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STATE-OF-THE-ART REVIEW

# The mitochondrial coenzyme Q junction and complex III: biochemistry and pathophysiology

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**Keywords**
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Coenzyme Q (CoQ, ubiquinone) is the electron-carrying lipid in the mitochondrial electron transport system (ETS). In mammals, it serves as the electron acceptor for nine mitochondrial inner membrane dehydrogenases. These include the NADH dehydrogenase (complex I, CI) and succinate dehydrogenase (complex II, CII) but also several others that are often omitted in the context of respiratory enzymes: dihydroorotate dehydrogenase, choline dehydrogenase, electron-transferring flavoprotein dehydrogenase, mitochondrial glycerol-3-phosphate dehydrogenase, proline dehydrogenases 1 and 2, and sulfide:quinone oxidoreductase. The metabolic pathways these enzymes are involved in range from amino acid and fatty acid oxidation to nucleotide biosynthesis, methylation, and hydrogen sulfide detoxification, among many others. The CoQ-linked metabolism

**Abbreviations**
ACADL, acyl-CoA dehydrogenase long chain; ACADM, acyl-CoA dehydrogenase; ACADM, acyl-CoA dehydrogenase medium chain; ACADS, acyl-CoA dehydrogenase short chain; ALDH18A1, aldehyde dehydrogenase 18 family member A1; ALDH4A1, aldehyde dehydrogenase 4 family member A1; ALDH7A1, aldehyde dehydrogenase 7 family member A1; AOX, alternative oxidase; BCS1L, BC1 (ubiquinol-cytochrome c reductase) synthesis-like; BHMT, betaine-homocysteine S-methyltransferase; CAD, carbamoyl phosphate synthetase 2; CEPT1, choline/ethanolamine phosphotransferase 1; CHDH, choline dehydrogenase; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CK, choline kinase; CoQ, coenzyme Q; COX, cytochrome c oxidase; CPT2, carnitine palmitoyltransferase 2; CYC1, cytochrome c1; DHODH, dihydroorotate dehydrogenase; DMGDH, dimethylglycine dehydrogenase; ECHS1, enoyl-CoA hydratase; ETFA, electron-transferring flavoprotein subunit alpha; ETFB, electron-transferring flavoprotein subunit beta; ETFDH, electron-transferring flavoprotein dehydrogenase; ETHE1, ETHE1 persulfide dioxygenase; ETS, electron transport system; FA, fatty acid; FAO, fatty acid oxidation; FMN, flavin adenine mononucleotide; GCDH, glutaryl-CoA dehydrogenase; GK, glycerol kinase; GLUL, glutamate-ammonia ligase; GPD1, glycerol-3-phosphate dehydrogenase; GPD2, mitochondrial glycerol-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSH, oxidized glutathione; HADH, hydroxyacyl-CoA dehydrogenase; HADHA, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha; HADHB, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta; IMS, intermembrane space; LYRM7, LYR motif containing 7; MIM, mitochondrial inner membrane; MT-CYB, mitochondrially encoded cytochrome b; mTHF, 5-methyltetrahydrofolate; MTP, mitochondrial trifunctional protein; $NAD^+$, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; Ndi1, rotenone-insensitive NADH-ubiquinone oxidoreductase, mitochondrial; Ndufs4, NADH:ubiquinone oxidoreductase subunit s4; OXPHOS, oxidative phosphorylation; PARK2, parkin RBR E3 ubiquitin protein ligase; Parl, presenilin-associated rhomboid-like; PCYT1A, phosphate cytidylyltransferase 1A; Pdss2, decaprenyl diphosphate synthase subunit 2; PEMT, phosphatidylethanolamine N-methyltransferase; PPi, pyrophosphate; PRODH, proline dehydrogenase; PRPP, phosphoribosyl pyrophosphate; Q, oxidized coenzyme Q; QH2, reduced coenzyme Q; RISP, rieske iron-sulfur protein; ROS, reactive oxygen species; SARDH, sarcosine dehydrogenase; SQOR, sulfide:quinone oxidoreductase; SQSTM1, sequestosome 1; SUOX, sulfite oxidase; TCA cycle, tricarboxylic acid cycle; THF, tetrahydrofolate; TPI1, triosephosphate isomerase 1; TST, thiosulfate sulfurtransferase; TTC19, tetratricopeptide repeat domain 19; UMP, uridine monophosphate; UMPS, uridine monophosphate synthetase; UQCC, ubiquinol-cytochrome c reductase complex assembly factor; UQCRB, ubiquinol-cytochrome c reductase binding protein; UQCRC2, ubiquinol-cytochrome c reductase core protein 2; UQCRFS1, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1; UQCRQ, ubiquinol-cytochrome c reductase complex III subunit VII; URA1, URAcil requiring 1.



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depends on CoQ reoxidation by the mitochondrial complex III (cytochrome $bc_1$ complex, CIII). However, the literature is surprisingly limited as for the role of the CoQ-linked metabolism in the pathogenesis of human diseases of oxidative phosphorylation (OXPHOS), in which the CoQ homeostasis is directly or indirectly affected. In this review, we give an introduction to CIII function, and an overview of the pathological consequences of CIII dysfunction in humans and mice and of the CoQ-dependent metabolic processes potentially affected in these pathological states. Finally, we discuss some experimental tools to dissect the various aspects of compromised CoQ oxidation.

## Introduction

The basic mechanisms of chemiosmotic energy conversion are analogous in all life on Earth. Reduced substrate molecules (such as NADH) initiate an electron transfer process through a series of membrane-embedded or membrane-associated enzymes and mobile electron carriers toward a terminal electron-accepting substrate such as oxygen. The electron transfer is coupled to translocation of protons ($H^+$) across the membrane, generating an electrochemical membrane potential [1]. Quinones are mobile lipid molecules that shuttle electrons and protons between the redox enzyme complexes in the membrane during the chemiosmotic energy conversion. A class of quinones called menaquinones have been suggested as the original electron carrier even before the last universal common ancestor (LUCA) of all life. After the dramatic rise in atmospheric oxygen, approximately 2.3 billion years ago ubiquinones (coenzyme Q, CoQ) evolved and took over as specific electron carriers in most bacteria and all mitochondria today [2].

The mammalian mitochondrial electron transport system (ETS) comprises the electron transfer-initiating dehydrogenases such as complex I (CI, NADH-ubiquinone oxidoreductase) and complex II (CII, succinate dehydrogenase), the membrane-embedded mobile electron carrier CoQ, the CoQ-cytochrome *c* oxidoreductase (complex III, CIII, cytochrome $bc_1$ complex), the soluble mobile electron carrier cytochrome *c*, and the terminal oxidase the cytochrome c oxidase (complex IV, CIV) (Fig. 1). CI is a NADH dehydrogenase, which oxidizes NADH to $NAD^+$ and reduces—that is, transfers electrons to—CoQ with concomitant proton translocation to the mitochondrial intermembrane space (IMS). CII oxidizes succinate to fumarate and transfers electrons to CoQ via its FAD cofactor. In addition, at least seven other mitochondrial inner membrane dehydrogenases mediate electron transfer to CoQ. The CoQ, having been reduced ($QH_2$, Fig. 1) by the dehydrogenases, enters CIII for reoxidation, in which one electron per CoQ passes on to the soluble IMS protein cytochrome *c*. CIV then transfers the electrons to molecular oxygen, reducing it to water (terminal oxidation). In the literature, this process is also called respiratory chain or electron transport chain, but here we use the term ETS in order to emphasize the multienzyme convergent electron flow to the CoQ pool, a process that is not a linear chain of electron transfer reactions. In mitochondria, CoQ pool forms a junction through which all electrons flow, and which connects multiple entry points into the ETS [3]. In this review, we shall discuss the biochemistry and pathophysiology of the mitochondrial CoQ junction, with an emphasis on CIII and seven of the nine dehydrogenases that depend on CoQ oxidation by CIII.

## The mitochondrial CoQ junction

CIII, or more generally cytochrome $bc_1$ complex, is an evolutionarily ancient dimeric enzyme and its three catalytic subunits are highly conserved among bacteria and eukaryotes [4]. In its simplest form, such as in the prokaryote *Rhodobacter capsulatus*, the cytochrome $bc_1$ complex contains only cytochrome *b*, Rieske iron-sulfur protein (RISP), and cytochrome $c_1$. Here, we limit our focus on the mitochondrial (eukaryotic) $bc_1$ complex, in which additional subunits (e.g., in mammals altogether 11) have evolved with largely unknown functions [5]. As a remnant of the evolutionary history predating the birth of the eukaryotic cell, mtDNA still encodes the cytochrome *b* subunit (MT-CYB) while all the other subunits, including the ancient RISP and CYT-C1, are encoded by the nuclear genome and imported into the mitochondria. The assembly of the 11-subunit complex is an intricate process the details of which have been reviewed quite extensively elsewhere [5]. A large fraction of CIII dimers interacts

