

# Mitochondrial transport and metabolism of the vitamin B-derived cofactors thiamine pyrophosphate, coenzyme A, FAD and NAD<sup>+</sup>, and related diseases: A review

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## Abstract

Multiple mitochondrial matrix enzymes playing key roles in metabolism require cofactors for their action. Due to the high impermeability of the mitochondrial inner membrane, these cofactors need to be synthesized within the mitochondria or be imported, themselves or one of their precursors, into the organelles. Transporters belonging to the protein family of mitochondrial carriers have been identified to transport the coenzymes: thiamine pyrophosphate, coenzyme A, FAD and NAD<sup>+</sup>, which are all structurally similar to nucleotides and derived from different B-vitamins. These mitochondrial cofactors bind more or less tightly to their enzymes and, after having been involved in a specific reaction step, are regenerated, spontaneously or by other enzymes, to return to their active form, ready for the next catalysis round. Disease-causing mutations in the mitochondrial cofactor carrier genes compromise not only the transport reaction but also the activity of all mitochondrial enzymes using that particular cofactor and the metabolic pathways in which the cofactor-dependent enzymes are involved. The mitochondrial transport, metabolism

**Abbreviations:** ALS, acetolactate synthase; BCAA, branched-chain amino acid; BCKDH(c), branched-chain  $\alpha$ -ketoacid dehydrogenase (complex); CHO, chinese hamster ovary; CoA, coenzyme A; COASY, coenzyme A synthase; EPRA, expression, purification, reconstitution and transport assay; FAD, flavin adenine dinucleotide; FADS, flavin adenine dinucleotide synthase; FMN, flavin mononucleotide; MADD, multiple acyl-CoA dehydrogenase deficiencies; MC, mitochondrial carrier; NAAD, nicotinic acid adenine dinucleotide; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; NAMN, nicotinate mononucleotide; NUDIX, nucleoside diphosphate-linked moiety X; OADH(c), 2-oxoadipate dehydrogenase (complex); OGDH(c), oxoglutarate dehydrogenase (complex); PANK, pantothenate kinase; PAP, adenosine 3',5'-diphosphate; PDC, pyruvate decarboxylase; PDH(c), pyruvate dehydrogenase (complex); RDA, recommended daily allowance; SAM, S-adenosylmethionine; SM, synthetic minimal; TCA, tricarboxylic acid; ThMP, thiamine monophosphate; ThPP, thiamine pyrophosphate; ThTP, thiamine triphosphate; THTPA, thiamine triphosphatase; TKT, transketolase; TPC, thiamine pyrophosphate carrier; TPK1, thiamine pyrophosphatase 1.

Ferdinando Palmieri and Magnus Monné contributed equally to this work.

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and diseases of the cofactors thiamine pyrophosphate, coenzyme A, FAD and NAD<sup>+</sup> are the focus of this review.

#### KEYWORDS

coenzyme, coenzyme A, flavin adenine dinucleotide, mitochondria, mitochondrial carrier family SLC25, nicotinamide adenine dinucleotide, thiamine pyrophosphate

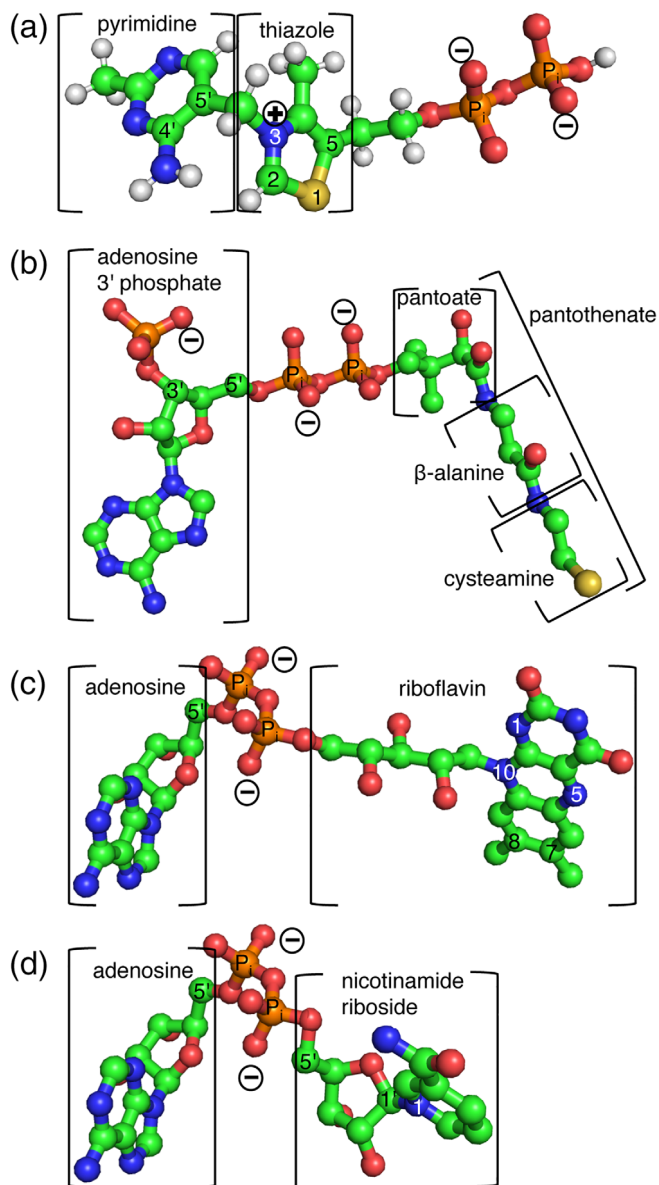
## 1 | INTRODUCTION

Many enzymes use different non-proteic cofactors, such as metal ions and organic coenzymes, that have an important role in catalysis, for example, by activating the substrates, transferring chemical groups and donating/accepting electrons or protons. Whereas many microorganisms and plants have biosynthesis capabilities for the coenzymes, animals depend on diet absorption of most of them or their precursors, in the form of vitamins or essential amino acids. Subsequently, the cofactors or their precursors and derivatives are distributed, taken up by various tissue cell types, and the precursors are modified to assume their active forms. Many of these cofactors are required in the mitochondrial matrix for enzymatic activities of vital importance in central metabolism. Some of the mitochondrial cofactors are synthesized or assembled, at least partly, in mitochondria, such as ubiquinone, lipolate, iron-sulfur clusters and heme.<sup>1-4</sup> Others have to be imported into mitochondria, such as thiamine pyrophosphate (ThPP), FAD, NAD<sup>+</sup>, CoA, pyridoxal phosphate, biotin, tetrahydrofolate and cobalamin, that are all B-type vitamins or their derivatives: B1 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B9 (folic acid or folate) and B12 (cobalamins), respectively. Until now, mitochondrial coenzyme transporters have been identified for ThPP, CoA, FAD (and folate) and NAD<sup>+</sup>, and they all belong to the mitochondrial carrier protein family.<sup>5-13</sup>

Mitochondrial carriers (MCs or SLC25 in higher animals) are found in all eukaryotes in variable numbers: 35 in *Saccharomyces cerevisiae*, 53 in *Homo sapiens* and 58 in *Arabidopsis thaliana*.<sup>14-17</sup> They all have the N- and C-termini exposed towards the cytosolic side of the mitochondrial membrane and a triple-repeated sequence of about 100 amino acids, which contains two transmembrane segments that are linked by a signature motif sequence PX[DE]XX[KR]X[KR]X<sub>20-30</sub>[DE]GXXXX[WYF][KR]G (PROSITE PS50920, PFAM PF00153 and IPR00193).<sup>18-23</sup> With a few exceptions, MCs are localized to the inner membrane of mitochondria, where their about 300-residue structure is arranged in a barrel of six-transmembrane  $\alpha$ -helices (H1-H6) around a central substrate translocation pore with gates towards the

intermembrane space (cytoplasmic c-gate) and matrix (m-gate) sides of the membrane.<sup>24-26</sup> With this structural arrangement, MCs transport their specific substrates across the mitochondrial inner membrane with a similar mechanism (named “single binding center-gating”,<sup>27</sup> “ping pong”<sup>20,28-30</sup> and recently “alternating access transport” mechanism<sup>25</sup>) by binding the substrates in the central part of the pore and alternatively opening and closing the gates for entry and exit of the transported solute on the opposite side of the membrane.<sup>23,26,31-35</sup> Besides the above-mentioned cofactors, different MCs have been shown to transport specific substrates, for example, phosphate, sulfate, 2-oxoglutarate, 2-oxoadipate, malate, citrate, aspartate, glutamate, ornithine, arginine and methylarginines, branch chain amino acids (BCAA), purine and pyrimidine nucleotides and deoxynucleotides.<sup>36-43</sup> In phylogenetic analysis, MCs are divided into three main clusters that coincide with the type of substrate they transport: nucleotides, carboxylated metabolites and amino acids.<sup>32,44</sup> It has been shown that there is a co-variation between the type of substrate and the MC residues in the central translocation pore approximately halfway between the two gates on H4 in the so-called contact point II (motifs G[IVLM] for nucleotides, R[QHNT] for carboxylated metabolites, and R[DE] for amino acids), which are part of the substrate-binding site.<sup>45-48</sup> The MCs for B-vitamin derived cofactors all belong to the nucleotide-transporting carriers, both considering the contact II residues and their clustering in phylogenetic analysis, with the exception of the animal NAD<sup>+</sup> transporter SLC25A51.<sup>32,36,49</sup> The fact that they belong to nucleotide-transporting carriers is not surprising given that ThPP, CoA, FAD and NAD<sup>+</sup> all enclose nucleotide or nucleotide-like moieties (Figure 1).

The substrates of many human, *S. cerevisiae* and *A. thaliana* MCs have been identified through an approach comprising their recombinant expression, purification and reconstitution into liposomes that are used in transport assays (EPRA method).<sup>50,51,36</sup> Besides determining the substrate specificity, the transport assays have been used to identify mitochondrial carrier inhibitors, characterize their kinetic parameters and their modes of transport, that is, uniport, symport and antiport, of which the latter is most common,<sup>52</sup> quantify the importance of



**FIGURE 1** Coenzymes represented in ball-and-stick. (a) Thiamine pyrophosphate, (b) coenzyme A, (c) FAD and (d)  $\text{NAD}^+$ . These cofactors have nucleotide and nucleotide-like moieties: coenzyme A, FAD and  $\text{NAD}^+$  contain an adenosine, whereas ThPP lacks the ribose but contains a pyrimidine. All four of them contain a pyrophosphate

each residue along their structure as well as to hypothesize their physiological roles.<sup>16,53–60</sup> To understand the physiological role of MCs, their characterized transport function is integrated with the knowledge of the identified substrates in metabolism, position in metabolic pathways, subcellular location of the substrates and enzymes using them. Many important clues also come from human diseases caused by mutations in MCs.<sup>61–64</sup> By using this kind of analysis, the importance for mitochondrial and general metabolism of MC-mediated transport

of the B-vitamin derived cofactors thiamine pyrophosphate, coenzyme A, FAD and  $\text{NAD}^+$  is demonstrated in this review.

## 2 | THIAMINE PYROPHOSPHATE

Thiamine pyrophosphate (ThPP), also known as thiamine diphosphate, is an essential cofactor that is required in the general metabolism of all organisms. It is a derivative of thiamine (vitamin B1 or aneurin) that consists of a six-membered pyrimidine ring linked through a methylene to a five-membered thiazole ring, in which the carbon-5 substitution may be covalently linked to one, two or three phosphates, forming thiamine monophosphate (ThMP), diphosphate (i.e., ThPP, Figure 1a) or triphosphate (ThTP), respectively. ThPP is the active cofactor form and corresponds to about 80% of thiamine found in the human body as free thiamine, ThMP, ThPP, and ThTP.<sup>65</sup> When ThPP is bound to enzymes its phosphates are coordinated by  $\text{Mg}^{2+}$  and function as a handle to keep the cofactor in the right position in the active site. The reactivity of ThPP depends on the activation of carbon-2 (C2) between the nitrogen and the sulfur in the thiazole ring that, upon donation of a hydrogen ion, forms a carbanion. This acts as a nucleophile attacking a carbonyl-carbon of the first substrate.<sup>66</sup> In most reactions, the C2-proton is donated to the N'4 amino group of the pyrimidine moiety, being in proximity in the *v*-conformation of ThPP (Figure 1a). The metastable reaction intermediate formed by a covalent bond, between the thiazole-C2 of ThPP and the carbonyl-carbon of the first substrate, is then resolved by reacting with the second substrate and giving rise to the subsequent formation of the products. In this second “half”-reaction ThPP thiazole C2 gets re-protonated and returns to its initial state (Figure 1a). Although ThPP-dependent enzymes have different 2-oxocarboxylate or 2-hydroxycarboxylate substrates and catalyse diverse reactions that break or make C–C, C–N, C–O, C–H, and C–S bonds, from a mechanistic point of view their reactions may all be categorized into decarboxylations and non-decarboxylations (i.e., transferase or transketolase reactions).<sup>67</sup> Active site residues and additional cofactors or prosthetic groups further contribute to ThPP reactivity, catalysis and substrate binding.

