescence-Activated Cell Sorting, Ctrn: Citrin, mGPD: mitochondrial Glycerol-3-Phosphate Dehydrogenase. Image created with BioRender.com.

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