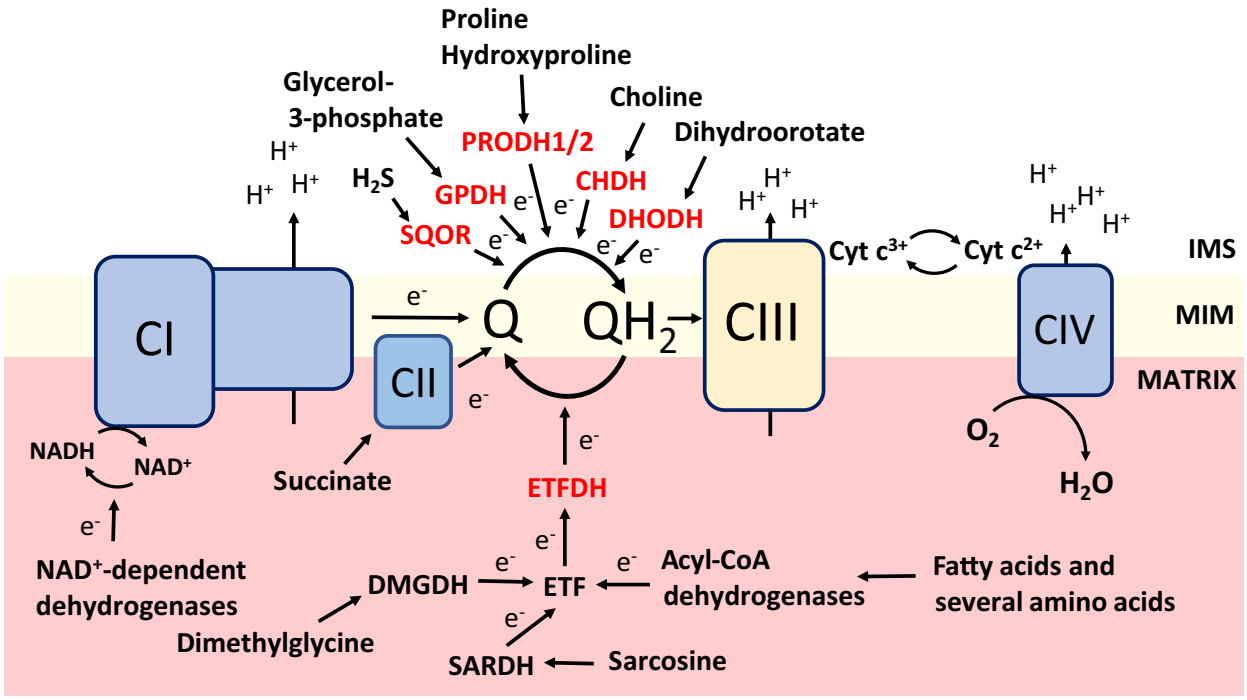


**Fig. 1.** The mammalian mitochondrial electron transport system (ETS) comprises complexes I-IV and enzymes (in red font) linked directly or via electron-transferring proteins to the CoQ pool. For clarity, CHDH, DHODH, ETFDH, GPD2, PRODH1/2, and SQOR are depicted in the intermembrane space (IMS), but they are all associated peripherally with the mitochondrial inner membrane (MIM). (CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CHDH, choline dehydrogenase; DHODH, dihydroorotate dehydrogenase; ETFDH, electron-transferring flavoprotein dehydrogenase; GPD2, mitochondrial glycerol-3-phosphate dehydrogenase; PRODH1/2, proline dehydrogenase 1/2; SQOR, sulfide:quinone oxidoreductase; DMGDH, dimethylglycine dehydrogenase; ETF, electron-transferring flavoprotein; SARDH, sarcosine dehydrogenase).

physically and relatively stably with CI and CIV to form so-called respiratory supercomplexes [6]. A recent review gives an extensive overview on the organization of supercomplexes in cells with defective CIII [7]. Some recent reports suggest that also the mitochondrial fatty acid oxidation enzymes MTP and ETFDH can interact with supercomplexes via binding to CI and CIII, respectively [8]. Supercomplexes have been proposed to compartmentalize the reaction centers of CoQ and cytochrome *c* to provide kinetic advantage and minimize harmful side reactions leading to superoxide production. However, it remains possible that supercomplexes are merely an inevitable consequence of the high protein density in the MIM [9]. Further structural and biochemical studies will undoubtedly clarify this in future.

Mammalian CIII has four key functions: (a) electron transfer from CoQ to cytochrome *c* (and further to molecular oxygen via CIV, the terminal oxidase), (b) re-oxidization of CoQ to maintain the quinone pool redox status, (c) proton translocation to the intermembrane space (IMS), and (d) superoxide production. The general mechanism by which cytochrome $bc_1$ complexes catalyze a net reaction leading to quinol oxidation is termed the Q cycle [4,10]. The cycle starts at the quinol oxidation site ($Q_o$), where the Fe-S cluster of RISP accepts the first electron from a quinol molecule, converting it to an unstable semiquinone. Subsequently, a low-potential heme (heme $b_L$ of cytochrome *b*) accepts the second electron from the semiquinone, restoring quinone. RISP transfers the first electron to the CIII subunit cytochrome $c_1$, which then reduces the heme of the soluble electron carrier cytochrome *c*. Simultaneously, the second electron is passed on from heme $b_L$ via heme $b_H$ to a second quinone molecule at the quinone reduction site ($Q_i$). Thus, both quinol oxidation and quinone reduction take place, but because only one of the two electrons entering CIII passes on to quinone reduction, the net reaction results in quinol oxidation.

Electrons can leak from several sites of the ETS, and this results in partial reduction of molecular oxygen to superoxide ($O_2^{\cdot -}$), an oxygen radical and a precursor of other reactive oxygen species (ROS) [11,12].

The semiquinone at the $Q_o$ site of CIII is one of the 11 known mitochondrial sources of superoxide. Uniquely, CIII can emit superoxide to both sides of the MIM. In the IMS, superoxide is rapidly dismutated to hydrogen peroxide ($H_2O_2$), which can exit from the cytoplasm and participate in cellular signaling. Mutations and chemical inhibitors that affect CIII can either increase or decrease ROS production. Typically, mutations or inhibitors (e.g., antimycin A) that block the $Q_i$ site enhance superoxide production, whereas $Q_o$ site blockade (e.g., with stigmatellin) results in decreased ROS production [11].

In summary, compromised CIII function may result in.

- Decreased proton motive force, causing loss of membrane potential and thus compromised ATP production by the ATP synthase, as well as the import/export of many molecules. At the cellular level, this may be partially compensated by increasing glycolysis or other means of ATP production.
- Inhibition of electron flow from CI and CII to CoQ, resulting in compromised $NAD^+$ generation at CI and inhibition of the TCA cycle, which is linked to CII. The former can lead to problems in a multitude of $NAD^+$-dependent processes, such as redox reactions, protein ADP-ribosylation (essential in e.g., DNA damage repair), and sirtuin-mediated protein deacetylation [13]. The latter can impede anaplerosis, that is, the replenishment of TCA cycle intermediates that have been redirected for various biosynthetic pathways.
- Increased or decreased ROS production via electron leak or reverse electron transfer due to an overreduced CoQ pool [14], which may cause oxidative damage to the ETS and other proteins, lipids, and nucleic acids or alter cellular redox balance and compromise ROS signaling, respectively. Altered ROS production may be compensated by modulating multiple cellular ROS detoxification and defense mechanisms.
- General inhibition of CoQ-dependent mitochondrial dehydrogenases. Theoretically, this may impair a wide variety of metabolic processes from pyrimidine nucleotide biosynthesis to cellular redox ($NAD^+$/NADH) balance, methylation reactions, hydrogen sulfide detoxification, and fatty acid oxidation in a tissue-specific manner. We shall discuss these enzymes, often overlooked in discussion on ETS dysfunction, in more detail below.

Collectively, impairment of these molecular and cellular-level processes leads to the pathology in OXPHOS diseases in humans, but their relative contribution to the widely varying and tissue-specific manifestations is only beginning to be understood. At the tissue and organ level, an important role has emerged for mitochondria in cell fate determination and development, as well as stem cell maintenance [15]. For example, during adipogenic differentiation of human mesenchymal stem cells, there is an increase in oxidative metabolism and mitochondrial biogenesis. Increased CIII-mediated ROS production and subsequent activation of peroxisome proliferator-activated receptor gamma (PPARγ) transcriptional response was suggested to be essential for adipocyte differentiation [16]. Again, very little is known about the involvement of stem cell dysfunction in OXPHOS diseases.

In the next two sections, we shall move on to the consequences of CIII dysfunction *in vivo* by discussing human CIII deficiencies and their animal models.

## Human CIII deficiencies

Diseases caused by mutations in genes encoding CIII subunits or its assembly factors are collectively called CIII deficiencies. These diseases have been reviewed in detail already [17]. Briefly, mutations in the mtDNA-encoded *MT-CYB* subunit were the first identified mutations underlying CIII deficiency in 1999 [18,19]. Extremely rare mutations in the *UQCRB*, *UQCRQ*, *UQCRC2*, *UQCRFS1* (*RISP*), and *CYC1* subunit genes have been thus far identified in patients with primarily juvenile-onset mitochondrial diseases (Table 1). The most common cause of CIII deficiency is mutations in the gene coding for the BCS1L assembly factor. BCS1L is an ATPase located in the inner mitochondrial membrane and acts as a translocase for the Rieske iron-sulfur protein (RISP) subunit of CIII [20–22]. In yeast and mammalian cells with mutated BCS1L, RISP assembly into CIII is compromised, which leads to loss of CIII activity [23]. The homoheptameric translocase BCS1L (bcs1 in yeast) performs the astonishing task of retrotranslocating the folded RISP from the mitochondrial matrix across the MIM by an airlock-like mechanism that preserves the membrane potential, as shown in two recent elegant structural biology papers [22,24–26]. So far, 35 different *BCS1L* mutations have been reported in various compound heterozygous combinations and ten of them as homozygous mutations. The resulting phenotypes vary from the neonatal lethal GRACILE syndrome or GRACILE-like disease, usually caused by homozygous mutations, to the mild Björnstad syndrome with neurosensory hearing loss and an apparently normal life span [17,27]. The most thoroughly characterized of these is the GRACILE