### 2.1 | Biosynthesis and distribution of ThPP

Thiamine, which is necessary for ThPP formation, is synthesized *de novo* in most prokaryotes and some

eukaryotes, such as fungi and plants, but not in animals. The biosynthesis of thiamine requires different enzymes in different species; however, the thiazole and pyrimidine moieties are always synthesized by separate pathways before they are conjugated, as described in detail in several reviews.<sup>68–70</sup> In prokaryotes, the thiazole and pyrimidine rings are linked by thiamine phosphate synthase to form ThMP. ThMP is subsequently phosphorylated by thiamine phosphate kinase to form ThPP. In bacteria, cytoplasmic ThPP is used directly by, for example, transketolase, and other enzymes, that in eukaryotes are located in mitochondria, such as the E1 components of pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and acetolactate synthase. In yeast too, the thiazole ring and the pyrimidine ring are unified by thiamine phosphate synthase to ThMP.<sup>68</sup> ThMP is then dephosphorylated to thiamine, which is diphosphorylated to produce ThPP. Whereas the yeast enzymes involved in ThMP biosynthesis are all located in the cytoplasm, in plants ThMP production takes place in chloroplasts and only the last two reactions needed to convert ThMP into ThPP take place in the cytoplasm, as in yeast.<sup>70</sup> In all thiamine-producing organisms additional enzymes taking part in thiamine salvage pathways have been identified, that is, various bacteria have several enzymes for thiazole, pyrimidine and thiamine recuperation<sup>69</sup> as well as active and facilitated transporters for thiamine uptake<sup>71,72</sup>; yeast may import thiamine from the environment by Thi7p (Thi10p)<sup>73</sup> and convert it into ThPP<sup>68</sup>; Arabidopsis too have various thiamine salvage pathways that bypass the energetic costs of de novo biosynthesis of ThPP.<sup>70</sup> In thiamine-synthesizing organisms, the expression levels of several enzymes involved in ThPP biosynthesis are regulated by feed-back inhibition mechanisms involving mRNA riboswitches, which sense increased concentrations of ThPP leading to reduced transcription and translation.<sup>74</sup>

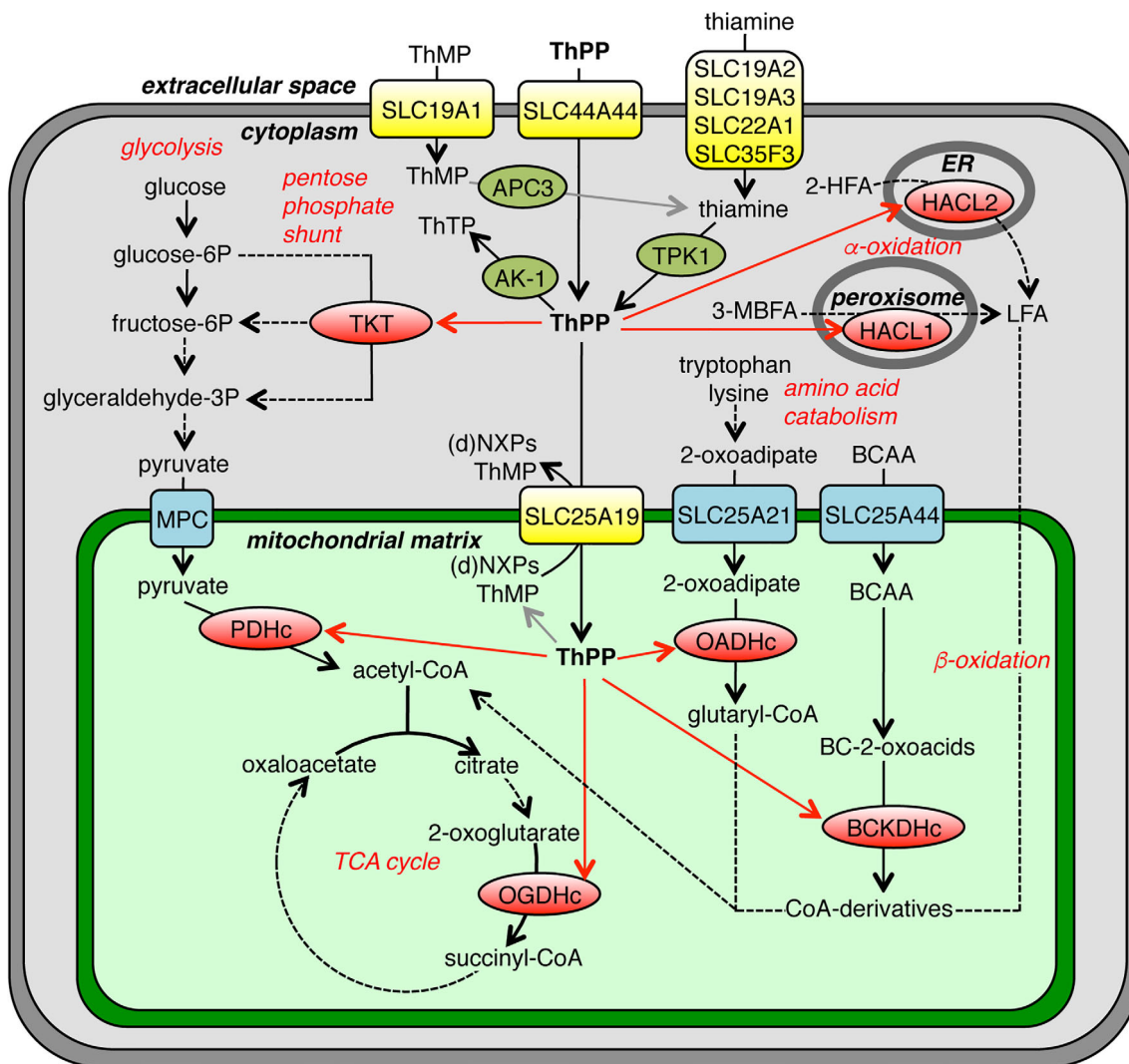
Animals, which are all unable of ThPP biosynthesis, rely on the absorption of the essential vitamin thiamine and its derivatives from the diet (RDA about 1.2 mg for adults), mainly depending on plant-produced thiamine and animal/gut microflora-synthesized ThPP.<sup>75</sup> In humans, it has been estimated that about 95% of the thiamine intake (normally <2 mg) is absorbed by the body. Most of thiamine absorption is thought to take place in the proximal part of the small intestine by the major high-affinity thiamine transporters SLC19A2 (ThTR-1)<sup>76,77</sup> and SLC19A3 (ThTR-2),<sup>78,79</sup> which have about 48% sequence identity and belong to the 12 transmembrane  $\alpha$ -helix major facilitator family of membrane transporters. Furthermore, a homolog of these two proteins, SLC19A1, which is mainly a folate transporter, may contribute to the transport of ThMP in the gastrointestinal

tract to some extent.<sup>80</sup> In colon, thiamine may also be taken up by the unspecific organic cation transporters (OCTs), and ThPP specifically by SLC44A4.<sup>81,82</sup> The thiamine and thiamine derivatives absorbed across the apical membrane of intestinal epithelial cells are exported through the basolateral membrane into the blood, probably via some of the above-mentioned transporters. Subsequently, thiamine is distributed throughout the body by erythrocytes, which assimilate >80% of the total blood thiamine (total body content of thiamine and its derivatives about 25–30 mg) and is found especially in skeletal muscles, heart, brain, liver and kidneys.<sup>83</sup> To import thiamine or its derivatives, cells are thought to use several different transporters, many of which are expressed in numerous cell types at different levels; these transporters are SLC19A2 (highly expressed in skeletal muscle),<sup>76,77</sup> SLC19A3 (adipose tissue, breast and placenta), SLC19A1 (blood and brain), SLC35F3 (brain)<sup>84</sup> and SLC22A1 (OCT1, liver)<sup>85</sup> (Figure 2). Excess thiamine from intestinal absorption and cell secretion (9–18 days turnover time) is eliminated from circulation mainly through urine by glomerular filtration and in minor quantities in faeces and sweat in the form of free thiamine, ThMP, ThPP or more than 20 different degradation intermediates.<sup>75,86,87</sup>

Once imported into the cytoplasm of animal cells, thiamine is transformed into ThPP by thiamine pyrophosphokinase (TPK1, thiamine pyrophosphotransferase or thiamine diphosphokinase) by transfer of a pyrophosphate from ATP onto thiamine (Figure 2).<sup>88</sup> Most probably cell-imported ThMP is converted into free thiamine and then to ThPP as in yeast and plants. ThPP is rapidly bound to proteins or imported into organelles leaving only a small cytoplasmic fraction free, which has a fast turnover although not being very reactive by itself.<sup>89</sup> In most eukaryotic cells, cytosolic ThPP is a cofactor of transketolase (TKT), which is an enzyme of the pentose phosphate shunt.<sup>90</sup> Cytosolic ThPP may also be used by adenylate kinase 1 (AK-1) to form ThTP, which corresponds to approximately 5–10% of total thiamine in the human body and has a regulatory role activating the chloride channels and acting as a phosphate donor in some phosphorylation reactions.<sup>89,91,92</sup> Moreover, in plant and yeast, cytosolic ThPP is used as a cofactor by pyruvate decarboxylases (PDC) for the non-oxidative conversion of pyruvate (and other 2-oxo acids) to acetaldehydes (and other aldehydes) and CO<sub>2</sub>.<sup>93,94</sup> The existence of other ThPP-dependent enzymes and riboswitches in many non-mammalian organisms have made them interesting targets for drugs/pesticides.<sup>95</sup>

In eukaryotic cells, as mentioned above, cytoplasmic ThPP is distributed into the subcellular compartments; in most human cells, about 50% of total thiamine is found





**FIGURE 2** Thiamine metabolism and transport in a typical human cell. The schematic overview shows thiamine/thiamine-derivative transporters (yellow), enzymes using ThPP as a cofactor (red), enzymes metabolizing ThPP (olive green), enzymatic reactions (black arrows), reactions with unidentified enzymes (grey arrows) and distribution (red arrows). 2-HFA, 2-hydroxy-fatty acids; 3-MBFA, 3-methyl-branched-chain fatty acids; AK-1, adenylate kinase 1; APC3, prostatic acid phosphatase; BC-2-oxoacids, branched-chain 2-oxoacids; BCAA, branched-chain amino acids; BCKDHc, branched-chain  $\alpha$ -ketoacid dehydrogenase; (d)NXP, (deoxy)nucleotides; HACL1 and HACL2, 2-hydroxyacyl-CoA lyase 1 and 2; LFA, long fatty acids; MPC, mitochondrial pyruvate carrier; OADHc, 2-oxoadipate dehydrogenase complex; OGDHc, 2-oxoglutarate dehydrogenase complex; P, phosphate; PDHc, pyruvate dehydrogenase complex; ThMP, thiamine monophosphate; ThPP, thiamine pyrophosphate; ThTP, thiamine triphosphate; TKT, transketolase; TPK1, thiamine pyrophosphokinase

in cytoplasm, 35% in mitochondria, 10% in nucleus and 5% in ER<sup>75</sup> although in rat brain a much higher thiamine level has been measured in mitochondria versus cytoplasm.<sup>96</sup> In fact, ThPP is known to be a cofactor not only of enzymatic reactions in cytoplasm but also in ER, peroxisomes and mitochondria (Figure 2) as well as in chloroplasts. In ER, ThPP is required for 2-hydroxyacyl-CoA lyase 2 (HACL2 or ILVBL) in the phytosphingosine (and other 2-hydroxy-fatty acids)  $\alpha$ -oxidation degradation pathway.<sup>97</sup> In animal and probably also in plant peroxisomes, ThPP is a coenzyme of 2-hydroxyacyl-CoA lyase 1 (HACL1), which catalyses the cleavage of phytanic acid

(and other 3-methyl branched fatty acids) in  $\alpha$ -oxidation.<sup>98</sup> Mitochondria require import of ThPP for the following enzymes: pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA which enters the tricarboxylic acid (TCA) cycle<sup>99</sup>; 2-oxoglutarate dehydrogenase (OGDH), an enzyme of the TCA cycle<sup>100</sup>; 2-oxoadipate dehydrogenase (OADH)<sup>101</sup>; branched-chain  $\alpha$ -ketoacid dehydrogenase (BCKDH in animals and plants), which is involved in the degradation of BCAA<sup>102</sup> (Figure 2); and acetolactate synthase (ALS in yeast), which is involved in BCAA biosynthesis. In plants, ThPP serves as a cofactor for chloroplastic pyruvate dehydrogenase to produce

acetyl-CoA and NADH for fatty acid synthesis, transketolase, an enzyme in the Calvin cycle, 2-deoxyxylulose 5-phosphate synthase (DXPS) for the biosynthesis of isoprenoids via the mevalonate-independent pathway, and aceto-hydroxyacid synthase (AHAS, similar to ALS) for BCAA production.<sup>70</sup> Whereas no transporters for ThPP or other forms of thiamine have been identified for their import into ER, peroxisomes or chloroplasts, mitochondrial ThPP transporters have been identified in yeast, insect, human and plants.

