Mitochondrial proteins containing coiled-coil-helix-coiled-coil-helix (CHCH) domains in health and disease

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Abstract

Members of the coiled-coil-helix-coiled-coil-helix (CHCH) domain-containing protein family that carry (CX_9C) type motifs are imported into the mitochondrion with the help of the disulfide relay-dependent MIA import pathway. These evolutionary-conserved proteins are emerging as new cellular factors that control mitochondrial respiration, redox regulation, lipid homeostasis or membrane ultrastructure and dynamics. Here, we discuss recent insights on the activity of known (CX_9C) motif-carrying proteins in mammals and review current data implicating the MIA40/CHCHD4 import machinery in the regulation of their mitochondrial import. Recent findings and the identification of disease-associated mutations in specific (CX_9C) motif-carrying proteins have highlighted members of this family of proteins as potential therapeutic targets in a variety of human disorders.

KEYWORDS:

Mitochondrion, respiratory chain, protein import, disulfide relay, metabolic disorder, cancer

Mitochondrial protein import: Historical background and its discovery

Mitochondria play a quintessential role in normal metabolism [1, 2] and lethal signaling processes in the context of physiological or pathological cell death [3, 4]. Mutations in mitochondrial proteins, be they encoded by the mitochondrial or nuclear genomes, have been associated with multiple diseases including cancer, metabolic disorders, neurodegenerative pathologies, diabetes and premature aging [5-8].

A hypothetical reconstruction of cellular evolution postulates that some two billion years ago the organelle appeared in the eukaryotic cell by the engulfment of an endosymbiotic prokaryotic organism. This endosymbiotic relationship was consolidated by the progressive incorporation of originally bacterial genes into the nuclear genome [9, 10], thereby improving the integration of mitochondria in various aspects of cellular metabolism [1, 2, 6, 9-12]. Modern cells control mitochondrial function at several levels by regulating (i) the transcription of nuclear genes, (ii) the cytoplasmic translation of mRNAs, as well as (iii) the import of the proteins into one of the four subcompartments, namely the outer and inner membranes, the matrix and the intermembrane space [5, 13, 14].

The mitochondrial genome of human cells encodes only 13 proteins, while the function of the organelle requires the import of >1000 nuclear-encoded proteins [13, 14]. Studies in yeast revealed that most of the nuclear-encoded mitochondrial proteins pass into the organelle through one general entry gate at the outer membrane, the outer membrane translocase (TOM), which contains the protein Tom40 as its central protein-conducting channel. However, after engaging with the TOM translocon, mitochondrial proteins follow different sorting routes, dictated by specific targeting codes in their sequence or overall structure that guide them to their final destination. Additional translocase machineries like the sorting and assembly

machinery (SAM) complex in the outer membrane, the presequence translocase TIM23 complex and the carrier translocase TIM22 complex, both located in the inner membrane and the MIA40 complex in the intermembrane space (IMS) decode the import signals to finally dispatch each protein toward the appropriate submitochondrial compartment [1, 2, 11, 12]. Recently, the mitochondrial import (MIM) complex that is localized in the outer mitochondrial membrane was revealed to be necessary for the import of α-helical outer membrane proteins that bypass the TOM complex [2]. Mitochondrial import activity can be influenced by the differentiation and activation states of the cell or impacted by pathological conditions such as oxidative stress, aging and imminent cell death [2]. Human disease-associated mutations can affect either the mitochondrial-targeting segment of the nuclear-encoded proteins or specific components of the import machinery [2, 6, 8, 15, 16].

The characterization of the mitochondrial import process has led to the discovery of a family of nuclear-encoded proteins, the coiled-coil-helix-coiled-coil-helix (CHCH) domain (CHCHD) containing proteins, which are imported in the mitochondrial intermembrane space where they participate to activities such as mitochondrial biogenesis, bioenergetics, dynamics or quality control. These proteins are imported with the help of the evolutionary conserved redox active Mia40/CHCHD4 import machinery that catalyzes their oxidative folding through a disulfide relay system [1, 16] (**Figure 1, Key Figure**). Here, we review current knowledge on this fascinating family of proteins.