**Table 1.** Genes mutated in human CIII deficiency.

| Gene | Phenotype (no. of patients) | OMIM | References |
|---|---|---|---|
| CIII subunits | | | |
| *MT-CYB* | Cardiomyopathy, exercise intolerance, multisystem disorder (< 20) | 516020 | see OMIM |
| *UQCRB* | Hypoglycemia, lactic acidosis, transient liver dysfunction (1) | 191330 | [31] |
| *UQCRQ* | Severe psychomotor retardation, mildly elevated blood lactate (1) | 612080 | [32] |
| *UQCRC2* | Liver failure, lactic acidosis, hypoglycemia (4) | 191329 | [33,34] |
| *UQCRFS1* (RISP) | Lactic acidosis, cardiomyopathy, alopecia totalis (2) | 191327 | [35] |
| *CYC1* | Ketoacidosis, recurrent metabolic crises, hyperglycemia (2) | 123980 | [36] |
| Assembly/accessory factors | | | |
| *BCS1L* | Varies from severe visceral (GRACILE syndrome) to mild sensorineural (Björnstad syndrome) (80) | 603647 | [17,37] |
| *TTC19* | Leigh syndrome, progressive neurodegeneration, cerebellar ataxia (13) | 613814 | [17,38–42] |
| *LYRM7* | Leukoencephalopathy, lactic acidosis (12) | 615831 | [43–46] |
| *UQCC2* | Intrauterine growth retardation, lactic acidosis, delayed psychomotor development (2) | 614461 | [47,48] |
| *UQCC3* | Lactic acidosis, hypoglycemia, hypotonia, developmental delay (1) | 616097 | [49] |

syndrome (growth restriction, aminoaciduria, cholestasis, iron overload, lactic acidosis, early death), described in Finland [23,28,29]. A single homozygous missense mutation (*c.A232G*, Ser78Gly) in *BCS1L* causes GRACILE syndrome [30].

Mutations in the other assembly or ancillary factor-encoding genes *TTC19*, *LYRM7*, *UQCC2*, *UQCC3* have been identified in a few patients and the clinical pictures are similar to the other CIII deficiencies (Table 1).

## Mouse models with CoQ-CIII-CIV segment dysfunction

The mitochondrial CoQ pool can be compromised by defects in the biosynthesis of CoQ or due to a blockade in ETS upstream of CoQ. The polymerization of the CoQ isoprenoid tail and the quinone head group modifications take place in a multiprotein complex of at least 12 proteins in MIM (reviewed in [50] and [51]). Constitutive knockouts of enzymes directly involved in the CoQ biosynthesis have proven embryonically lethal in mice [52]. An adult-onset global knockout of the essential CoQ biosynthesis gene *Coq7* showed that mouse liver and kidney have a surprisingly high amount of reserve CoQ relative to levels required to sustain normal ETS function [53]. In this study, adult mice survived longer than 6 months after the induction of CoQ biosynthesis blockade and a considerable decrease (~ 80% within 2 months) in CoQ levels. Dietary CoQ has poor bioavailability and cells thus rely on *de novo* biosynthesis [51]. Studies employing cell type-specific disruption of CoQ biosynthesis illustrate a strong cell autonomous requirement for the CoQ biosynthesis. Conditional knockouts of *Pdss2*, required for the CoQ isoprenyl tail polymerization, in cerebellar neurons or Purkinje cells cause loss of vitality of the targeted cells [54]. Likewise, targeted knockout of *Coq6* in podocytes compromises this vital cell type in the kidney leading to glomerulosclerosis [55]. Mice with a whole-body knockout of *Coq8a* (previously known as *Adck3*), an enzyme required for the stability of the CoQ biosynthetic complex, are viable, show relatively mild CoQ deficiency, and recapitulate the most frequent form of human CoQ deficiency, autosomal-recessive cerebellar ataxia type 2 [56]. In contrast, the *Coq8a* paralogue *Coq8b* (*Adck4*) is indispensable in mice for postnatal viability [57]. Podocyte-specific *Coq8b* knockout mice are viable and serve as a model of glomerulopathy [57].

Three further physiological mouse models of CoQ deficiency have been reported. A spontaneous missense mutation in *Pdss2* causes an isolated renal disease [58]. López's group has generated two *Coq9* knock-in mutant mouse models carrying patient mutations ($Coq9^{p.R239X}$ and $Coq9^{p.Q95X}$) [59,60]. The exact function of the COQ9 protein remains unclear, but it is needed for the stability and function of the hydroxylase COQ7 [61]. Of these models, $Coq9^{p.R239X}$ mice have a more severe CoQ deficiency and predominant encephalomyopathy as a phenotype while the $Coq9^{p.Q95X}$ mice have a mild myopathy [60]. Despite relatively similar CoQ depletion in different tissues, the $Coq9^{p.R239X}$ mice showed defects in mitochondrial bioenergetics, and interestingly also in the assembly of supercomplexes, only in the brain [59], suggesting a highly tissue-specific thresholds for CoQ depletion.

The introduction of the GRACILE syndrome mutation ($Bcs1l^{c.A232G}$, $Bcs1l^{p.S78G}$) into the mouse genome some 10 years ago led to the successful generation of the first viable mouse model of CIII deficiency [62]

(Table 2). The homozygous *Bcs1l*$^{p.S78G}$ mice are born healthy, but from the fourth week of age recapitulate the human syndrome including growth failure, hepatopathy, renal tubulopathy, and loss of white adipose tissue [62–64]. In 2017, our group noticed that in a slightly different congenic genetic background (C57BL/6JCrl), the survival of the *Bcs1l* mutant mice was dramatically longer, up to 200 days in contrast to the 30-40 days on the original congenic background [65,66]. Whole genome sequencing revealed a novel homoplasmic mtDNA variant (*m.G14904A*), in the gene encoding the cytochrome *b* subunit of CIII (*mt-Cyb*$^{p.D254N}$), in the original mouse colony. We showed that this variant further decreases the CIII activity and aggravates the disease progression in *Bcs1l*$^{p.S78G}$ mice, leading to lethal juvenile metabolic crisis [67]. Most interestingly, analyses of CIII activity, ETS function, whole-body energy metabolism, and gene expression showed that the *mt-Cyb*$^{p.D254N}$ variant is not silent on its own, but has clear metabolic effects [67]. This suggests that these mice qualify as the first *mt-Cyb* mutant mouse model and one of the very few mouse models with a mtDNA mutation in general [68].

*BCS1L* mutations affect CIII indirectly via the assembly of its RISP subunit. Two mutant mouse models targeting RISP directly have been described in the literature (Table 2). Moraes' group generated a conditional (floxed) *Risp* allele (CKO) [69]. Although they did not report this, Cre-excision of this allele in the whole body is likely embryonic lethal. The excision of *Risp* in neurons resulted in severe encephalopathy and short survival of about 3 months [70]. This allele has been utilized by others in studies of specialized cell types such as T cells, in which loss of *Risp* resulted in ROS signaling-dependent defect in T cell activation [71]. In another study, the loss of *Risp* in mouse hematopoietic stem cells disrupted differentiation and induced aberrant cell cycle entry in this cell population, leading to severe anemia [72]. Hekimi's group generated a *Risp* knock-in allele harboring a point mutation (*Risp*$^{p.P224S}$) at the same conserved site that extends life span in *C. elegans* [73]. This mutation was homozygous lethal in mice while the heterozygotes had slightly decreased CIII activity and were healthy. The effect of heterozygous *Risp*$^{p.P224S}$ on lifespan was minimal, conflicting with the *C. elegans* data. The

**Table 2.** Mouse models of complex III deficiency. Abbreviations KI, knock-in, n.d., not determined, n.a., not applicable, CKO, conditional knockout, KO, knockout, HO, homozygous, HE, heterozygous.

| Mutations(s) | CIII activity (% of WT) | Survival | Phenotype | References |
|---|---|---|---|---|
| *Bcs1l*$^{p.S78G}$ KI Ser78Gly | 25–35 liver<br>50–65 kidney<br>50 heart<br>35 muscle<br>(30 days of age) | 6 months | Growth restriction, hepatopathy, tubulopathy, loss of WAT, hypoglycemia, cardiomyopathy, localized cerebral astrogliosis | [62,65,66] |
| *mt-Cyb*$^{p.D254N}$ spontaneous Asp254Asn | 75-100 | n.d. | Decreased energy expenditure and whole-body respiratory exchange ratio (P30) | [67] |
| *Bcs1l*$^{p.S78G}$; *mt-Cyb*$^{p.D254N}$ | 10–25 liver<br>30–40 kidney<br>30–35 heart<br>25 muscle | 35 days | Growth restriction, hepatopathy, tubulopathy, loss of WAT, hypoglycemia, lactic acidosis | [62,67] |
| *Risp* CKO (Δexon2) in neurons (CaMKIIα-Cre) | 25 cortex<br>14 hippo-campus | 3–3.5 months | Neuronal cell death and mild astrogliosis at end stage | [69,70] |
| *Risp*$^{p.P224S}$ KI Pro224Ser | HO n.a.<br>HE 80-85 liver | HO lethal<br>HE males 10% decrease | HO embryonic lethal<br>HE males 10% decrease in life span | [73] |
| *Uqcrq* CKO (Δexon 1) in endothelium (Cdh5CreERT2) | n.d. | 15–30 days | Tamoxifen at P0-4: severely compromised angiogenesis, lethal by P30 | [74] |
| *Ttc19* KO (Δexon7) | 40–50<br>brain, liver, skeletal muscle | >18 months | Motor dysfunction, astrogliosis, decreased energy expenditure (females) | [76] |
| *Parl* KO and CKO (Δexon2-3) | ~ 70 brain | 2 months | Leigh-like severe encephalomyelopathy | [77,81] |

identification of the first patient mutations in *RISP* as recently as in 2020 [35] gives the possibility to attempt to generate a more useful mouse model based on these mutations. Recently, Chandel's group published a *Uqcrq* CKO allele in which the first exon is flanked by loxP sites [74]. They only excised the allele postnatally in the endothelium using a tamoxifen-inducible VE cadherin promoter, but this alone caused juvenile lethality (P15-30) and severe angiogenesis defects.