## 2.2 | Mitochondrial transporters of ThPP

In *S. cerevisiae*, a mitochondrial transporter for ThPP has been identified and named the ThPP carrier (Tpc1p), which is encoded by YGR096w and belongs to the MC family.<sup>7</sup> Tpc1p is responsible for the import of cytosol-synthesized ThPP into the mitochondrial matrix, where it is required as a cofactor for a few enzymes.

Several pieces of evidence from in vivo and in vitro experiments led to the annotation of this function to this carrier.<sup>7</sup> The localization of Tpc1p to the mitochondrial inner membrane was demonstrated by immunoblot of mitochondria and co-fractionation with an inner membrane protein marker.<sup>7</sup> The function of Tpc1p was especially associated with anaerobic metabolism in yeast, because of its higher expression levels on fermentable than on non-fermentable carbon sources. In fact, only on synthetic minimal media (SM) supplemented with fermentable carbon sources, the yeast *TPC1* knockout strain (*tpc1Δ*) displayed a requirement for thiamine or BCAA for growth. This finding was interpreted as an indication that Tpc1p was involved in mitochondrial ThPP import to supply the cofactor for ALS. Therefore, ALS and OGDH activities were measured in mitochondrial extracts from the *tpc1Δ* strain grown under the above-mentioned conditions (SM with a fermentable carbon source without thiamine) and found to be 20% and 25%, respectively, of those of the wild-type extracts. Interestingly, both the activities could be restored by adding ThPP to the reaction. In contrast, the activity of PDC, which is localized in the cytoplasm, was equal in *tpc1Δ* and wild-type cells. The levels of ThPP were eight times higher in mitochondria of wild-type cells compared with *tpc1Δ* cells, whereas the cytosolic levels were similar in both of them. It is noteworthy that no growth defect of the *tpc1Δ* strain was observed with non-fermentable carbon sources, although substantial mitochondrial requirement for ThPP would also be expected. This finding could indicate that in aerobic conditions another mitochondrial ThPP transporter exists or that an uncharacterized mitochondrial ThPP salvage pathway is

activated. In conclusion, these in vivo experiments suggest that Tpc1p is involved in ThPP import into yeast mitochondria.

The EPRA method was applied to confirm the identification of Tpc1p and characterize its transport properties by several sets of experiments<sup>7</sup>: (a) substrate homo-exchange (with the proteoliposomes loaded with a substrate and the same radioactively-labelled substrate added externally) to identify transportable radioactive substrates; (b) hetero-exchange (with different substrates loaded in the proteoliposomes and an identified transportable radioactive substrate added externally) to identify exchangeable substrates (without the need of having all substrates radioactively labelled); (c) and efflux (with the identified transportable radioactive substrate loaded into the proteoliposomes and various externally added unlabeled substrates). Furthermore, the conditions of these experiments were also varied, for example, times of reaction (for time-courses of transport), concentrations of substrate (for kinetic parameter determinations), pH values, omission of the non-radioactive substrate (to study uniport), addition of competitive substrates or various inhibitors (specific inhibitors or general protein modifiers). At the time of Tpc1p characterization by the EPRA method, no radioactive ThPP was available. However, radioactive dATP was shown to be transported by Tpc1p in the experimental set (a) and exchanged for ThPP in the (b) and (c) procedures.<sup>7</sup> Furthermore, non-radioactive ThPP efflux from proteoliposomes was assayed enzymatically. Taken together, the in vitro results obtained by the EPRA method demonstrated that Tpc1p is able to transport ThPP, ThMP, and to a lower extent PAP, various ribonucleotides and deoxyribonucleotides, but not many compounds such as thiamine, adenosine, CoA or S-adenosylmethionine (SAM). The Tpc1p-catalysed ThPP transport was observed to be faster in antiport than in uniport mode. The kinetic parameters  $K_m$  and  $V_{max}$  for dATP uniport uptake were determined to be 0.5 mM and about 40  $\mu\text{mol/g}$  protein per minute at 25°C, respectively. Competitive dATP transport inhibition by addition of ThPP, ThMP, dADP, ADP and AMP was also studied to determine their constants ( $K_i$ ), which are about 0.2, 0.3, 0.6, 0.7, and 0.6 mM, respectively. Based on these results, it was suggested that the main function of Tpc1p is to import ThPP into the mitochondrial matrix by uniport transport or in exchange for ThMP or one of the other substrates.<sup>7</sup>

In *H. sapiens* the closest homolog of yeast Tpc1p is called SLC25A19 (TPC). To this carrier was originally, even before the characterization of Tpc1p, attributed deoxynucleotide transport ability (and therefore named deoxynucleotide carrier, DNC).<sup>103</sup> Deoxynucleotides were investigated by the EPRA method because phylogenetic

analyses showed that TPC clusters together with the adenine nucleotide carrier. The conclusion of the initial experiments was that TPC transports deoxynucleoside diphosphates and ADP very efficiently, GDP, CDP, UDP and dideoxynucleoside triphosphates less effectively, and NTPs, dNTPs, dNMPs and phosphate at low levels, whereas no transport was observed with NMPs.<sup>103</sup> Due to the findings with *S. cerevisiae* Tpc1p, which has 28% sequence identity with human TPC, the transport capability of TPC was investigated further.<sup>8</sup> *SLC25A19* knockout mice displayed elevated 2-oxoglutarate in the amniotic fluid and did only survive the early embryonic stage, but their isolated mitochondria exhibited normal NTP and dNTP levels. On the contrary, mitochondrial ThPP and ThMP levels were reduced, whereas their cytosolic levels were increased in knockout compared to wild-type cells. In the knockout cells, PDH and OGDH activities were diminished and could be rescued by addition of ThPP. Furthermore, EPRA approach results with human TPC showed that *SLC25A19* (TPC) is a strict antiporter with transport capacity for dATP (radioactive form available), ThPP and ThMP. Thus, it was clear that TPC can import ThPP into mitochondria; however, given its strict antiport mode of transport, what mitochondrial matrix substrate is ThPP exchanged with, among all the substrates transported by TPC? It should be noted that it had been observed previously that isolated rat-liver mitochondria may convert ThPP to ThMP<sup>104</sup>; however, a mitochondrial phosphatase responsible for this reaction has not been identified yet. In addition, in certain physiological conditions mitochondria require a net import of ThPP necessitating another matrix counter-substrate than ThMP. Therefore, also human TPC has a similar function as yeast Tpc1p: mitochondrial import of ThPP in exchange for ThMP or another of its substrate (Figure 2).<sup>8</sup>

A mitochondrial ThPP carrier in *Drosophila melanogaster*, *DmTpc1p*, has also been characterized by the EPRA method.<sup>105</sup> *DmTpc1p*, which has 53 and 24% sequence identity with human TPC and yeast Tpc1p, respectively, transports ThPP and, to a lesser extent, (d) NTPs, (d)NDPs and pyrophosphate, whereas ThMP, thiamine, (d)NMPs or phosphate are not transported. Furthermore, *DmTpc1p* expression complements the growth defects of the *S. cerevisiae tpc1Δ* strain grown on fermentable carbon sources. These results suggest that also *DmTpc1p* has the function of importing ThPP into mitochondria in exchange for another of its substrates.

In *A. thaliana*, two isoforms of mitochondrial ThPP carrier were found, AtTpc1 (At3g21390) and AtTpc2 (At5g48970) that share 74% sequence identity. They were characterized together with their maize homologs, ZmTpc1 (GRMZM2G118515) and ZmTpc2 (GRMZM2G124911)<sup>106</sup>;

and all of these four plant Tpcs display 23–24%, 34–36% and 29–32% sequence identity with the mitochondrial ThPP carrier from *S. cerevisiae*, *H. sapiens* and *D. melanogaster*, respectively. Consistently, in phylogenetic analysis, the four plant proteins cluster together with the human, *Drosophila* and yeast ThPP carriers.<sup>32</sup> Their subcellular localization was determined by two approaches; the dual import assay,<sup>107</sup> in which radioactively-labelled protein is incubated with isolated plant chloroplast or mitochondria and then treated with proteases, and confocal microscopy of suspension-cultured tobacco BY-2 cells in which GFP-fused carrier constructs were expressed.<sup>108</sup> Both approaches indicated that all four proteins are mitochondrial and not localized to chloroplasts, peroxisomes or any other organelle.<sup>106</sup> Unfortunately, although AtTpc1, AtTpc2 and ZmTpc1 were expressed in *E. coli* with the purpose of being used for characterization by the EPRA method, no activity was detected under any of the reconstitution and transport conditions tested. However, the expression of each of the four proteins in the yeast *tpc1Δ* strain rescued the growth rate on fermentable carbon sources to wild-type levels. Furthermore, ThPP transport in mitochondria isolated from the yeast *tpc1Δ* strain was restored by the expression of each of the four plant proteins. In addition, ALS activity was rescued in the AtTpc- and ZmTpc-complemented *tpc1Δ* strain. Taken together, although the biochemical characterization of the isolated proteins remains to be determined, the above-mentioned results strongly support the notion that AtTpc1, AtTpc2, ZmTpc1 and ZmTpc2 have a similar physiological function to those of the mitochondrial ThPP carriers identified in other organisms.

### 2.3 | Role of ThPP in mitochondria

As illustrated in Figure 2, in animal cells, ThPP is involved in important enzyme reactions in carbohydrate, lipid and amino acid catabolism. ThPP imported into mitochondria is used by four different 2-oxoacid dehydrogenase complexes (PDHc, OGDHc, OADHc and BCKDHc) that, although function in distinct metabolic pathways, have similar macromolecular assembly structures and catalyse quite similar decarboxylation reactions.<sup>109</sup> The E1 components (also called PDH, OGDH, OADH and BCKDH) of the four complexes bind ThPP and the initial 2-oxoacid substrates, whereas the other components E2 and E3 bind the other cofactors: lipoate, CoA, FAD and NAD<sup>+</sup>. In all four enzymes, ThPP is directly involved in the decarboxylation of the incoming substrate and the transfer of the remaining decarboxylated acyl intermediate to lipoate bound to E2.

The subsequent reaction steps lead to the final formation of acyl-CoA while the electrons are transferred onto  $\text{NAD}^+$ . The role of ThPP in PDH will be described in the next paragraph and, subsequently, compared with that in the other enzyme complexes. In yeast, mitochondria contain another ThPP-dependent enzyme, ALS.

PDHc exists in all known organisms and links glycolysis to the TCA cycle by catalysing the conversion of pyruvate into acetyl-CoA and  $\text{CO}_2$  (Figure 2).<sup>110</sup> In humans, this large complex consists of multiple copies of the E1, E2 (dihydrolipoyl transacetylase) and E3 (dihydrolipoyl dehydrogenase) components, with a total of 114 polypeptides and a mass of 9 MDa. Each human E1 component is a heterotetramer of two  $\alpha$ - and two  $\beta$ -subunits (PDHA1/2 and PDHB). ThPP is bound in the active site of PDH (E1 component) with the pyrimidine ring hydrogen-bonded at the interface between the  $\alpha$ - and  $\beta$ -subunits and the pyrophosphate moiety penetrating into the  $\alpha$ -subunit where a  $\text{Mg}^{2+}$  is bound leaving the reactive thiazole at the centre of the active pocket where the substrate pyruvate enters.<sup>111</sup> In all known ThPP-requiring enzymes, a special motif takes part in binding the cofactor: in  $^{166}\text{GDGX}_{26}\text{NN}^{196}$  of the  $\alpha$ -subunit of human PDH Asp167 and Asn196 coordinate the  $\text{Mg}^{2+}$ , and Gly168 and Ala169 make hydrogen bonds with the pyrophosphate moiety.<sup>111,112</sup> The dissociation constant of ThPP in pig heart PDH has been estimated to be about 7  $\mu\text{M}$ , which supposedly is above the mitochondrial concentration of ThPP.<sup>113</sup> ThPP is directly involved in two consecutive steps of the PDH-catalysed reaction: (a) the deprotonated thiazole-C2 attacks the 2-oxo-carbon of pyruvate leading to the decarboxylation of pyruvate and the formation of a covalently linked intermediate, (b) the transfer of this intermediate in the reductive acetylation onto the lipoyl group bound to E2 and the return of ThPP to its normal protonated state.<sup>110</sup> Subsequently, E2 catalyses the transfer of the acetyl-moiety to CoA to form acetyl-CoA, and E3 oxidizes the E2 lipoyl group by electron transfer to FAD and then to  $\text{NAD}^+$ . The binding of ThPP in PDH orders  $\alpha$ -subunit loops localized at the entrance of the substrate-binding pocket containing serines, which are phosphorylated/dephosphorylated by kinases/phosphatases and play an important role in the metabolic regulation of PDH activity.<sup>110</sup>

OGDH is the E1 component of OGCHc, which catalyses the conversion of 2-oxoglutarate into succinyl-CoA, a reaction that is most commonly known for its role in the TCA cycle, where it is a rate-limiting step (Figure 2).<sup>114</sup> Besides from carbohydrate metabolism, 2-oxoglutarate may also derive from amino acid catabolism. ThPP is bound to OGDH in a very similar way as to PDH and the reaction mechanism is also very similar: deprotonated C2 of the ThPP thiazole ring attacks the

2-oxo-carbon of 2-oxoglutarate,  $\text{CO}_2$  leaves and the metastable intermediate is resolved by the transfer of succinyl via lipoate onto CoA by E2 (dihydrolipoyl succinyltransferase), while the electrons are handled by E3 (dihydrolipoyl dehydrogenase).