Import into the mitochondrial intermembrane space

The intermembrane space (IMS) constitutes an essential crossroad for the physiological communication of mitochondria with the rest of the cell [16, 17], as well as for lethal signaling processes [4]. Upon mitochondrial outer membrane permeabilization (MOMP),

which is generally associated with apoptosis [3], a series of proteins that are normally confined in the IMS such as apoptosis-inducing factor (AIF), cytochrome *c,* endonuclease G, HtrA2 peptidase and Diablo homolog are released from the IMS to the extramitochondrial space, where they contribute to cellular dismantling [18, 19].

Proteomic analyses conducted in *S. cerevisiae* [20] or in human cells [21] revealed that all IMS-confined proteins are nuclear-encoded, with their number ranging between 50 to 130. IMS proteins that carry an N-terminal mitochondrial localization sequence (MLS) engage with the matrix-targeting TIM23 complex, but then either become attached to the inner membrane facing the IMS or get released into the IMS as soluble proteins, following the proteolytic cleavage of their N-terminal presequence [1, 2]. A second class of IMS-localized proteins does not possess such an N-terminal targeting sequence. Instead, their import is coupled to cofactor-triggered or redox-regulated folding events that stabilize and trap them in the IMS [16, 17, 22]. Cofactor-triggered IMS entrapment is exemplified by the heme-dependent import and maturation of cytochrome *c,* which is orchestrated by the activity of cytochrome *c* heme lyase (CCHL) [17]. The oxidation-driven import of cysteine motif-carrying proteins depends on a "disulfide relay" pathway that is controlled by the evolutionary conserved oxidoreductase Mia40/CHCHD4 [1, 16, 17, 22].

The Mia40/CHCHD4 protein import machinery and its substrates from yeast to man

The oxidative folding-coupled, Mia40-dependent import of small nuclear-encoded cysteine rich proteins into the mitochondrial IMS was initially discovered in the yeast *Saccharomyces cerevisiae*. The oxidoreductase Mia40, its central component [23-27], acts as an import receptor that performs two functions. During an initial 'sliding step' the peptide-binding cleft of Mia40 specifically binds to the internal IMS-specific Targeting Signal (called ITS or

MISS) of the incoming precursor proteins via hydrophobic interactions. In the subsequent 'docking step', Mia40 binds covalently via an intermolecular disulfide bond engaging the active site CPC tripeptide of the Mia40 to one cysteine of the substrate [28]. Thus, Mia40 acts as a chaperone that induces the folding of the substrate in the first 'sliding' step, followed by a disulfide donor function in the second 'docking' step. The end result of this process is the catalyzed oxidative folding and entrapment of the substrate in the IMS [16, 22, 29, 30]. At the end of its catalytic cycle, after donating a disulfide to the substrate, the Mia40 CPC motif is reduced. The reoxidation of the CPC motif is ensured by the FAD-linked sulfhydryl oxidase Erv1 (human equivalent: ALR) [31-33]. This step is mediated specifically by the natively disordered N-terminal segment of Erv1 [34] that contains a CX_2C motif. The pair of electrons that are released during the formation of the disulfide bond are subsequently transferred in a cascade of reactions from the N-terminal CX_2C motif of Erv1 (C30/C33) to its FAD-proximal CX_2C pair (C130/C133), onto the flavin moiety, and then to cytochrome *c*, cytochrome oxidase (COX) and finally molecular oxygen. Alternatively, Erv1-released electrons can be absorbed by cytochrome C peroxidase (Ccp1) [35]. Although it is known that this pathway can operate under anaerobic conditions, the final electron acceptor in these situations is still elusive.

Even though the principal catalytic and structural features of Mia40, in particular within the segment that contains the redox active cysteine-proline-cysteine (CPC) and the $(CX_9C)_2$ motifs, have been conserved throughout eukaryotic evolution [27, 36-40], there are important differences between yeast and mammals. In contrast to yeast Mia40 (~40 kDa), human CHCHD4 does not possess an N-terminal presequence and hence lacks a membrane anchor [27, 36-38, 40]. Thus, CHCHD4 is a relatively small (16 kDa) soluble protein that is no longer imported via the TIM23 translocase but rather relies on a self-catalytic import process