Two further mouse models, with an indirect decrease in CIII activity, have been established (Table 2). Mutations in the *TTC19* gene were first identified as a cause of CIII deficiency in 2011 [75]. A later mechanistic study proposed that TTC19 is required for the clearance of RISP N-terminal cleavage fragments from CIII, completing the assembly of the complex [76]. *Ttc19* KO mice have a fairly mild and slowly progressing disease characterized by motor dysfunction and astrogliosis [76]. The CIII activity in these mice is 40–50% of wild-type in the brain, liver, and skeletal muscle. Finally, knockout of the conserved rhomboid family mitochondrial protease PARL was found to cause a Leigh syndrome-like severe encephalopathy [77]. PARL was previously shown to proteolytically cleave several mitochondrial proteins, including PINK1 and PGAM5, but Spinazzi *et al.* showed that these substrates are not causally linked to the encephalopathy. In contrast, their data suggest that impaired proteolytic processing of TTC19 (see above), combined with loss of several CoQ biosynthetic enzymes (COQ3, 4, 5), leads to the CIII deficiency in *Parl* KO mice. They further showed that the total CoQ concentration was decreased and the ratio reduced/oxidized CoQ increased in the mutant brain tissue already presymptomatically, indicating compromised CoQ oxidation.

The respiratory electron transfer continues from CIII to the soluble electron carrier cytochrome *c* and through CIV finally to oxygen. Disruption of this segment of the ETS necessarily compromises also CIII function and CoQ oxidation, although the CoQ redox ratio has rarely been measured in CIII-CIV segment deficiencies. *Cytochrome c* mutations have not been found in patients with mitochondrial disease. A knock-in mutation targeting the apoptotic role of cytochrome *c* causes embryonic lethality or severe dysgenesis leading to early neonatal lethality [78]. Postnatal ablation of cytochrome *c* in mouse neurons causes a severe neurodegeneration phenotype [79]. Unlike other ETS complexes, CIV harbors several subunits with tissue-specific isoforms, the ablation of which retains viability but causes tissue-specific pathology in mice [80]. Constitutive knockouts of single-isoform subunits or assembly factors of CIV generally lead to embryonic lethality, and therefore, most viable CIV deficiency models carry conditional knockout alleles. A recent review exhaustively covers CIV deficiencies in humans and model organisms [80]. The outcome of both CIII and CIV deficiencies, in theory, should be compromised CoQ oxidation, but how the blockade of these two different sites of ETS differ respective to CoQ pool redox status, ROS production, OXPHOS efficiency, and mitochondrial membrane potential remains poorly understood.

## CoQ-dependent dehydrogenases

Altogether, seven mitochondrial noncanonical ETS enzymes use oxidized CoQ directly as electron acceptor (Tables 3 and 4) and are therefore dependent on CoQ oxidation by CIII. Although some of these enzymes have been widely studied with respect to the metabolic routes they are part of, there has been relatively little attention to how ETS dysfunction, such as in mitochondrial diseases, affects them. In addition, the converse question, how the impairment of the Q pool-dependent enzymes might contribute to the pathology in mitochondrial diseases, deserves more attention. Next, we turn to the function of these enzymes and the biochemical pathways involved, concentrating on the mammalian enzymes and their associated disease phenotypes.

### CHDH—choline dehydrogenase

Mammalian choline dehydrogenase catalyzes the oxidation of choline to glycine betaine aldehyde, which is further oxidized to betaine (also known as trimethylglycine) [93] (Fig. 2). Choline metabolism mainly takes place in the liver. Choline is a conditionally essential nutrient for animals, meaning that it must be obtained from the diet as such or in the form of choline phospholipids. *De novo* biosynthesis via a hepatic phosphatidylethanolamine N-methyltransferase pathway exists in humans and other animals, but this is insufficient to satisfy the need. In the 1930s, choline deficiency was found to lead to fatty liver disease in animals [94]. Currently, choline-deficient or methionine and choline-deficient diets are widely used to induce experimental liver injury resembling human nonalcoholic steatohepatitis in mice [95]. The main physiological use of choline is in the CDP-choline pathway of nucleus and endoplasmic reticulum, which synthesizes choline phospholipids, vital components in cell membranes, and very low-density lipoproteins. Choline is also the precursor of the essential neurotransmitter

**Table 3.** CoQ pool-dependent human mitochondrial enzymes. Abbreviations PQQ, pyrroloquinoline quinone, FMN, flavin mononucleotide, FAD, flavin adenine dinucleotide, 4Fe-4S - iron-sulfur cluster.

| Enzyme | Abbr | EC no. (human) | Cofactor | Reaction | CoQ-independent ortholog/ alternative |
|---|---|---|---|---|---|
| Choline dehydrogenase | CHDH | 14.03.2001 | PQQ (?) | choline + Q => betaine aldehyde + $QH_2$ | *Arthrobacter globiformis* choline oxidase (codA) [82] *Arabidopsis thaliana* choline monooxygenase [83] |
| Dihydroorotate dehydrogenase | DHODH | 1.3.5.2 | FMN | (S)-dihydroorotate + Q => orotate + $QH_2$ | *Saccharomyces cerevisae* URA1 [84] *Lactococcus lactis* pyrDB [85] |
| Electron-transferring flavoprotein dehydrogenase | ETFDH | 1.5.5.1 | FAD, 4Fe-4S | reduced ETFA + Q => oxidized ETFA + $QH_2$ + $H^+$ | ? |
| Glycerol-3-phosphate dehydrogenase, mitochondrial | GPD2 | 1.1.5.3 | FAD | glycerol-3-phosphate + Q => dihydroxyacetone phosphate + $QH_2$ | *Lactobacillus brevis* NADH oxidase (LbNOX) [74] |
| Proline dehydrogenase 1 | PRODH1 | 1.5.5.2 | FAD | L-proline + Q => (S)-1-pyrroline-5-carboxylate + $QH_2$ + $H^+$ | Bacterial dye-linked L-PRODH enzymes? [86] |
| Proline dehydrogenase 2 (hydroxyproline dehydrogenase) | PRODH2 (HYPDH) | 1.5.5.3 | FAD | trans-4-hydroxy-L-proline + Q => (3R,5S)-1-pyrroline-3-hydroxy-5-carboxylate + $QH_2$ + $H^+$ | ? |
| Sulfide:quinone oxidoreductase | SQOR | 1.8.5.4 | FAD | $H_2S$ + $2H^+$ + $SO_3^{2-}$ + Q => $S_2O_3^{2}$ + $QH_2$ | ? |

**Table 4.** Molecular genetics of CoQ pool-dependent enzymes.

| Enzyme | Highest expression[a] | Biological processes | Human diseases (MIM#) | Mutant mouse phenotype |
|---|---|---|---|---|
| CHDH | Kidney, liver | Methionine cycle, one-carbon metabolism, lipid biosynthesis | None reported | Ko: viable, male and female infertile[2] |
| DHODH | Liver | DNA replication & repair, transcription, ribosome biogenesis, hexosamine biosynthesis, glycosylation | Miller syndrome, postaxial acrofacial dysostosis (MIM 263750) | KO: preweaning lethality (100%)[2] |
| ETFDH | Liver, heart, skeletal muscle, endocrine tissues | β-oxidation, generation of acetyl-coa | Multiple acyl-coa dehydrogenase deficiency, glutaric aciduria IIC (MIM 231680) | KO: preweaning lethality (100%)[2] |
| GPD2 | BAT, skeletal muscle, brain, pancreatic β cells | Glycerol phosphate shuttle, $NAD^+$/ NADH balance, lipid metabolism | Type 2 diabetes mellitus, susceptibility to (MIM 125853) | KO: decreased viability, fertility and WAT mass in C57BL/6 background [87], slightly decreased body weight [88] |
| PRODH | Small intestine, skin, brain, lung | ATP production by proline oxidation, protein turnover | Hyperprolinaemia, type I (MIM 239500) Schizophrenia, susceptibility to, 4 (MIM 600850) | Spontaneous mutant strain (PRO/Re): viable, fertile, defective sensorimotor gating [89] |
| PRODH2 | Kidney, liver, testis | Dietary and endogenous hydroxyproline degradation | None reported | Ko: viable, healthy, decreased urinary oxalate and glycolate [90] |
| SQOR | Large intestine, leukocytes, lung | $H_2S$ signaling, intestinal $H_2S$ detoxification | Leigh-like disease [91] | $Sqor^{\Delta N}$: severe post-weaning growth defect, ataxia, death by 10 weeks of age [92] |

[a]According to Human Protein Atlas (https://www.proteinatlas.org/) and references, [2]International Mouse Phenotyping Consortium (https://www.mousephenotype.org/), MIM, Mendelian Inheritance in Man databasewww.omim.org/.