In animals and maybe in plants but not in yeast, OADHc is involved in the catabolism of lysine and tryptophan (Figure 2),<sup>114</sup> from which 2-oxoadipate is derived directly in mitochondria or imported into mitochondria via ODC (SLC25A21).<sup>115</sup> OADHc has its own specific E1 component (encoded by *DHTKD1*-dehydrogenase E1 and transketolase domain-containing protein 1), whereas the E2 and E3 components are identical to those of OGDHc.<sup>109</sup> The substrate 2-oxoadipate and product glutaryl-CoA are only one  $\text{CH}_2$  longer than those of OGDHc, 2-oxoglutarate and succinyl-CoA, which is reflected by the slightly larger substrate-binding pocket in OADHc. The reaction mechanism of OADHc is thought to be very similar to that of OGDHc and PDHc.

BCKDHc, which is found in human and plants but probably not in yeast, is involved in the catabolism of BCAA (leucine, isoleucine and valine), which are imported into mitochondria by SLC25A44<sup>43</sup> and deaminated into their corresponding 2-oxoacids (2-oxoisocaproate, 2-oxo-3-methylvalerate and 2-oxovalerate) (Figure 2). These 2-oxoacids react with ThPP bound to component E1 (2-oxoisovalerate dehydrogenase) of BCKDHc, and the reactions catalysed by E2 (dihydrolipoyl transacylase) and E3 (dihydrolipoyl dehydrogenase) lead to the formation of isovaleryl-CoA, 2-methyl-buteryl-CoA and isobutyryl-CoA, which are further transformed to metabolic intermediates and acetyl-CoA that may enter the TCA cycle.<sup>116</sup>

ALS (encoded by *ILV2* and *ILV6* in *S. cerevisiae*) is a ThPP-requiring enzyme, which catalyses the first step out of four in the biosynthetic pathway of BCAA in yeast.<sup>117</sup> Whereas yeast ALS is found in the mitochondrial matrix and the corresponding enzyme AHAS of plants is localized to chloroplasts and that of bacteria in the cytoplasm, no counterpart of these enzymes exists in animals. ALS condenses two molecules of pyruvate producing  $\text{CO}_2$  and 2-acetolactate (which is used for the synthesis of valine and leucine) or one molecule of pyruvate and one of 2-oxobutyrate forming 2-aceto-2-hydroxybutyrate for isoleucine synthesis. Thus, unlike all the other mitochondrial above-mentioned 2-oxoacid dehydrogenase complexes that have similar supramolecular structures, ALS is different and belongs, together with PDC, to the pyruvate oxidase protein family. In fact, ALS consists of a catalytic subunit (CSU, ILV2), containing the cofactors ThPP,  $\text{Mg}^{2+}$  and FAD, and a regulatory subunit (RSU, ILV6).<sup>118</sup>



## 2.4 | Diseases related to thiamine and ThPP

Several diseases, both acquired and genetic, caused by alterations in genes encoding thiamine transporters, thiamine-converting and ThPP-dependent enzymes, have been described. Insufficient intake of thiamine causes beriberi and Wernicke's encephalopathy.<sup>119</sup> Disease-causing mutations have been found in SLC19A2, SLC19A3, SLC44A4 and TPK1 as well as in the E1 subunits of the mitochondrial ThPP-dependent enzymes PDHc, OGDHc, OADHc and BCKDHc.<sup>120</sup> SLC25A19 (TPC) deficiency is caused by mutations in the gene encoding the mitochondrial ThPP importer. Until now, two autosomal recessive disorders have been disclosed: Amish microcephaly (OMIM 607196) and thiamine metabolism dysfunction syndrome 4 (progressive polyneuropathy type, OMIM 613710). Amish microcephaly is the more severe form and is caused by the mutation p.Gly177Ala, which affects a highly conserved residue of the second signature motif sequence. The recombinant protein harbouring this mutation was found to display a negligible transport activity in proteoliposomes by the EPRA method.<sup>8,121</sup> This mutation has been traced back several generations in Amish family in Pennsylvania.<sup>121</sup> Amish microcephaly symptoms are severe congenital microcephaly, 2-oxoglutaric aciduria and premature death in many patients. In an attempt to create an animal model for SLC25A19 deficiency, mice with the single knockout of this gene appeared normal, whereas the double knockout displayed mitochondrial ThPP and ThMP depletion, embryonic lethality, brain malformations and anaemia.<sup>8</sup> The diminished mitochondrial ThPP content caused reduced OGDHc and PDHc activities resulting in 2-oxoglutaric aciduria and accumulation of lactate. Thiamine metabolism dysfunction syndrome 4 is caused by mutations of non-conserved residues (p.Gly125Ser and p.Gln192His, inside the substrate translocation pore, and p.Ser194Pro and p.Leu290Gln outside the pore) as well as by the mutation p.Glu304Lys, which affects a conserved residue of the cytoplasmic gate (for a review see Reference 64). This disease is characterized by increased lactate levels in cerebrospinal fluid or serum, chronic progressive polyneuropathy and fever-triggered acute episodes of flaccid paralysis and encephalopathy with bilateral striatal necrosis.<sup>122-125</sup> In contrast to Amish microcephaly, patients with this disorder have normal head circumference, development and cognitive skills, and oral thiamine administration seems to improve the clinical situation.<sup>123,125</sup> The severe neuronal syndromes including encephalopathy associated with thiamine metabolism dysfunction syndrome 4 are also symptoms linked to mutations in SLC19A3, TPK1 and PDHc.

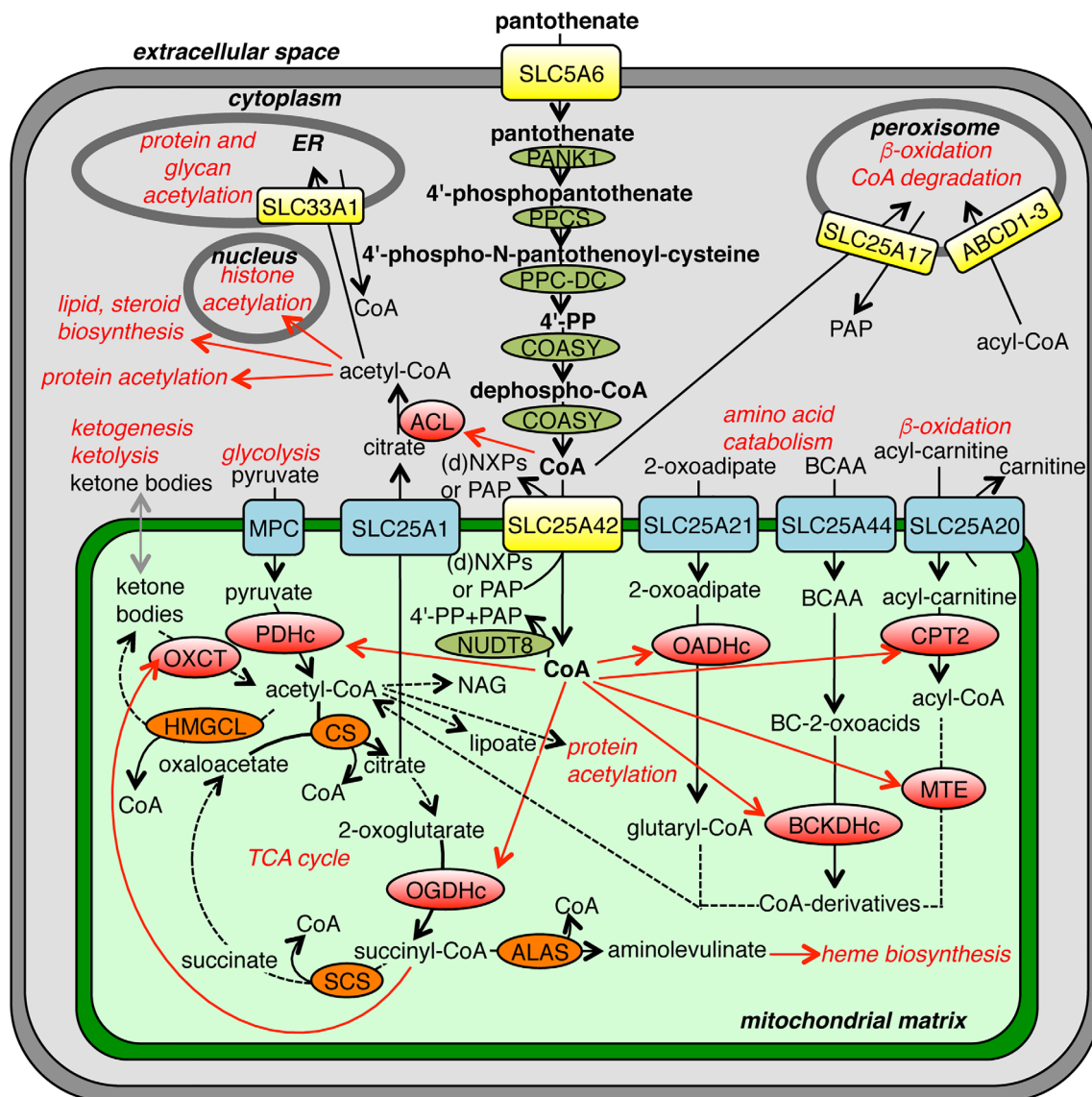
Notably, all these genetic variants affect four enzymatic steps on the linear pathway from thiamine absorption to ThPP-dependent PDHc activity (Figure 2).<sup>120</sup>

## 3 | COENZYME A

Coenzyme A (CoA, SHCoA, CoASH) is a cofactor present in all organisms of the three domains of life: Archaea, Bacteria and Eukarya. It is involved in approximately 4% of all enzymatic reactions, most of which play key roles in the metabolism of fatty acids, carbohydrates, amino acids and ketone bodies.<sup>126</sup> CoA is composed of 3'-phosphoadenosine-5'diphosphate linked to pantothenate (vitamin B5), which consists of pantoate amide bonded to  $\beta$ -alanine, which in turn is connected to cysteamine by a second amide bond (Figure 1b). The terminal thiol group of cysteamine has the property of forming high-energy thioester bonds with carboxylated intermediates leading, for example, to the formation of acetyl-CoA and various size acyl-CoA, which can be transferred to various C, O, N and S acceptors in enzymatic reactions, where the CoA moiety is used as a handle. Due to its size and hydrophilic nature, CoA does not penetrate membranes.

### 3.1 | Biosynthesis and distribution of CoA

Pantothenate (vitamin B5), which is essential for the formation of the CoA molecule, is synthesized *de novo* in plants, some bacteria and fungi. In these organisms, pantoate- $\beta$ -alanine ligase (PanC) catalyses the condensation of  $\beta$ -alanine with pantoate to form pantothenate.<sup>127</sup> In non-pantothenate-producing bacteria and fungi, pantothenate is imported by transporters of the major facilitator family with 12 transmembrane helices, such as PanF and Fen2p. In animals, which are unable to synthesize pantothenate, the uptake of this vitamin is essential. It is found in food such as meat, vegetables, cereal grains, legumes, eggs, and milk as well as is produced by the intestinal flora. Intestinal CoA is hydrolysed into the intermediates dephospho-CoA, 4'-phosphopantetheine and pantetheine.<sup>128</sup> In the small intestine, pantothenate is taken up by the sodium-dependent multivitamin transporter SLC5A6 predicted to contain 13 transmembrane segments.<sup>129</sup> This transporter is also expressed in the cellular plasma membrane of heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas, and is therefore probably responsible for the import of pantothenate in most cells (Figure 3).<sup>129</sup> In all living organisms pantothenate, cysteine and four molecules of ATP are used to synthesize



**FIGURE 3** CoA metabolism and transport in a typical human cell. The schematic overview shows CoA/CoA-derivative transporters (yellow), enzymes adding CoA on a metabolite (red), enzymes taking away CoA from a metabolite (orange), enzymes metabolizing CoA (olive green), enzymatic reactions (black arrows), reactions with unidentified enzymes (grey arrows) and distribution (red arrows). (d)NXPs, (deoxy)nucleotides; ACL, ATP-citrate lyase; ALAS, aminolevulinic acid synthase; BC-2-oxoacids, branched-chain 2-oxoacids; BCAA, branched-chain amino acids; BCKDHc, branched-chain  $\alpha$ -ketoacid dehydrogenase; COASY, coenzyme A synthase; CPT2, carnitine *O*-palmitoyltransferase 2; CS, citrate synthase; HMGCL, Hydroxymethylglutaryl-CoA lyase; MPC, mitochondrial pyruvate carrier; MTE, mitochondrial trifunctional enzyme; NAG, *N*-acetyl-glutamate; OADHc, 2-oxoadipate dehydrogenase complex; OGDHc, 2-oxoglutarate dehydrogenase complex; OXCT, 3-oxoacid-CoA transferase; PANK1, pantothenate kinase 1; PAP, adenosine 3',5'-diphosphate; PDHc, pyruvate dehydrogenase complex; PPC-DC, phosphopantothenoylcysteine decarboxylase; PPCS, phosphopantothenoylcysteine synthetase; SCS, succinyl-CoA synthetase