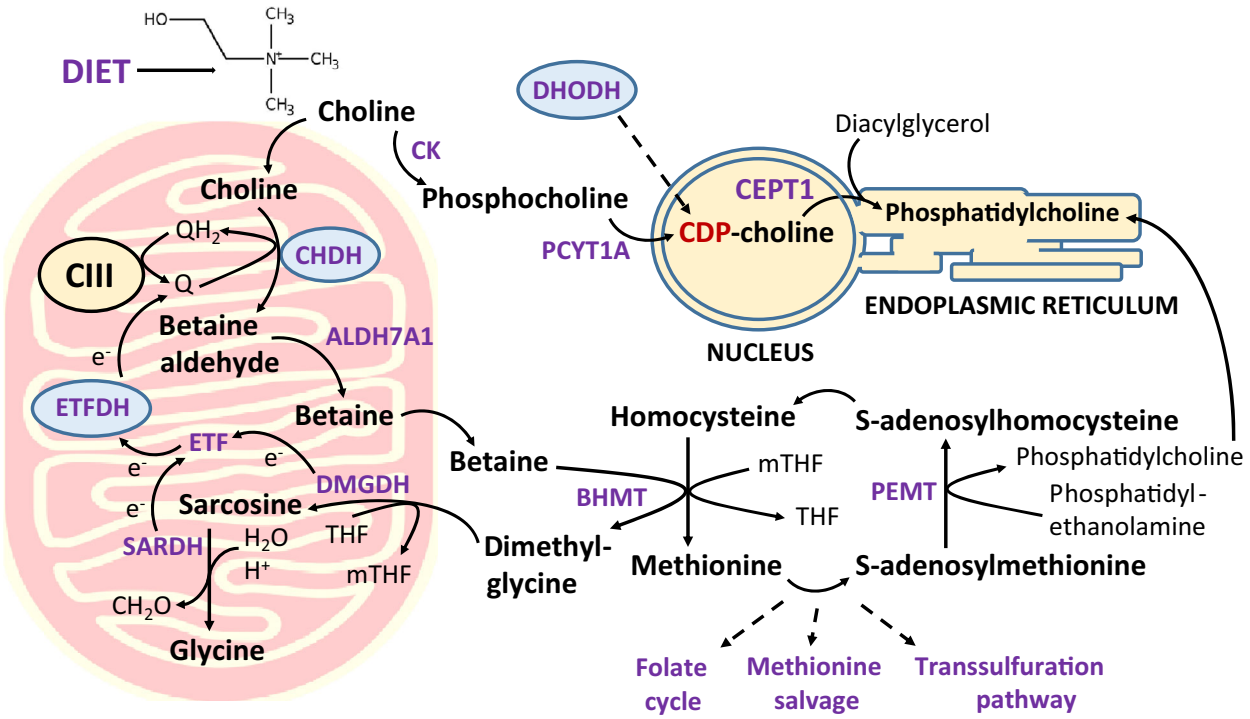


**Fig. 2.** Choline degradation produces betaine, an important methyl donor. The methionine cycle is an ancient, evolutionarily conserved metabolic pathway, which recycles the amino acids methionine and homocysteine to synthesize S-adenosylmethionine, the main methyl donor in the methylation of nucleic acids, histones and nonhistone proteins and many other molecules. The cartoon is modified from Teng and Zeisel [99]. The drawing representing a mitochondrion was modified from an image downloaded from a public png library (https://www.pngegg.com/en/png-yncgz). (CK, choline kinase; PCYT1A, phosphate cytidylyltransferase 1A, choline; CDP, cytidine diphosphate; ALDH7A1, aldehyde dehydrogenase 7 family member A1; BHMT, betaine-homocysteine S-methyltransferase; THF, tetrahydrofolate; mTHF, 5-methyltetrahydrofolate; PCYT1A, phosphate cytidylyltransferase 1A, choline; CEPT1, choline/ethanolamine phosphotransferase 1; PEMT, phosphatidylethanolamine N-methyltransferase).

acetylcholine [96]. Betaine is an important methyl donor in the remethylation of homocysteine to methionine in the methylation cycle. The cytoplasmic enzyme betaine-homocysteine S-methyltransferase (BHMT) catalyzes this step and thus links choline oxidation to the methionine cycle and further to the transsulfuration pathway (which generates the sulfur metabolites cysteine, glutathione, and hydrogen sulfide), the folate cycle, and methionine salvage [97]. Perhaps surprisingly, *CHDH* knockout mice are viable and overtly healthy, although both males and females are infertile (Table 4). While mutations in genes coding for choline transporters and enzymes in other branches of choline-related metabolism cause severe childhood-onset neurological and metabolic disease, no disease-causing *CHDH* mutations have been reported in humans [96]. There are hardly any cell biological studies related to CHDH, but one paper suggested that CHDH is required for PARK2-mediated mitophagy via interaction with the mitophagy receptor SQSTM1 on the mitochondrial outer membrane [98].

## DHODH—dihydroorotate dehydrogenase

DHODH was first isolated in 1953 from the bacterium *Clostridium oroticum* [100] and since then more than 500 publications have investigated the structure, function, and roles in pyrimidine nucleotide biosynthesis and metabolism of DHODHs from numerous organisms. In the early 1980s, studies on chick embryo cells exposed to chemical mitochondrial DNA (mtDNA) depletion ($\rho^0$ or rho zero cells) revealed that respiration-deficient cells are auxotrophic for pyrimidine nucleosides. Moreover, Grégoire *et al.* showed that the critical respiration-dependent enzyme was DHODH, the activity of which could be restored with the artificial electron acceptor menadione (vitamin K3) in respiration-deficient cells [101]. The first human $\rho^0$ cell lines, reported in 1989, turned out to be auxotrophic for uridine as well [102]. Since these discoveries, it has become customary to supplement the medium of respiration-defective cells, such as fibroblasts from patients with a mitochondrial disease, with uridine.

A large trifunctional enzyme named CAD (carbamoyl phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) catalyzes the first three steps in the pyrimidine biosynthesis pathway [103] (Fig. 3). The reactions involved take in glutamine, bicarbonate, ATP, and aspartate and generate dihydroorotate, which is the crucial precursor for pyrimidine biosynthesis. The third step is, however, reversible and favors carbamoyl-aspartate over dihydroorotate [104]. The flavoenzyme DHODH catalyzes the fourth enzymatic step, the CoQ-coupled oxidation of dihydroorotate to orotate [105]. Orotate then undergoes ribosylation and decarboxylation by the fusion enzyme uridine monophosphate synthase (UMPS) to yield uridine monophosphate, a precursor for all pyrimidine nucleosides [106]. Balanced levels of pyrimidine nucleotides are vital for RNA and DNA synthesis. Apart from this, uridine nucleotides are also critical for numerous other metabolic pathways in the form of UDP-activated sugars such as UDP-glucose (glycogen synthesis), UDP-galactose (protein N-glycosylation), UDP-glucuronic acid (detoxification, proteoglycan synthesis), and UDP-N-acetylglucosamine (protein O-N-acetylglucosaminylation) [107]. Cytidine nucleotides, derived from uridine, serve as similar activator molecules in the biosynthesis of phospholipids, glycolipids, cardiolipin, and some glycoproteins [108].

DHODH inhibitors have been extensively investigated as a treatment for cancer, immunological disorders, bacterial and viral infections, and parasitic diseases [103]. Brequinar, a highly potent DHODH inhibitor, failed in phase II clinical trials for cancer [109]. Another DHODH inhibitor leflunomide and its active metabolite teriflunomide are currently indicated in rheumatoid and psoriatic arthritis, in which they moderate the excessive proliferation of rapidly dividing lymphocytes and alleviate inflammation [110]. In mice, leflunomide is teratogenic, causing fetal death and skeletal and visceral malformations [111]. In line with this, *DHODH* missense mutations cause Miller syndrome, or postaxial acrofacial dysostosis (Table 4), a rare monogenic condition manifesting as malformations in the craniofacial area, arms, hands, and/or feet [112].

Most knowledge of the cellular effects of direct or indirect DHODH inhibition derives from proliferating cultured cells and tumor grafts. Inhibition of DHODH depletes cellular UMP, UDP, and UTP in cultured cells, mitogen-activated T cells, embryonic tissues, and tumor grafts [111,113,114], but nonproliferating cells are less affected *in vivo* [113]. Curiously, DHODH inhibition can also partially deplete purine nucleotides in some cell types [114–116]. In line with these changes, direct DHODH inhibition causes replication and ribosomal stress [113,117]. Studies employing $\rho^0$ or CIII-deficient cells in combination with an alternative oxidase (*AOX*) xenogene to bypass CoQ oxidation blockade have shown that the only essential function of ETS for the growth of some cancer cells is to allow DHODH activity [118,119]. Compromised CoQ oxidation can inhibit DHODH, but some evidence suggests that DHODH inhibition can also cause mitochondrial dysfunction on its own [116,120,121]. Interestingly, DHODH inhibition is sufficient to induce the expression of GDF15 [122], one of the best circulating markers of mitochondrial dysfunction [123].

Taken together, DHODH function is dependent on functional CoQ oxidation, but to what extent is pyrimidine nucleotide biosynthesis compromised in mitochondrial diseases and if inadequacy of this vital

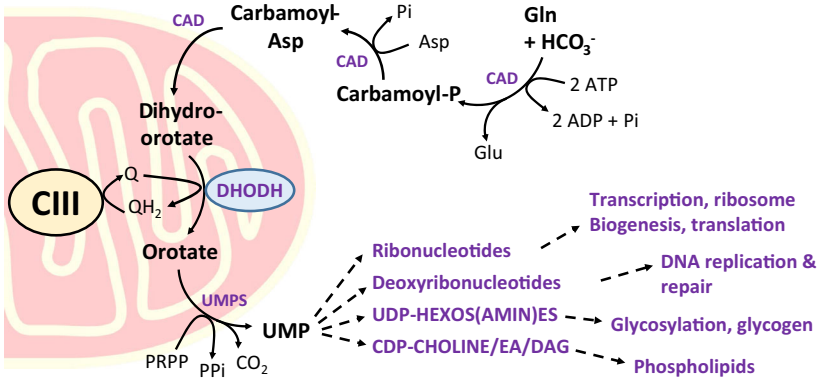


**Fig. 3.** DHODH links pyrimidine nucleotide biosynthesis to the mitochondrial ETS. UMP is the precursor of all uridine, thymidine, and cytidine nucleotides, the corresponding deoxyribonucleotides, UDP-hexoses and UDP hexosamines. Pyrimidine nucleotide biosynthesis and choline metabolism converge at the level of CDP-choline/ethanolamine/diacylglycerol needed for the phospholipid biosynthesis. (CAD, carbamoyl phosphate synthetase 2, aspartate transcarbamylase and dihydroorotase; UMPS, uridine monophosphate synthetase; PRPP, phosphoribosyl pyrophosphate; PPi, pyrophosphate; UMP, uridine monophosphate).

biosynthetic route explains some of the mitochondrial dysfunction-related tissue phenotypes remains poorly understood.

## ETFDH—electron-transferring flavoprotein dehydrogenase

Fatty acid (FA) β-oxidation (FAO) is important for the production of ATP and biosynthetic precursors in many cell types [124]. In mammals, FAO becomes crucial during fasting, when glucose supply is limited and FAs mobilized from the white adipose tissue. Some tissues such as heart, skeletal muscle, and kidney utilize FAO extensively even in the fed state. A prolonged fasting stimulates FAO to the extent that the acetyl-CoA generated diverts to the production of ketone bodies, primarily in the liver, which then circulate to other organs for a local acetyl-CoA regeneration [125].

Plasma membrane fatty acid transporters, such as CD36 and fatty acid transport proteins, facilitate and regulate FA uptake into the cell, while several cytoplasmic fatty acid-binding proteins assist in intracellular FA trafficking [126]. The metabolism of FAs requires their activation with CoA. Acyl-CoA synthetases (ACSLs) perform this job in an ATP-consuming reaction immediately upon FA entry into the cell or on the surface of cytoplasmic organelle membranes. Fatty acyl-CoAs diffuse to the MIM, where those with a chain length of >13 carbons translocate to the mitochondrial matrix for β-oxidation via the carnitine shuttle. Shorter fatty acyl-CoA do not need assisted translocation, and very long-chain (>21 carbons) fatty acids do not enter mitochondria at all, but instead undergo oxidation in peroxisomes [124].

The mitochondrial FAO is a cyclic process started by oxidation of the α and β carbons of the fatty acyl-CoA (Fig. 4). The enzymes responsible are acyl-CoA dehydrogenases (ACADs), and they are specific to chain length: long-chain (ACADL), medium-chain (ACADM), and short-chain (ACADS) dehydrogenases [124]. This is a step at which FAO connects to the CoQ pool via two proteins: the heterodimeric electron-transferring flavoprotein (ETF, encoded by *ETFA* and *ETFB* genes) and ETFDH. ETF is also the entry point into the ETS for electrons from dehydrogenases involved in one-carbon metabolism (sarcosine and dimethylglycine dehydrogenases) [127,128], and catabolism of the amino acids lysine, hydroxylysine, and tryptophan (glutaryl-CoA dehydrogenase) [129]. The mitochondrial trifunctional protein (MTP), which consists of two subunits, HADHA and HADHB, performs the next three steps in the cycle of

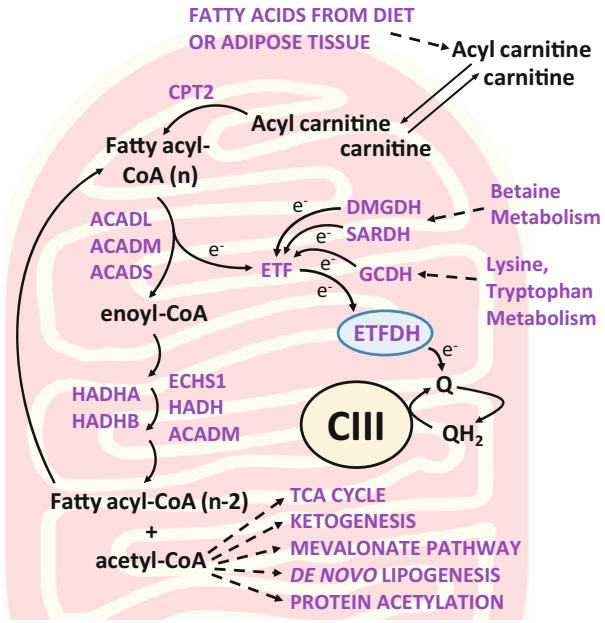


**Fig. 4.** The catabolism of fatty acids, betaine, lysine, and tryptophan feed electrons to CoQ via the electron-transferring flavoprotein ETF and its dehydrogenase ETFDH. The end product of fatty acid oxidation, acetyl-CoA, is a precursor for various biosynthetic processes and acetylation reaction. (CPT2, carnitine palmitoyltransferase 2; ACADL, acyl-CoA dehydrogenase long chain; ACADM, acyl-CoA dehydrogenase medium chain; ACADS, acyl-CoA dehydrogenase short chain; CoA, coenzyme A; HADHA, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha; HADHB, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta; ECHS1, enoyl-CoA hydratase, short chain 1; HADH, hydroxyacyl-CoA dehydrogenase; ACADM, acyl-CoA dehydrogenase, medium chain; GCDH, glutaryl-CoA dehydrogenase; TCA cycle, tricarboxylic acid cycle).

mitochondrial FAO [124]. A different set of enzymes perform the corresponding reactions for short-chain FAs, and the oxidation of unsaturated fatty acyl-CoAs requires additional enzymatic steps. Each cycle reduces one CoQ via the ETF-ETFDH path and generates one NADH and one acetyl-CoA. While NADH is a direct substrate for CI, acetyl-CoA can enter the TCA cycle and generate three more NADH and reduce one CoQ via CII. Overall, mitochondrial FAO connects to the CoQ pool at three different sites of ETS: ETFDH, CI, and CII.

Mutations in many genes encoding FAO enzymes cause rare metabolic diseases. *ETFA*, *ETFB*, and *ETFDH* mutations cause multiple acyl-CoA dehydrogenase deficiencies, also named glutaric aciduria types IIA-C, respectively, with severe multiorgan pathology and metabolic crises [130]. Interestingly, some ETFDH mutations have been associated with a secondary CoQ

deficiency [131]. No reported phenotypes of *Etfdh* knockout mice exist in the literature, but mouse phenotyping databases report 100% neonatal lethality. Published data on secondary ETDH deficiency in disorders of CoQ oxidation are scarce and indirect. Some patients with isolated CIII, CIV, or ATP synthase deficiency have shown blood acyl-carnitine profile suggestive of compromised ETFDH function [19,132,133]. Hypoketotic hypoglycemia, a highly typical phenotype in the disorders of FAO, is also a phenotype of some mitochondrial diseases involving defects in CIII, CIV or mtDNA maintenance [134–136]. Unfortunately, more detailed studies in model organisms are largely yet to be performed. In juvenile CIII-deficient mice ($Bcs1l^{p.S78G}$), metabolomics showed an extensive hepatic accumulation of acyl carnitines [63]. Hypoketotic hypoglycemia is also a phenotype of these mice on standard chow [65,137]. However, the $Bcs1l^{p.S78G}$ mice tolerate, and even benefit, from ketogenic diet, which implies that they are able to adapt for a metabolic state requiring a high rate of FAO [65]. These mice also have a rather normal systemic lipid mobilization when stressed with a short fasting [137].

## GPD2—mitochondrial glycerol-3-phosphate dehydrogenase

The mitochondrial enzyme GDP2, identified as early as in 1936 [138], and the cytoplasmic GDP1 form the glycerol-3-phosphate (GP) shuttle, a simple metabolic loop that regenerates $NAD^+$ from glycolysis-derived NADH in the cytoplasm and donates electrons via CoQ to the ETS [139] (Fig. 5). Mammalian tissues express highly variable levels of GPD2. Its highest levels are in brown adipose tissue (BAT), skeletal muscle, and brain and the lowest in liver and heart. It is of historical interest that studies of the insect flight muscle, which possesses extremely high GDP2 activity, led to the proposal of the existence of the GP shuttle in the 1950s [140,141]. The main metabolic role of the GP shuttle is the reoxidation of NADH produced by glycolysis, which enables cytosolic ATP production without the accumulation of an intermediate by-product such as lactic acid. Both GDP1 and GPD2 are highly active and in BAT, where the GP shuttle supports glycolysis via NADH reoxidation. Glycolytic ATP generation is important in BAT despite the high rate of fatty acid oxidation, because almost all energy generated by OXPHOS is released as heat via uncoupling protein 1. Simultaneously, the GP shuttle may support BAT thermogenesis by feeding additional reducing equivalents to mitochondria. Finally, the GP shuttle may be involved in the regulation triglyceride synthesis, because esterification of glycerol-3-phosphate to acylglycerol-phosphate is the first and rate-limiting step of lipid synthesis [139].