CoA in a five-step process (Figure 3): (a) pantothenate is phosphorylated to 4'-phosphopantothenate by pantothenate kinase (PANK1), which is the committed step in CoA biosynthesis, requires ATP and is regulated by product inhibition; (b) cysteine is added to 4'-phosphopantothenate by phosphopantothenoylcysteine synthetase (PPCS) to form 4'-phospho-N-pantothenoylcysteine in an ATP-dependent reaction;

(c) the latter metabolite is decarboxylated by phosphopantothenoylcysteine decarboxylase (PPC-DC) to 4'-phosphopantetheine; (d) to which AMP is added by phosphopantetheine adenylyl transferase (PPAT or COASY) to form dephospho-CoA; (e) which is phosphorylated by dephosphocoenzyme A kinase (DPCK or COASY) to form CoA.<sup>130</sup> In humans, a single polypeptide chain constitutes CoA synthase COASY, a

bifunctional enzyme catalysing both PPAT and DPCK (step [d] and [e]) activities. In humans, all of these enzymes are localized outside mitochondria with the exception of PANK2, one of several PANK isoforms, which has been suggested to be present in the intermembrane space of mitochondria.<sup>131</sup> Furthermore, the subcellular localization of COASY is under debate, because it has been found in association with mitochondrial outer membrane,<sup>132,133</sup> in mitochondrial matrix<sup>134–136</sup> and partly in the cytosol.<sup>134,137</sup> Later, it was found that COASY is expressed as two splice variants:  $\alpha$ , which is expressed ubiquitously, and  $\beta$ , which is predominantly expressed in brain.<sup>136</sup> Unfortunately, in these later studies, the cellular localization of these two variants was not investigated. However, looking at the pre-sequences, it should be noted that  $\alpha$ -COASY has a predicted N-terminal mitochondrial targeting peptide, whereas  $\beta$ -COASY has a 29 residue N-terminal extension preceding the above-mentioned peptide rendering the latter variant of COASY to be predicted non-mitochondrial. It has been suggested that CoA may also be synthesized through alternate routes when its intracellular levels are reduced and the de novo pathway is impaired.<sup>138</sup> Furthermore, Daugherty et al.<sup>139</sup> identified a human gene on chromosome 17, named *DCAKD*, encoding a putative monofunctional human DPCK enzyme (Accession Numbers: gi13623688 and Q8WVC6 [UniProt]), which shared 35% sequence identity with the *E. coli* DPCK over its entire length. Orthologs of monofunctional *DCAKD* are also present in *D. melanogaster* (Q9VI57) and *C. elegans* (P34558).<sup>140</sup> Interestingly, monofunctional *DCAKDs* display a greater sequence identity to the *E. coli* and other bacterial DPCK sequences than to the paralogous DPCK portions of the bifunctional PPAT/DPCK sequences in each organism. Submitochondrial fractionation studies revealed that most of the *DCAKD* enzymatic activity is associated with the outer membrane of mitochondria.<sup>134</sup>

Excess CoA may be degraded intracellularly, mainly by mitochondrial and peroxisomal NUDIX (nucleoside diphosphate-linked moiety X) hydrolases, or extracellularly, which leads to its conversion into pantothenate that may be recycled by cellular uptake and de novo CoA synthesis.<sup>141</sup>

CoA is distributed within the mammalian cells between the different compartments: cytosol, nucleus, ER, peroxisomes, lysosomes and mitochondria (Figure 3). Cytosolic/nucleic concentrations of CoA are estimated between 0.11 and 0.14 mM in liver and heart, in peroxisomes 0.3–0.7 mM CoA, whereas estimated mitochondrial CoA concentrations are significantly higher, about 5 mM.<sup>141</sup> Although these estimates are dependent on the purity and integrity of the subcellular fractionations, they

suggest that mitochondria contain 80–95% of total cellular CoA. In the cytosol and nucleus, CoA is used by ATP-citrate lyase to form acetyl-CoA for fatty acid and steroid synthesis, and for post-translational acetylation of thousands of sites on proteins, for example, histones.<sup>142</sup> The ratio of acetyl-CoA to CoA controls protein acetylation influencing key cellular processes, such as cell growth, regulated cell death and autophagy.<sup>143</sup> In the ER membrane, SLC33A1 imports acetyl-CoA in exchange for CoA for acetylation of proteins and glycans.<sup>144</sup> In peroxisomes, ABC transporters ABCD1–3 import a variety of acyl-CoAs,<sup>145</sup> and SLC25A17 (as well as its homologue PXN in plants) transport CoA, which is used, for example, in  $\beta$ -oxidation.<sup>146,147</sup> In mitochondria, CoA is imported into the matrix by SLC25A42,<sup>10</sup> where it plays key roles in metabolic processes, such as the oxidation of pyruvate, oxoglutarate, BCAA and fatty acids (Figure 3).

### 3.2 | Mitochondrial transport of CoA

The human mitochondrial carrier for CoA is SLC25A42, which clusters together with SLC25A16 as well as yeast Leu5p and YPR011cp, and Arabidopsis AT1G14560 and AT4G26180.<sup>32</sup> The EPRA approach was employed to characterize SLC25A42. Among the radioactive substrates tested, ATP, ADP and AMP were transported in homo-exchange (type i) experiments; subsequently, radioactive ADP was used in hetero-exchange (type ii) and efflux (type iii) transport experiments. The results showed that SLC25A42 transports CoA, adenosine 3',5'-diphosphate (PAP), dephospho-CoA and (deoxy-)adenine nucleotides by an obligatory counter-exchange mechanism.<sup>10</sup> Furthermore, radioactive ADP uptake by reconstituted SLC25A42 was inhibited competitively by PAP (Ki of 27  $\mu$ M), ADP (Ki of 54  $\mu$ M), CoA and dephospho-CoA (Ki about 110  $\mu$ M for both), the last Ki corresponding approximately to the cytosolic concentration of CoA. In addition, the expression of human SLC25A42 in a yeast strain with deleted Leu5, which had been suggested previously to be a mitochondrial CoA carrier,<sup>148</sup> complemented the growth defects on non-fermentable carbon sources that require mitochondrial CoA for their oxidation.<sup>10</sup> Finally, a fusion construct of SLC25A42-GFP expressed in CHO cells showed that the protein is localized to mitochondria.<sup>10</sup> SLC25A42 is expressed in almost all tissues.<sup>149</sup> Based on these results and assuming a cytosolic localization of the COASY, it was suggested that the main function of SLC25A42 is to import cytosolic CoA or dephospho-CoA into the mitochondrial matrix in exchange for PAP, which is generated from CoA in the mitochondrial matrix, or another substrate.<sup>10</sup> An alternative scenario should be taken into

consideration if COASY were localized in the matrix. In this case, SLC25A42 could be involved in the export of CoA, dephospho-CoA or PAP from the mitochondria, possibly by an exchange reaction with ADP.<sup>10</sup> Outside the mitochondria, dephospho-CoA would be converted to CoA by the monofunctional DCAKD localized in the mitochondrial outer membrane.<sup>134</sup> This hypothetical route might also be operating in *D. melanogaster*, where the SLC25A42 ortholog transports dephospho-CoA (and not CoA, see below) and DCAKD is present too. Although SLC25A42 can bi-directionally exchange CoA or dephospho-CoA with [<sup>14</sup>C]ADP in vitro,<sup>10</sup> no data are available about the direction of this exchange reaction in vivo until now. Answering this question may be of utmost importance in the light of the conflicting results on the sub-mitochondrial localization of COASY.

The functions of some of the SLC25A42 homologues have also been investigated. It is likely that yeast Leu5p is a mitochondrial transporter for CoA or one of its precursors, because deletion of its gene reduces mitochondrial CoA content and diminishes the activity of mitochondrial CoA-dependent enzymes.<sup>148</sup> The same conclusion was drawn for the human SLC25A16, which partly complements the alterations observed in the Leu5-knockout strain.<sup>148</sup> However, the substrate specificities of these two proteins have not yet been investigated. By using the EPRA method, yeast YPR011cp has been shown to transport not CoA or PAP but phosphate, sulfate, adenosine 5'-phosphosulfate (APS) and 3'-phospho-adenosine 5'-phosphosulfate (PAPS), the last two being structurally similar to PAP.<sup>150</sup> The homologue of SLC25A42 in *D. melanogaster* has been characterized using the EPRA method and shown to transport dephospho-CoA (and (deoxy-)adenine nucleotides) but not CoA; it also complements the growth defects of the yeast Leu5-deletion strain.<sup>151</sup> Arabidopsis AT1G14560 and AT4G26180 (and their two maize homologues), which are localized to mitochondria as shown by the dual import assay and confocal microscopy of tobacco BY-2 cells with GFP-fused proteins, also rescue the growth defects and the reduced mitochondrial CoA content of the yeast Leu5-deletion strain.<sup>152</sup> Taken together, these findings suggest that the SLC25A42 homologues, with the exception of YPR011cp, have a similar physiological function in importing CoA and/or dephospho-CoA into mitochondria.

### 3.3 | Role of CoA in mitochondria

Mitochondrial CoA is used by a long list of matrix enzymes involved in carbohydrate, lipid, amino acid and ketone body catabolism to form various length acyl-chain-CoA intermediates, most of which eventually end

up as acetyl-CoA (Figure 3). Glycolysis-derived and mitochondrially-imported pyruvate is transformed into acetyl-CoA by PDHc (as described in Section 2.3); the E2 component transfers the acetyl-moiety (originally donated from pyruvate in E1) from the lipoyl group onto CoA, followed by re-oxidation of lipoate by the E3 component.<sup>110</sup> In the TCA cycle, CoA is used by the E2 component of OGDHc to form succinyl-CoA. In the mitochondrial  $\beta$ -oxidation, CoA substitutes the carnitine moiety of mitochondrially-imported carnitine-linked fatty acid chains by the action of carnitine O-palmitoyltransferase 2, and functions as a handle in the subsequent steps catalysed by acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and  $\beta$ -ketothiolase, of which the latter adds a new CoA molecule to the remaining acyl chain after the exit of the terminal acetyl-CoA. In amino acid catabolism, the E2 component (lipoamide acyltransferase) of BCKDHc adds CoA to the 2-oxoacids derived from BCAAs; branched-chain specific acyl-CoA dehydrogenases convert the carbon skeletons into acetyl-CoA; and the E2 component of OADHc adds CoA onto 2-oxoadipate, which is derived from lysine and tryptophan, and is further converted to acetyl-CoA. Finally, ketone bodies, such as acetoacetate and  $\beta$ -hydroxybutyrate, are utilized for ATP production by conversion into acetyl-CoA in the mitochondrial matrix of non-hepatic tissues when glucose and glycogen stores are diminished.<sup>153</sup>

Once mitochondrial acetyl-CoA has been formed it may be used in various ways. When the cellular demand for ATP is high, most acetyl-CoA enters the TCA cycle by citrate synthase, which condenses acetyl and oxaloacetate into citrate, leaving CoA free. In the same metabolic cycle, succinyl-CoA is converted into succinate and free CoA by the action of succinyl-CoA synthetase. Matrix succinyl-CoA may also be condensed with glycine by 5-aminolevulinic synthase to form free CoA and aminolevulinic, which is the first step in heme synthesis.<sup>154</sup> Especially in liver during fasting, mitochondrial acetyl-CoA derived from  $\beta$ -oxidation may be used for the biosynthesis of the ketone bodies acetoacetate and  $\beta$ -hydroxybutyrate, which are activated and oxidized in other tissues with the production of ATP.<sup>153</sup> Furthermore, acetyl-CoA (via malonyl-CoA) is utilized in mitochondrial type II fatty acid synthesis for the production of lipoate, which acts as a cofactor in the E2 component of the matrix dehydrogenase complexes.<sup>155</sup> In addition, mitochondrial acetyl-CoA may be used for regulatory purposes through acetylation of proteins and the production of the urea cycle regulator N-acetylglutamate.<sup>156,157</sup>

The above-mentioned mitochondrial enzymes associate or disassociate CoA from acyl chains without changing the CoA moiety itself. However, mitochondria also contain the



NUDIX protein NUDT8, which hydrolyses CoA (or acyl-CoAs) to phosphopantetheine (or acyl-phosphopantetheine) and PAP, exhibiting the same function in CoA degradation as its peroxisomal homologues NUDT7 and NUDT19.<sup>158</sup> It seems most likely that NUDT8 is localized in the matrix, but it cannot be excluded that it is in the intermembrane space.<sup>141</sup> The fact that also COASY may be located in the matrix implies that the resulted phosphopantetheine may be recycled into CoA; in this case, however, NUDT8 and COASY activities must be regulated to avoid a futile cycle of CoA synthesis and breakdown.<sup>158,141</sup> Moreover, mitochondrial type II fatty acid synthesis of lipoate requires acyl carrier protein (ACP), which is modified covalently with a phosphopantetheinylate group derived from CoA, by phosphopantetheinyl transferase producing PAP as a leaving group.<sup>155</sup> In yeast, phosphopantetheinyl transferase is localized in mitochondria, whereas the corresponding enzyme in human appears to be cytoplasmic. Besides being an intermediate in lipoate synthesis, acetylated ACP (via the phosphopantetheine group) seems to have a role in mitochondrial translation, iron-sulfur cluster biogenesis and assembly of the respiratory chain complexes.<sup>155</sup> Thus, if human NUDT8 and phosphopantetheinyl transferase are located in the mitochondrial matrix, PAP, the breakdown product of CoA, may be exported from the mitochondrial matrix by SLC25A42.<sup>10</sup>

### 3.4 | Diseases related to CoA

In humans, pantothenate deficiency due to low intake of the vitamin (RDA 5 to 10 mg) is a very rare disorder that causes asthenia, headaches, nausea and vomiting, as well as some forms of neuropathies similar to those of other vitamin B deficiencies.<sup>159</sup> Mutations in PANK2 and COASY give rise to neurodegeneration with brain iron accumulation.<sup>160</sup> Homozygous mutations in SLC25A42 cause variable symptoms with wide spectrum of severity, even among patients with the same mutation in the same family, including lactic acidosis, mitochondrial myopathy with muscle weakness, encephalopathy, developmental regression and epilepsy.<sup>161–163</sup> The identified disease-causing mutations are a splice site and a point mutation (p.Asn291Asp) close to the substrate-binding site (for a review, see Reference 64).