*Gdp2* KO mice show several interesting genetic background-dependent phenotypes. Brown *et al.* reported that in mixed 129X1/SvJ × C57BL/6J genetic background, homozygous KO animals appeared at the expected frequency (25%). After five to seven generations of backcrosses to C57BL/6J, the frequency of KO animals dropped to 14% [87]. The cause of the embryonic or juvenile lethality has not been determined to date.

## PRODH1 and PRODH2—proline dehydrogenase 1 and 2

Mammalian PRODH1 catalyzes the oxidation of L-proline to Δ1-pyrroline-5-carboxylate leading to electron transfer to CoQ [142] (Fig. 6). This intermediate

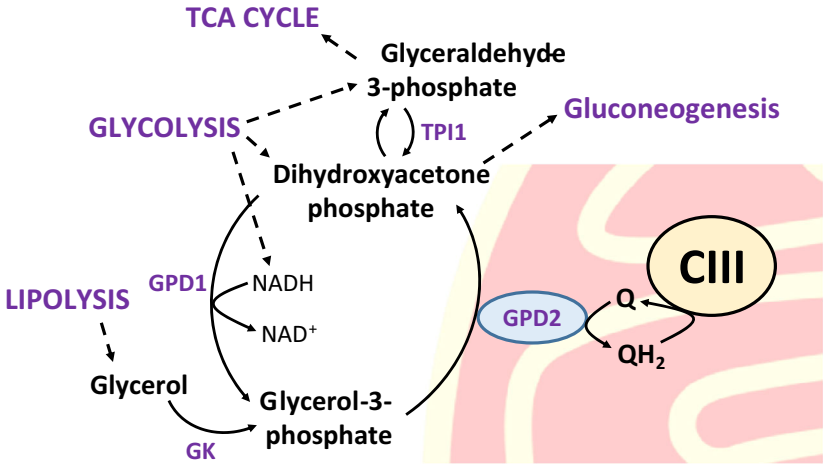


**Fig. 5.** GDP1 and GDP2 run the glycerol phosphate shuttle between the cytoplasm and mitochondria. (GK, glycerol kinase; GPD1, glycerol-3-phosphate dehydrogenase, cytoplasmic; TPI1, triosephosphate isomerase 1).

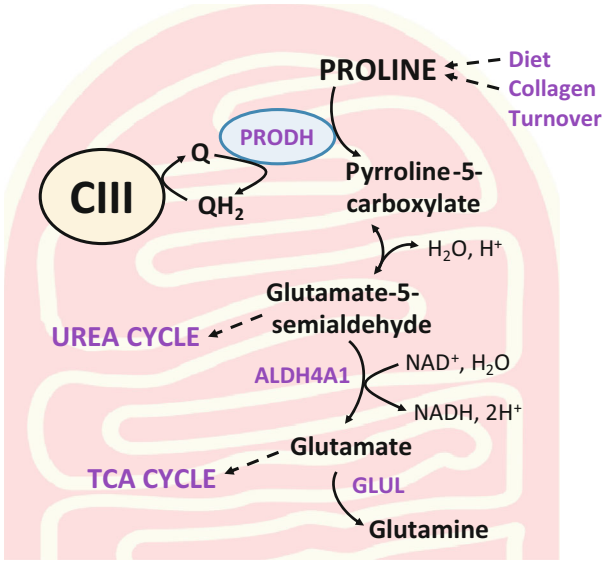


**Fig. 6.** Diet- and collagen turnover-derived proline is an efficient fuel for mitochondria. Its degradation products can be utilized by the urea cycle and the TCA cycle. (ALDH4A1, aldehyde dehydrogenase 4 family member A1; GLUL, glutamate-ammonia ligase).

hydrolyzes nonenzymatically to glutamic semialdehyde, which is further oxidized to glutamate in an $NAD^+$-dependent reaction catalyzed by ALDH4A1. Proline oxidation is an excellent source of energy as oxidation of one molecule of proline can yield 30 ATP equivalents. Some flying insects like the honeybee (*Apis mellifera*) have a high proline content in their hemolymph and this is rapidly consumed during flight, possibly providing a rapid energy source at the beginning of a flight. Therefore, proline metabolism is especially important in nutrient stress because proline is available in large amounts from the breakdown of extracellular matrix, particularly the proline-rich collagens, and can under such conditions be used to drive the ETS for ATP production [142]. PRODH activity has been suggested to be an important contributor to cellular ROS production. However, this likely takes place indirectly via other mitochondrial dehydrogenases [143].

The importance of proline catabolism is underscored by the fact that *PRODH1* and *ALDH4A1* mutations cause hyperprolinaemia type 1 and 2, respectively. The clinical pictures of both can be vague and variable, but neurological manifestations such as mental retardation, seizures, and behavioral problems have been reported, as well as renal abnormalities in some patients (Table 4).

Collagens, the main class of extracellular matrix proteins, contain a very large proportion of hydroxyproline, which is formed post-translationally during collagen maturation. Thus, there is free hydroxyproline derived from dietary collagen and from the turnover of muscle, connective tissue, and bone. Most of this is catabolized in the renal proximal tubules, and minor amounts in the liver. The enzymatic steps are partially different from proline degradation. The initial step is catalyzed by PRODH2 whereas the next step, conversion of 3-hydroxy-1-pyrroline-5-carboxylate into 4-hydroxyglutamate is catalyzed by ALDH41, the same enzyme that acts in proline degradation. The last two enzymes again differ: glutamic-oxaloacetic transaminase 2 and 4-hydroxy-2-oxoglutarate aldolase 1 catalyze the formation of glyoxylate and pyruvate instead of glutamate, which is the end product of proline degradation. No *PRODH2* mutations have been reported in humans and the mouse phenotype is mild [90], implying that there are alternative catabolic routes for hydroxyproline degradation.

As for the involvement of proline oxidation in OXPHOS diseases, Terburgh *et al.* showed in their convincing metabolomics analyses of CI-deficient (*Ndufs4* KO) muscle that proline oxidation (along with the GP shuttle, the ETF/ETFDH system, and CII) compensate the CI deficiency by fueling the Q cycle [144]. Intriguingly, proline biosynthesis was shown to be highly upregulated at mRNA and protein level (e.g., ALDH18A1 protein > 1000-fold increased) and the ratio of proline to its precursor glutamate increased in the heart of several different KO mouse models with defective mtDNA maintenance [145]. We found robust accumulation of proline in the heart of end-stage $Bcs1l^{p.S78G}$ mice and a modest upregulation of ALDH18A1 (1.8-fold) at mRNA level [66].

## SQOR—sulfide quinone oxidoreductase

Hydrogen sulfide was abundant in the prebiotic Earth, suggesting that it was a key molecule to the origin of life, and also a major source of energy for primitive life for billions of years [146]. The dramatic increase in atmospheric oxygen about 600 million years ago ended the era of $H_2S$ as an abundant energy source, but the long evolutionary history was not lost and $H_2S$ remains an important metabolite even in humans today. In mammals, $H_2S$ is a gaseous signaling molecule with multiple established and proposed physiologic functions in the cardiovascular system, in the nervous system, and as a protectant against reactive nitrogen species [146].

$H_2S$ is generated intracellularly from the sulfur-containing amino acids cysteine and methionine in several enzymatic reactions (Fig. 7). In most tissues, the

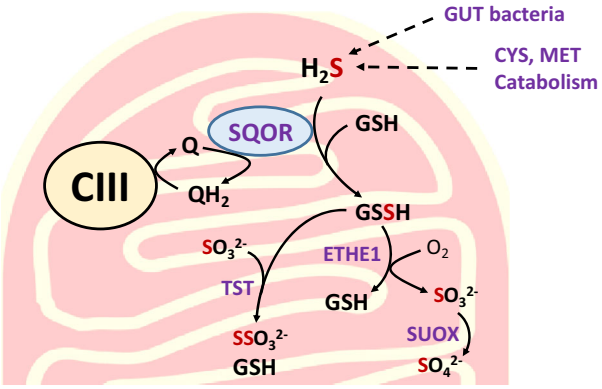


**Fig. 7.** Hydrogen sulfide is both an important gaseous signaling molecule and a toxin that is detoxified by the ETS-linked enzyme SQOR. (GSH, reduced glutathione; GSSH, oxidized glutathione; ETHE1, ETHE1 persulfide dioxygenase; TST, thiosulfate sulfurtransferase; SUOX, sulfite oxidase).

majority of $H_2S$ is produced by cystathionine γ-lyase upon conversion of L-cysteine to L-serine. $H_2S$ is also formed by cystathionine β-synthase in the reaction converting homocysteine to cystathionine, the first step in the transsulfuration pathway. Further, cysteine aminotransferase transaminates L-cysteine to 3-mercaptopyruvate, which is then acted on by mercaptopyruvate sulfurtransferase in a reaction releasing $H_2S$. In the brain, D-cysteine oxidation can also serve as a source of 3-mercaptopyruvate and ultimately $H_2S$. Finally, gut bacteria produce considerable amounts of $H_2S$, which is rapidly metabolized in the intestinal epithelium by the CoQ-linked enzyme the sulfide:quinone oxidoreductase (SQOR), fueling the ETS and protecting the neighboring tissues from toxicity [147]. The first steps of $H_2S$ catabolism in humans and many bacteria are identical, in line with its ancient evolutionary origin [148]. Some controversy exists over the physiological sulfur acceptor in the reaction [149,150]. SQOR readily utilizes sulfite and cyanide as sulfur acceptors *in vitro*. However, the much more abundant cellular metabolite glutathione (GSH) most likely serves as the physiological sulfur acceptor, yielding glutathione persulfide oxidized glutathione [150]. The next proposed steps in the $H_2S$ catabolic pathway involve cleavage of the disulfide bond in oxidized glutathione by either persulfide dioxygenase (SDO, gene *ETHE1*) or thiosulfate sulfurtransferase (rhodanese, gene *TST*) in reactions releasing sulfite or thiosulfate, respectively [150]. Sulfite can be further oxidized to sulfate by sulfite oxidase (SUOX), which, interestingly, can utilize cytochrome *c* of ETS as an electron acceptor [151].

Mutations in several genes encoding enzymes involved in $H_2S$ metabolism cause diseases in humans. Mutations in *ETHE1* cause ethylmalonic encephalopathy, a severe neurological and metabolic disease of childhood onset [152], and utilizing an *Ethe1* KO mouse model and patient material, this was shown to be due to toxic sulfide accumulation [153]. S*UOX* mutations lead to severe neurological abnormalities, seizures, and mental retardation [154] and SQOR mutations to Leigh syndrome-like encephalopathy [91]. Interestingly, studies in the fission yeast *S. pombe* first revealed that strains with defects in CoQ biosynthetic genes accumulated $H_2S$ [155]. More recent studies in mammalian cells and tissues have shown that CoQ deficiency decreases SQOR protein amount and impairs SQOR-driven respiration and $H_2S$ detoxification, leading to its toxic accumulation [156,157]. Conversely, supraphysiological CoQ levels transcriptionally upregulate SQOR in cultured human fibroblasts, especially in combination with CI defects [158]. Paradoxically, repression of the transsulfuration pathway, a major source of $H_2S$, accompanied SQOR upregulation in these cells or upon ectopic SQOR overexpression. The connection between CoQ levels, SQOR, and the transsulfuration pathway warrants further studies especially considering that the upregulation of the transsulfuration pathway is a part of the integrated mitochondrial stress response [159].

The acute toxicity of $H_2S$ is thought to be mainly due to inhibition of cytochrome c oxidase (COX, CIV) and mitochondrial respiration [147]. Indeed, COX-inhibiting concentrations of $H_2S$ are similar to those of cyanide ($K_i$ = 0.2 μM) [160]. Full knockout of *Sqor* has not been reported, but Marutani *et al.* recently used CRISPR/Cas9 editing to develop an interesting strain, in which the mitochondrial targeting sequence of SQOR is deleted but protein expression not affected, apparently resulting in loss of mitochondrial SQOR. These mice display severe post-weaning growth defect, cachexia, ataxia, and death by 10 weeks of age [92].

## Tools to bypass or modify CIII-dependent enzymes

### Alternative oxidases

The first observations of respiration that is resistant to cytochrome pathway inhibitors were made in plants already in the 1920s, and in the 1950s this new alternative respiration activity was localized to mitochondria [161]. In the 1970s, the canonical and alternative pathways were shown to divert at the level of CoQ. Finally, a cyanide-resistant quinol oxidase was purified

from plant tissue and named alternative oxidase (AOX) [162]. Today, AOXs are known to be mitochondrial inner membrane proteins of plants, many single-celled eukaryotes like *Trypanosoma*, and some lower animals like tunicates. They are also widely present in yeasts, which is often overlooked because the standard laboratory model *Saccharomyces cerevisae* lacks an AOX gene [163]. AOXs transfer electrons directly from quinols to oxygen, thus bypassing the CIII-CIV axis of ETS [164]. Thus, if CIII is blocked, AOX can restore upstream electron flow through CI-CII and other CoQ-dependent dehydrogenases. However, it does not rescue the full capacity of mitochondrial ATP production as it bypasses the two proton translocases. It is not known why AOX was lost from the vertebrate lineage. However, plentiful data from the fruit fly (*Drosophila melanogaster*), cultured mammalian cells, and mice show that xenogenically expressed AOX is correctly targeted to mitochondria and is apparently inert and harmless in healthy cells and tissues. As predicted, it can render cells and even whole organisms resistant to inhibitors of cellular respiration such as cyanide, as well as by genetic manipulations affecting CoQ oxidation [165–167]. Apart from AOX being an excellent tool to probe the ETS experimentally, another motivation for the studies employing xenogenic AOX expression has been the prospective benefit in OXPHOS diseases. Two transgenic mouse lines expressing tunicate (*Ciona intestinalis*) AOX have been generated and characterized [168,169]. The first demonstration that AOX expression can be dramatically beneficial in a physiologically relevant mouse model of a human mitochondrial disease came in 2019 [66], when our and Jacobs' group published a study in which we crossed the CIII-deficient $Bcs1l^{p.S78G}$ mice with the single-copy $Rosa26^{AOX}$ transgenic mice. Stunningly, AOX expression extended the survival of the homozygotes from median 200 to almost 600 days. This was due to complete prevention of lethal cardiomyopathy. Kidney tubulopathy and cerebral astrogliosis were also ameliorated, while the liver disease was apparently unaffected by AOX in the late stages of disease analyzed [66]. We have since then analyzed the mice at juvenile age (4–5 weeks) and found transient amelioration of the hepatic pathology as well (unpublished). The mechanism(s) of the beneficial AOX effects beyond mitochondria remain largely unknown, but several analyses suggested that amelioration of the increased ROS production due to ETS blockade was not a significant factor in *Bcs1l* mutant mice. Because we did not measure the $QH_2/Q$ ratio in that study, it remains to be investigated to what degree the CoQ pool was overreduced by the CIII deficiency in the affected tissues of the $Bcs1l^{p.S78G}$ mice and what was the effect of AOX on it.

Surprisingly, Dogan *et al.* reported harmful effects of AOX in a skeletal muscle-specific *Cox15* knockout model of complex IV deficiency [170]. In these mice, AOX expression exacerbated some phenotypes of the myopathy. This is puzzling since AOX showed no adverse effects in the CIII-deficient mice [66], including in the skeletal muscle, which has extremely low CIII activity [67]. It is possible that the response to CIII-CIV bypass by AOX depends on the exact nature of the mutation and respiration defect. Nevertheless, the mechanisms and metabolic consequences of xenogenic AOX expression in various models of OXPHOS diseases are only beginning to be understood and many interesting questions remain to be studied.

## CoQ pool-independent enzyme orthologs

Restoring CoQ oxidation with an AOX is a general strategy to relieve some consequences of CIII dysfunction in experimental models. Here, we propose an additional, more specific bypass strategy: xenogenic expression of the CoQ-independent orthologs, that is, functionally equivalent enzymes from other phyla, of the enzymes reviewed here. While the vast variety of plant and microbial orthologs is out of the scope of this review, we shall bring up some examples that might be useful in experimental work (Table 3). Firstly, the *Arthrobacter globiformis* genome encodes a choline oxidase (codA) and the thale cress (*Arabidopsis thaliana*) a choline monooxygenase that are both quinone-independent [82,83]. Ectopic expression of these in mammalian, yeast, or insect cells might help dissect the effects of compromised CoQ oxidation on choline-related metabolism.

Secondly, some single-celled eukaryotes harbor an exclusively cytoplasmic DHODH enzyme, such as URA1 in the baker's yeast (*Saccharomyces cerevisae*) [84]. The microbial DHODHs are divided into two subclasses: homodimeric DHODHs that use fumarate as the electron acceptor (e.g., URA1), and heterotetrameric DHODHs that use $NAD^+$ as the electron acceptor. The latter, for example, DHODH from the dairy bacterium *Lactococcus lactis*, consist of a catalytic subunit pyrDB and an electron-transferring subunit pyrK, which contains an iron-sulfur cluster and a FAD cofactor [85]. We could not find any literature on the expression of nonmitochondrial DHODHs in mammalian cells, but our own data suggest that bakers' yeast DHODH localizes to the cytoplasm and has enzymatic activity when stably expressed in mammalian cells (unpublished). Further studies should investigate whether it can restore uridine autotrophy in

mammalian cells when the mitochondrial DHODH is inhibited or CoQ oxidation compromised, such as in rho$^0$ cells lacking mtDNA. If cytoplasmic DHODH activity can rescue uridine autotrophy, these enzymes could turn out useful experimental tools, in analogy to AOX, in the wide spectrum of topics related to pyrimidine nucleotide biosynthesis.

## Conclusions

The understanding of the structure and function of mitochondria, including the ETS and the CoQ junction, as well as of the molecular pathogenesis of human mitochondrial diseases is progressing at an unprecedented pace. This is largely due to the fast development and refinement of technologies and methodology. However, as we can perhaps conclude from the discussion above, the ETS-linked metabolism is complex and how it is disturbed in mitochondrial diseases is only beginning to be understood. Studies in *Drosophila* and mammalian cells and our work showing dramatic beneficial effects of transgenic AOX expression in CIII-deficient mice indicate that the tissue pathology in OXPHOS diseases is not simply due to ATP deficiency or ROS-related damage but that other CoQ pool-dependent processes must play a major role. Measuring the CoQ redox status in tissues reliably is technically demanding, requiring skillful tissue collection and special analytical equipment. Furthermore, because several enzymes converge at the CoQ junction, it is difficult to simulate the *in vivo* conditions in the activity assays of the CoQ pool-linked enzymes to obtain physiologically meaningful data. Thus, dissecting the CoQ pool-linked metabolism is challenging, but new experimental models and methodologies will undoubtedly help find answers to the question how ETS dysfunction leads to the wide spectrum of manifestations in human OXPHOS diseases and how these can be treated.

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## Conflict of interest

The authors declare no conflicts of interest.

## Author contributions

JK wrote the manuscript draft and prepared the tables and figures. JP and RB assisted in the literature searches, writing, and editing. All authors revised the manuscript and have contributed substantially.

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