## 4 | FLAVIN ADENINE DINUCLEOTIDE (FAD)

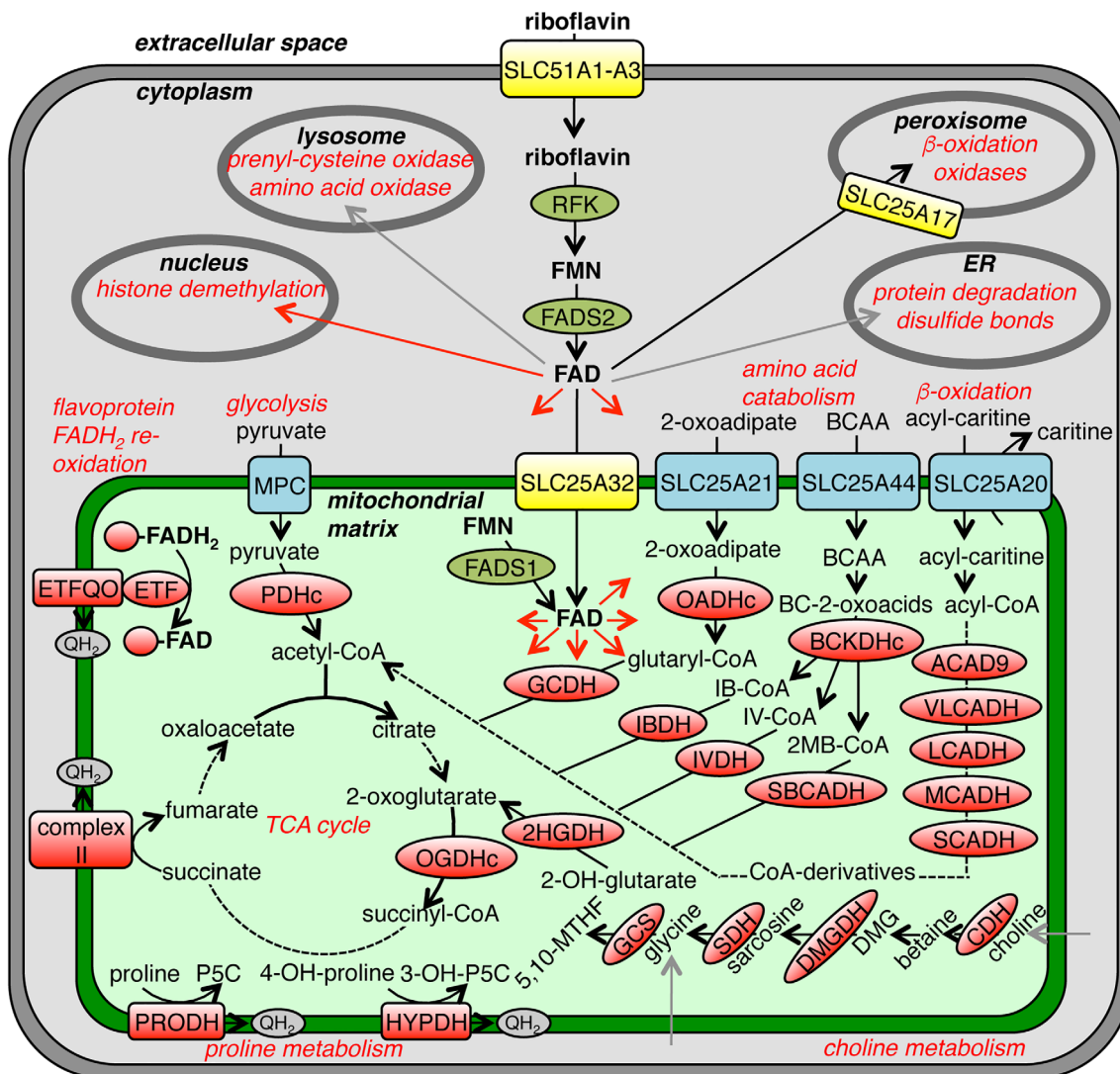
FAD is an important cofactor in redox reactions catalysed by flavoproteins, which contain FAD, or its mononucleotide form FMN, and play key roles in cell metabolism. FAD

consists of a riboflavin moiety (vitamin B2 or previously vitamin G), a flavin isoalloxazine ring bound to the reduced pentose ribitol, that with one phosphate attached forms FMN or two phosphates and adenosine forms FAD (Figure 1c). Two nitrogen atoms (in positions 1 and 5) in the conjugated 3 six-membered ring system of riboflavin have the ability of accepting a proton and an electron, each giving the fully reduced form of FAD and FMN. FAD works as a prosthetic group in flavoproteins, to which it is most often bound non-covalently.

### 4.1 | FAD biosynthesis and distribution

The FAD precursor riboflavin is not synthesized by animals but by bacteria, fungi and plants where its synthesis starts from GTP and ribulose-5-phosphate.<sup>164</sup> In humans riboflavin is mainly taken up in small intestine from food such as meat, cereals, fish, dark-green vegetables, egg, nuts, mushrooms, legumes, milk and dairy products.<sup>165</sup> Food processing and exposure to UV light may reduce riboflavin content. In the small intestine, FAD and FMN are hydrolysed to riboflavin, which is mainly absorbed by SLC52A3 (RFVT3) in the proximal small intestine and, to some extent, in the large intestine.<sup>166</sup> Also, the homologues of this transporter, SLC52A1 and SLC52A2, with 86% sequence identity between them and about 42% with SLC52A3, may contribute to intestinal absorption, enterocyte export, blood cell import/export and cellular uptake of riboflavin (Figure 4).<sup>167,168</sup> In blood riboflavin is transported bound to albumin or in erythrocytes and leukocytes.

Imported cellular riboflavin is phosphorylated by riboflavin kinase (RFK, ATP-riboflavin 5'phosphotransferase) to form FMN, which is subsequently adenylated by FAD synthase (FADS, ATP-FMN adenyltransferase) to FAD (Figure 4).<sup>169</sup> Whereas RFK is thought to be cytoplasmic, FADS has been suggested to have two isoforms in human, one cytoplasmic and one mitochondrial.<sup>170</sup> In yeast, there is only one isoform of FADS, which may be localized both to the cytoplasm and mitochondria due to a cis-acting mitochondrial localization motif present in the 3'-untranslated region of its mRNA.<sup>171,172</sup> FAD is distributed in different cellular compartments: cytoplasm, peroxisomes, ER, nucleus, lysosomes and mitochondria. Very limited examples of processes in which FAD is involved are: cytochrome P450 reductase and nitric oxide synthase 2 in the cytosol<sup>173,174</sup>;  $\beta$ -oxidation of fatty acids, D-amino acid and sarcosine oxidases in peroxisomes<sup>145</sup>; degradation of proteins and formation of disulfide bonds in the ER<sup>175,176</sup>; histone lysine demethylases in the nucleus<sup>177</sup>; amino acid and prenyl-cysteine oxidases in lysosomes<sup>178,179</sup>; and many enzymes involved in carbohydrate, fatty acid and amino



**FIGURE 4** FAD metabolism and transport in a typical human cell. The schematic overview shows FAD/FMN/riboflavin transporters (yellow), flavoenzymes (red), enzymes metabolizing FAD (olive green), enzymatic reactions (black arrows), reactions with unidentified enzymes (grey arrows) and distribution (red arrows). 2HGDH, 2-hydroxyglutarate dehydrogenase; 2-MB-CoA, 2-methylbutyryl-CoA; 2-OH-glutarate, 2-hydroxyglutarate; 3-OH-P5C,  $\Delta^1$ -pyrroline-3-hydroxy-5-carboxylate; 4-OH-proline, 4-hydroxyproline; 4'-PP, 4'-phosphopantetheine; 5,10-MTHF, 5,10-methylenetetrahydrofolate; ACAD9, acyl-CoA dehydrogenase 9; BC-2-oxoacids, branched-chain 2-oxoacids; BCAA, branched-chain amino acids; BCKDHC, branched-chain  $\alpha$ -ketoacid dehydrogenase; CDH, choline dehydrogenase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; ETF, electron transfer flavoprotein; ETFQO, electron transfer flavoprotein ubiquinone oxidoreductases; FADS, FAD synthase; GCDH, glutaryl-CoA dehydrogenase; GCS, glycine cleavage system; HYPDH, hydroxyproline dehydrogenase; IB-CoA, isobutyryl-CoA; IBDH, isobutyryl-CoA dehydrogenase; IV-CoA, isovaleryl-CoA; IVDH, isovaleryl-CoA dehydrogenase; LCADH, long-chain acyl-CoA dehydrogenase; MCADH, medium-chain acyl-CoA dehydrogenase; MPC, mitochondrial pyruvate carrier; OADHC, 2-oxoadipate dehydrogenase complex; OGDHc, 2-oxoglutarate dehydrogenase complex; PDHc, pyruvate dehydrogenase complex; P5C,  $\Delta^1$ -pyrroline-5-carboxylate; PROD, proline dehydrogenase; QH<sub>2</sub>, reduced ubiquinone; RFK, riboflavin kinase; SBCADH, short branched-chain acyl-CoA dehydrogenase; SCADH, short-chain acyl-CoA dehydrogenase; SDH, sarcosine dehydrogenase; VLCADH, very-long-chain acyl-CoA dehydrogenase

acid catabolism, such as succinate dehydrogenase (complex II), in mitochondria.<sup>168</sup> Moreover, in chloroplasts, FAD is used by acetolactate synthase and for fatty acid biosynthesis.<sup>70</sup> These compartmentalized FAD-requiring processes suggest that FAD, or one of its precursors, has to be imported into these organelles. SLC25A17 and SLC25A32 have

been identified as transporters for FAD (and/or FMN) in peroxisomes,<sup>146</sup> and in mitochondria,<sup>180,181</sup> respectively. SLC25A17 is better characterized than SLC25A32 biochemically; it transports FAD and FMN along with CoA and other substrates as shown by the EPRA method. In plants, the mitochondrial carriers NDT1 and NDT2, characterized

by the EPRA method, display the ability of transporting FAD and FMN although at a lower extent than their other substrates  $\text{NAD}^+$ , NAAD, NMN, ADP and AMP (described in more detail in Section 5.2).<sup>182</sup>

## 4.2 | Mitochondrial FAD transport

FLX1 (flavin exchange) has been suggested to import FAD into mitochondria and hence be the yeast mitochondrial carrier for FAD<sup>5</sup> for the following reasons: (a) a respiratory defective mutant strain of *S. cerevisiae* was shown to have low mitochondrial FAD/FMN ratio; (b) the gene responsible for this phenotype was identified as FLX1; (c) mitochondrial vesicles from wild-type cells displayed more efficient transport of FAD than those from the *flx1* mutant strain; and (d) yeast FADS was thought to be located in the cytoplasm.

The closest human homologue of FLX1, SLC25A32 (29% sequence identity), was originally suggested to transport folate (vitamin B9) and named mitochondrial folate carrier (MFT), because of its ability to complement glycine auxotrophy of a Chinese hamster ovary cell line (CHO GlyB cells) with diminished mitochondrial folate levels.<sup>6</sup> Later, human SLC25A32 was shown to rescue growth defects and succinate dehydrogenase activity of the *flx1* mutant strain.<sup>180</sup> The same authors suggested that mitochondrial FAD is necessary for dimethylglycine dehydrogenase and sarcosine dehydrogenase, which are involved in one-carbon metabolism in connection to the folate cycle, catalysing the conversion of choline to glycine. Furthermore, FAD is also the cofactor of other enzymes involved in folate metabolism such as methylene THF reductase or the glycine cleavage system. These grounds might explain the previous observations and the suggestion that SLC25A32 is a folate transporter. Also an Arabidopsis homologue of SLC25A32, At5g66380, which is located in chloroplasts, has been suggested to be a folate transporter based on complementation of CHO GlyB cells.<sup>183</sup> Unfortunately, no direct evidence for transport of FAD, FMN, folate or other substrates has yet been reported with isolated yeast FLX1, human SLC25A32 or Arabidopsis At5g66380. Therefore, it is not known whether these transporters are uniporters or antiporters and, given the possibility that FADS is located both in cytoplasm and mitochondria,<sup>171,170</sup> it is also not clear whether they have a role in mitochondrial FAD (or FMN) export.

## 4.3 | Role of FAD in mitochondria

FAD is an important cofactor for several mitochondrial matrix enzymes, mostly catabolic dehydrogenases. In these reactions, FAD is reduced to  $\text{FADH}_2$  by accepting two

electrons and two protons (Figure 4). The electrons of  $\text{FADH}_2$  in integral membrane flavoproteins are directly transferred onto ubiquinone, sometimes via other electron transfer groups. One example of membrane flavoprotein is complex II, succinate dehydrogenase, which catalyses the conversion of succinate to fumarate in the TCA cycle and to which FAD is covalently linked to a histidine residue,<sup>184</sup> whereas complex I NADH dehydrogenase contains FMN. Two other inner membrane bound mitochondrial flavoproteins, involved in proline/hydroxy-proline catabolism, are proline dehydrogenase (PRODH), which converts proline to  $\Delta^1$ -pyrroline-5-carboxylate, and hydroxyproline dehydrogenase (HYPDH), which transforms hydroxyproline, derived from collagen, to  $\Delta^1$ -pyrroline-3-hydroxy-5-carboxylate.<sup>185,186</sup>  $\text{FADH}_2$  of soluble flavoproteins is re-oxidized to FAD by electron transfer flavoprotein (ETF) onto ETF ubiquinone oxidoreductases (ETFQO), which transfers the electrons onto ubiquinone for the inner membrane-embedded electron transport chain. One example of a soluble matrix flavoprotein is the E3 component (dihydrolipoyl dehydrogenase, DLD), which is identical for all four different 2-oxoacid dehydrogenase complexes (PDHc, OGDHc, OADHc and BCKDHc) as discussed in Section 2.3. Dihydrolipoyl dehydrogenase is also part of the glycine cleavage system (GCS), involved in glycine metabolism.<sup>187</sup> Nine other soluble mitochondrial FAD-dependent enzymes are involved in (a) fatty acid  $\beta$ -oxidation, (b) BCAA catabolism and (c) amino acid catabolism. These include: (a) acyl-CoA dehydrogenases for various acyl-chain lengths (short-, medium-, long-, and very-long-chain; SCADH, MCADH, LCADH and VLCADH) as well as ACAD9 (acyl-CoA dehydrogenase 9); (b) isovaleryl-CoA, isobutyryl-CoA, and short branched-chain acyl-CoA dehydrogenases (IVDH, IBDH and SBCADH); and (c) glutaryl-CoA dehydrogenase (GCDH).<sup>168</sup> Other mitochondrial soluble flavoenzymes are L- and D-2-hydroxyglutarate dehydrogenases (2HGDH),<sup>188,189</sup> which are keeping the levels of toxic 2-hydroxyglutarate low, as well as choline dehydrogenase (CDH), dimethylglycine dehydrogenase (DMGDH)<sup>190</sup> and sarcosine dehydrogenase (SDH),<sup>191</sup> which are involved in choline metabolism. In addition, mitochondrial FAD is involved in heme biosynthesis (protoporphyrinogen oxidase PPOX), redox control (thioredoxin reductase TRXR2), sulfur metabolism (SQOR), complex I assembly/subcomplex (FXRD1, NDUFA10, NDUFA9 and ACAD9), ubiquinone biosynthesis (COQ6), adenosylcobalamin biosynthesis (MMAB) and mitochondrial tRNA modifications (MTO1).

## 4.4 | Diseases related to FAD

The RDA of riboflavin is about 0.9–1.3 mg/day for adults: a daily intake less than 0.6 mg may lead to vitamin B2

deficiency (ariboflavinosis), which is manifested by generalized weakness, angular stomatitis, glossitis, cheilosis, corneal vascularization, photophobia and, in severe cases, generalized seborrhoeic dermatitis.<sup>192–194</sup> Disease-causing mutations have been identified in the genes encoding the riboflavin transporters SLC52A1–3, mitochondrial SLC25A32, FADS1, ETF $\alpha$ , ETF $\beta$  and ETFQO.<sup>168</sup> Mutations in the latter three proteins, which are involved in the mitochondrial re-oxidation of FAD (Figure 4), give rise to a group of three severe disorders called multiple acyl-CoA dehydrogenase deficiencies (MADD I–III, respectively). These disorders involve multiple organs such as muscle, heart and liver, and are characterized by the accumulation of organic acids in urine, acylcarnitines and amino acids in plasma due to their compromised catabolism.<sup>168</sup>

SLC25A32 deficiency (OMIM 616839) has been found in two patients, of which one displayed milder symptoms and the other more severe. The milder case exhibited recurrent late-onset exercise intolerance and biochemical features similar to those of MADD patients.<sup>181</sup> The patient had compound heterozygous SLC25A32 mutations: a premature stop codon and a conservative missense mutation (p.Arg147His) in the second positively charged residue of the second signature motif sequence. The expression of the missense mutant protein could complement the defects in the yeast *flx1* mutant strain only partly.<sup>181</sup> The more severe case had a neuromuscular phenotype that manifested early onset ataxia, myoclonia and dysarthria besides muscle weakness, exercise intolerance and MADD metabolites.<sup>195</sup> This patient had a homozygous indel mutation causing loss of the translational start codon and consequent loss of SLC25A32 expression or the use of an alternative start codon and production of a dysfunctional SLC25A32. If the former possibility is true, it might be that matrix FAD is not only provided through this carrier. For MAADIII and the disorders caused by mutations in riboflavin/FAD transporters SLC25A32 and SLC52A1–3, diet supplemented with riboflavin is a quite effective treatment improving the prognosis of patients.<sup>168</sup>

## 5 | NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD<sup>+</sup>)

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) is a redox cofactor used by hundreds of enzymes because of its capacity to act as an electron donor and acceptor in its reduced (NADH) and oxidized (NAD<sup>+</sup>) form, respectively. In addition, NAD<sup>+</sup> is also used by non-redox enzymes, such as sirtuins and poly(ADP-ribose) polymerases for protein deacylation and ADP-ribosylation

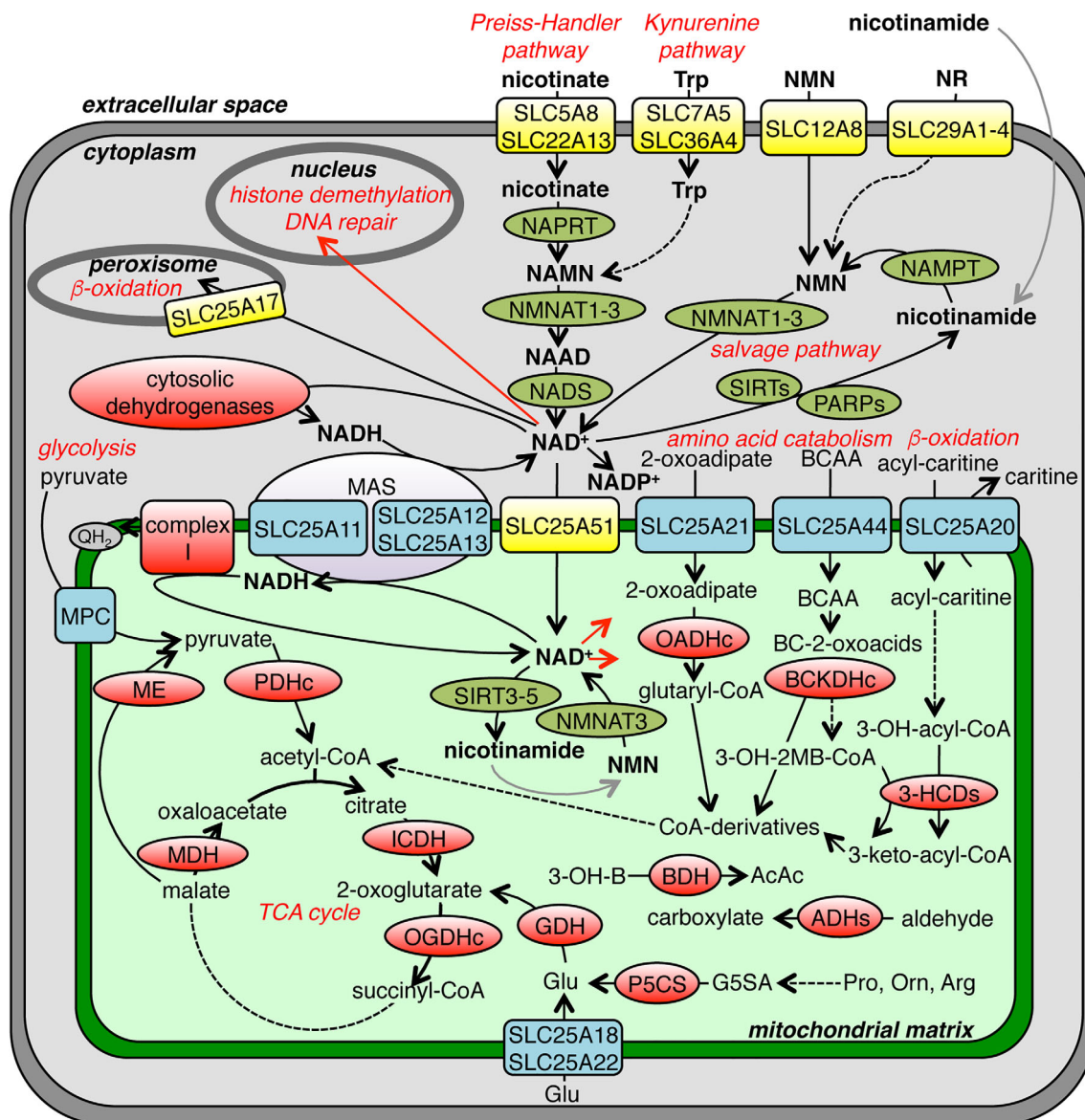
reactions, respectively. It is formed by AMP and nicotinamide mononucleotide (NMN) linked through their phosphates (Figure 1). NMN consists of a riboside, which is linked to nicotinamide through its 1'-carbon (together forming nicotinamide riboside), and a 5'-phosphate. Nicotinamide and nicotinamide riboside together with nicotinate (nicotinic acid or niacin) are different forms of vitamin B3. The oxidized form NAD<sup>+</sup> has a positive charge on N1 of nicotinamide (along with the two negative charges on the phosphates), whereas the reduced form NADH has accepted two electrons and one proton (together a hydride ion), giving rise to neutralized N1 and an additional hydrogen added onto C4 of the nicotinamide ring. Unlike FAD, which is most often tightly bound to their flavoproteins, NAD<sup>+</sup> is loosely bound to their proteins, and in many cases the NAD<sup>+</sup> binding domain has a Rossman fold.

### 5.1 | NAD<sup>+</sup> biosynthesis and distribution

There are several routes of NAD<sup>+</sup> biosynthesis in animals that require the absorption of at least one of the essential precursors: the different forms of vitamin B3 or the essential amino acid tryptophan. Intestinal absorption of vitamin B3 derivatives is mediated by SLC5A8, SLC22A13, SLC12A8 and SLC29 family members, whereas tryptophan is taken up by SLC7A5, SLC36A4 and other transporters (Figure 5).<sup>196,197</sup> The majority of NAD<sup>+</sup> is produced by the Preiss–Handler pathway initiating from nicotinate.<sup>198</sup> NAD<sup>+</sup> may also be synthesized de novo from tryptophan by the kynurenine pathway, which is thought to operate mainly in liver producing nicotinamide that can be exported to many different tissues and converted into NAD<sup>+</sup> by the NAD<sup>+</sup> salvage pathway. Nicotinamide is also formed by the action of extracellular NAD<sup>+</sup> glycohydrolases (NADases: CD38 and CD73/CD157) and by the intracellular non-redox NAD<sup>+</sup>-dependent enzymes.<sup>199</sup> Furthermore, NAD<sup>+</sup> may be reprinted by other salvage pathways from NMN and nicotinamide riboside.<sup>198</sup> It is noteworthy that NAD<sup>+</sup> homeostasis has been shown to be crucial in health, and several diseases are associated with its unbalance.<sup>200,201</sup>

Intracellular NAD<sup>+</sup> is especially involved in reactions catalysed by dehydrogenases in multiple catabolic pathways, including glycolysis, glutaminolysis and fatty acid  $\beta$ -oxidation (Figure 5). The reducing equivalents of cytosolic NADH, produced, for example, from glycolysis, are shuttled across the mitochondrial membrane by the classic malate–aspartate and glycerol-3-phosphate shuttles,<sup>202–204</sup> to intramitochondrial NAD<sup>+</sup>, which is re-oxidized to NAD<sup>+</sup> by complex I of the respiratory chain.





**FIGURE 5** NAD<sup>+</sup> metabolism and transport in a typical human cell. The schematic overview shows NAD<sup>+</sup>, vitamin B3 and tryptophan transporters (yellow), NAD<sup>+</sup>-using enzymes (red), enzymes metabolizing NAD<sup>+</sup> (olive green), enzymatic reactions (black arrows), reactions with unidentified enzymes (grey arrows) and distribution (red arrows). 3-HCDHs, 3-hydroxyacyl-CoA dehydrogenases; 3-OH-2 MB-CoA, 3-hydroxy-2-methylbutyryl-CoA; 3-OH-acyl-CoA, 3-hydroxy-acyl-CoA; 3-OH-B, 3-hydroxybutyrate; AcAc, acetoacetate; ADHs, aldehyde dehydrogenases; BC-2-oxoacids, branched-chain 2-oxoacids; BCAA, branched-chain amino acids; BCKDHc, branched-chain  $\alpha$ -ketoacid dehydrogenase; GDH, glutamate dehydrogenase; ICDH, isocitrate dehydrogenase; MAS, malate–aspartate shuttle; MDH, malate dehydrogenase; ME, malic enzyme; MPC, mitochondrial pyruvate carrier; NAAD, nicotinic acid adenine dinucleotide; NADS, NAD<sup>+</sup> synthase; NAMN, nicotinate mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, nicotinate phosphoribosyltransferase; NMN, nicotinamide mononucleotide; NMNAT, NMN adenylyl transferase; NR, nicotinamide ribonucleotide; OADHc, 2-oxoadipate dehydrogenase complex; OGDHc, 2-oxoglutarate dehydrogenase complex; PARPs, poly(ADP-ribose) polymerases; PDHc, pyruvate dehydrogenase complex; P5CS,  $\Delta^1$ -pyrroline-5-carboxylate synthase; QH<sub>2</sub>, reduced ubiquinone; SIRTs, sirtuins

NAD<sup>+</sup> is distributed in all cellular compartments; measures indicate a cytosolic free NAD<sup>+</sup> concentration of about 0.1 mM and twice as much in mitochondria.<sup>205</sup> However, other measures indicate 0.3–0.8 mM total cellular NAD<sup>+</sup>.<sup>206</sup> In the nucleus, NAD<sup>+</sup> is required for the de-acetylation of histones mediated by sirtuins and

poly(ADP-ribose) polymerases, which are involved in DNA damage repair.<sup>198</sup> MC family members have been identified to be responsible for mitochondrial and peroxisomal import of NAD<sup>+</sup> from the cytoplasm. In peroxisomes, human SLC25A17 and plant PXN have been shown to transport NAD<sup>+</sup> along with other nucleotides

and cofactors.<sup>146,147,207</sup> In animal mitochondria, net import of NAD<sup>+</sup> is catalysed by NAD<sup>+</sup> transporters that will be described briefly here, because they have been treated thoroughly in a recent review.<sup>49</sup>

## 5.2 | Mitochondrial NAD<sup>+</sup> transport

The EPRA method has been used to identify and characterize two NAD<sup>+</sup> transporter isoforms of the MC family in *S. cerevisiae* (Ndt1p and Ndt2p)<sup>9</sup> and in *A. thaliana* (AtNDT1 and AtNDT2),<sup>182</sup> whereas genetic approaches and indirect biochemical and complementation assays were used to identify the human SLC25A51 as NAD<sup>+</sup> transporter.<sup>11–13</sup>

Yeast Ndt1p and Ndt2p localize to mitochondria, as shown by GFP-fusion protein constructs. Ndt1p transports NAD<sup>+</sup> (Km of 0.38 mM) as well as (d)AMP, (d)GMP, NAAD and several other nucleotides to a lesser extent.<sup>9</sup> Both Ndt1p and Ndt2p deletion strains display growth defects on non-fermentable carbon sources and reduced mitochondrial NAD<sup>+</sup> levels; in double knockout cells these levels are even further decreased.<sup>9,208</sup> Based on these results, it was suggested that the main physiological role of Ndt1p and Ndt2p is mitochondrial NAD<sup>+</sup> import in exchange for (d)AMP or (d)GMP.<sup>9</sup>

The plant AtNDT1 and AtNDT2, which have 26–34% sequence identity with their yeast homologues, transport NAD<sup>+</sup> (Km values of 0.25 and 0.15 mM, respectively), NAAD, NMN, ADP and AMP, and less efficiently NAMN, FAD, FMN, and other nucleotides.<sup>182</sup> AtNDT1 and AtNDT2 are localized to mitochondria, and their expression in yeast Ndt1p- and Ndt2p-deletion strains complements the growth defect and mitochondrial NAD<sup>+</sup> transport.<sup>182,209</sup> The plant mitochondrial NAD<sup>+</sup> carriers are particularly expressed in energetically active and developing tissues, and their depletion leads to impaired reproduction.<sup>182,209,210</sup> Therefore, AtNDT1 and AtNDT2 most likely mediate the import of NAD<sup>+</sup> into mitochondria, possibly in exchange for ADP or AMP.<sup>182</sup>

Given that the closest human homologue of the yeast and plant NAD<sup>+</sup> carriers, SLC25A32, does not transport NAD<sup>+</sup><sup>211</sup> and the subcellular localization of the NAD<sup>+</sup> biosynthesis enzymes in animals is rather uncertain, the existence of an animal mitochondrial NAD<sup>+</sup> transporter has been enigmatic for quite a long time. Several pieces of evidence have recently pointed towards SLC25A51 as a mitochondrial NAD<sup>+</sup> importer (Figure 5), although this carrier has not yet been characterized by direct transport measurements. That SLC25A51, which is ubiquitously expressed in humans and has an isoform with 96% sequence identity (SLC25A52), has a central role in metabolism was demonstrated by SLC25A51 knockout

and knockdown in several cell lines causing defects in mitochondrial respiration, TCA cycle and fatty acid oxidation.<sup>11–13</sup> Furthermore, (a) its functional role was linked to other mitochondrial cofactor transporters, the plasma membrane glucose transporter SLC2A1 as well as to one-carbon and glutathione metabolism, (b) SLC25A51 knockdown led to a reduction of mitochondrial NAD<sup>+</sup> levels and transport, whereas overexpression of the protein had opposite effects, and (c) reduced mitochondrial respiration and NAD<sup>+</sup> levels of the human SLC25A51 knockout cells were rescued by expression of yeast Ndt1p and, vice versa, the growth defect, diminished mitochondrial NAD<sup>+</sup> levels and mitochondrial transport of NAD<sup>+</sup> was complemented by SLC25A51 expression in the yeast Ndt1/Ndt2 double knockout.<sup>11–13</sup> SLC25A51 lacks several of the conserved residues found in almost all MCs, and in particular, at variance with nucleotide-transporting MCs, it does not have G[IVLM] in contact point II nor does it cluster together with them in phylogenetic trees.<sup>49</sup> These sequence features make SLC25A51 different from the NAD<sup>+</sup> carriers of yeast and Arabidopsis as well as from the other MCs for B-vitamin-derived cofactors. Until now, no disease has been associated with SLC25A51. Based on the above-described results, SLC25A51 most likely mediates mitochondrial NAD<sup>+</sup> import.

## 5.3 | Role of NAD<sup>+</sup> in mitochondria

In the mitochondrial matrix, NAD<sup>+</sup> is used as a cofactor in redox reactions catalysed by numerous enzymes (Figure 5), for example, the E3 component (dihydrolipoamide dehydrogenase, DLDH), which is common to all the 2-oxoacid dehydrogenase complexes (PDHc, OGDHc, OADHc and BCKDHc) discussed in Section 2.3; other enzymes of, or connected to, the TCA cycle: isocitrate dehydrogenase, malate dehydrogenase (also involved in the malate–aspartate shuttle) and malic enzyme; of amino acid catabolism: glutamate dehydrogenase 2,  $\delta$ -1-pyrroline-5-carboxylate dehydrogenase (ALDH4A1) and 3-hydroxyacyl-CoA dehydrogenases (HCD2, DHB8 and HCDH); and of ketone body and alcohol metabolism: aldehyde dehydrogenases (ALDH2, AL1B1) and 3-hydroxybutyrate dehydrogenase (BDH). NADH, obtained from most of these reactions, is then re-oxidized to NAD<sup>+</sup> by complex I, which transfers two electrons to the electron transfer chain.

Sirtuins 3–5 are mitochondrial non-redox enzymes that require NAD<sup>+</sup> to remove various modifications (e.g., acetylations, lipoylations, biotinylations, succinylations and malonylations) on lysine residues of proteins producing nicotinamide as a byproduct.<sup>212</sup> Given

that the above-mentioned modifications generally cause inhibition of the protein activities, these regulatory sirtuins usually act as activators of enzymes, such as PDHc and LCAD, but also others involved in fatty acid oxidation, BCAA catabolism, TCA cycle, urea cycle, ATP production and ketone body synthesis. In humans, the nicotinamide produced by the sirtuins of the mitochondrial matrix might be recuperated into  $\text{NAD}^+$  by a salvage pathway; an NMN adenyl transferase (NMNAT3) has been localized to the matrix, but no nicotinamide phosphoribosyltransferase that would have been required for  $\text{NAD}^+$  recycling.<sup>213,214</sup> These observations evoke the questions of the other fates of mitochondrial nicotinamide and NMN (are they also transported?) and an alternative role of NMNAT3.<sup>49</sup>

## 6 | CONCLUSIONS/PERSPECTIVES

In the last two decades, a substantial progress has been made concerning the distribution of the vitamin B-derived cofactors and their metabolism/recycling enzymes in the various cellular compartments, and especially about their transporters across the cellular plasma membrane and the membranes of the intracellular organelles. In particular, a considerable advancement has been reached in the knowledge about how thiamine pyrophosphate, coenzyme A, FAD and  $\text{NAD}^+$  are transported into mitochondria, used by enzymes in alternative ways and recycled. However, as noted in this review, many pieces in the puzzle are missing to comprehend mitochondrial transport and metabolism of vitamin B-derived coenzymes. Some of the key points listed below should be explored to improve our understanding of this matter.

1. Some mitochondrial enzymes involved in cofactor metabolism/recycling have not yet been identified or their subcellular localization is unclear. These uncertainties have important consequences on the comprehension of the transporter functions in vivo. For example, in the case that ThPP phosphatase, which transforms ThPP in ThMP, does not exist in mitochondria, ThMP would be an unlikely counter-substrate for TPC-mediated ThPP mitochondrial import. Similarly, if nicotinamide phosphoribosyltransferase, which converts nicotinamide in NMN, is not localized within the mitochondria, a mitochondrial nicotinamide and/or NMN transporter would be required. Another example is COASY: if COASY is only localized to the cytoplasm, SLC25A42 most likely would mediate mitochondrial CoA import; if COASY is dually localized in mitochondrial matrix and cytoplasm, the

matrix variant could take part in a CoA salvage pathway; and, finally, if COASY is localized only to the matrix, 4'-phosphopantetheine would have to be imported into mitochondria and SLC25A42 would probably export CoA to the cytoplasm.

2. The substrate specificities and modes of transport (uniport/antiport) have not been characterized for the mitochondrial FAD carrier SLC25A32 and  $\text{NAD}^+$  carrier SLC25A51 by direct transport measurements (e.g., the EPRA method) using purified proteins. Given the presence of FADS1 in mitochondria, one could hypothesize that SLC25A32 catalyses FMN import and FAD export. Under the current state of knowledge, several hypotheses can be made about which  $\text{NAD}^+$ -derivatives are transported by SLC25A51 into the mitochondria and about which, if any, are the counter-substrates.
3. Other transporters for the same coenzymes in the inner mitochondrial membrane may exist, especially because other members of the MC family have been shown to have overlapping substrate specificity. There are already some clues suggesting that this is the case also for some of the vitamin B-derived coenzyme transporters. The close phylogenetic relationship between SLC25A42 and SLC25A16 as well as between SLC25A51, SLC25A52 and SLC25A53 might suggest similar substrates within each of these two groups of carriers. Furthermore, the fact that no growth defect of the *S. cerevisiae* *tpc1* $\Delta$  strain was observed when grown on non-fermentable carbon sources suggests the existence of another mitochondrial ThPP transporter. However, there is no close homologue of Tpc1p in yeast, which might indicate that a more distant member of the MC family or a transporter belonging to another protein family is involved in the import of ThPP into mitochondria of yeast cells grown on non-fermentable carbon sources.
4. The precise physiological roles of the vitamin B-derived coenzyme transporters in vivo remain to be investigated. These studies, which have already started by analyses of single gene overexpression or knockout in cells and model organisms, and are being much helped by the biochemical characterization of the purified transporters and their metabolic context, would benefit from the use of combined pairs/sets of gene knockouts and multiple-parameter analyses of their phenotypes. Furthermore, an improved and enlarged analysis of the phenotypes of the genetic variants causing vitamin B-related pathologies could contribute to a better understanding of the physiological roles of the transporters as well as the pathogenetic mechanisms underlying the major symptoms of these diseases. Such knowledge may further be used for

future improvement of disease diagnosis and development of new therapeutic protocols.

- For the vitamin B-derived coenzymes pyridoxal phosphate, biotin and cobalamin, no mitochondrial transporter has been identified yet. This is another interesting area of future research that might involve members of transporter families different from the MC family.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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