101 [36, 37]. This switch in the import pathway might have occurred during evolution to couple
102 the import of CHCHD4 more tightly to the IMS redox-signaling pathway, perhaps to achieve
103 rapid adaptive responses in mitochondria responding to ever changing metabolic needs.
104 CHCHD4 has lost its hydrophobic inner membrane anchor, yet has acquired the capacity to
105 interact with a new partner, the flavoprotein AIF [40], which is attached to the inner
106 membrane [41, 42]. The N-terminal 27 residues of CHCHD4 are necessary and sufficient to
107 establish a direct interaction with AIF [40]. Nuclear magnetic resonance (NMR) spectroscopy
108 indicates that the AIF-binding domain of CHCHD4 is part of an N-terminal unstructured lid
109 segment that includes the redox-active center CPC [27]. However, far-UV circular dichroism
110 (CD) spectroscopy analysis suggests that upon its interaction with AIF, the AIF-binding
111 segment of CHCHD4 undergoes important conformational rearrangements that may well
112 affect the interactions of the CHCHD4 CPC domain with its incoming substrates or its
113 reoxidizing partner ERV1/ALR [40]. The conformational status of AIF, which is determined
114 by the binding of its cofactor NADH [43], appears crucial for its interaction with CHCHD4.
115 Addition of NADH enhances the interaction between CHCHD4 and AIF, yet fails to do so
116 when AIF is mutated (G308E) in its NADH-binding domain [40]. Thus far it is not known
117 whether the redox activity of AIF (which is an NADH oxidase) directly affects CHCHD4 as
118 an electron acceptor.
119
120 Prototypic substrates of the redox-active MIA import pathway share conserved coiled coil-
121 helix1-coiled coil-helix 2 (CHCH) domains (CHCHD) containing within each helix two
122 cysteines that are organized as Cx_nC motifs [1, 44]. Typical Mia40 substrates are proteins of
123 less than 25 kDa that carry double cysteine-x_3-cysteine $(Cx_3C)_2$ or double cysteine-x_9-cysteine
124 $(Cx_9C)_2$ motifs [45-47] (**BOX 1**). Recent studies have revealed that Mia40 substrates are not

limited to CX_nC-containing precursors but also include proteins with alternate cysteine motifs that are not organized in a CHCH domain (**BOX1**).

C-X_9-C domain-containing proteins in health and disease

Mia40 substrates that carry $(CX_9C)_2$ motifs participate to a large array of activities that are related to the biogenesis of respiratory chain complexes, lipid homeostasis or mitochondrial dynamics [16, 46-48] (**Figure 1, Key Figure**). The doubling of the number of substrates during evolution from yeast to man [46, 47, 49] and the discovery of human disease-associated mutations affecting members of this family (**Table 2**) reflect the pathophysiological relevance of the MIA import pathway as they offer challenging opportunities to decipher the molecular activity of each of them.

Biogenesis of Respiratory chain complexes

Beyond the aforementioned connection between Mia40/CHCHD4 and Erv1/ALR (which establishes electron flow from Mia40/CHCHD4 to the respiratory chain) (**see BOX 1**), Mia40 has been linked to small $(CX_9C)_2$ motif-bearing proteins such as Cmc1, Cmc2, Cox6B, Coa4, Coa5, Coa6, Cox17, Cox 19 and Cox23 that are implicated in the biogenesis and assembly of respiratory chain complex CIV (cytochrome C oxidase, COX) [16, 46-50] (**Table 1**). Similarly, in mammals, the abundance of CHCHD4 (or that of its partner ALR) correlated with respiratory chain activity and the oxidation kinetics of subunits CMC1, COA4 (also called CMC3) and COX19 [39]. In human cells, the expression levels of CHCHD4 (or that of its binding partner AIF) and the CIV complex copper chaperone COX17 correlate [36, 40]. Indeed, NMR analyses indicate that human COX17 interacts with CHCHD4 via an intermolecular disulfide bond [29, 48]. Missense mutations in several of these CHCHD4 substrates are implicated in human diseases (**Table 2**). For instance, a missense mutation in

the human homolog of the yeast Cox12 (COX6B1), which carries an imperfect $(CX_9C)_2$ cysteine motif, is associated with a severe infantile encephalomyopathy [49, 51](**Tables 1 and 2**). Mutations in the CIV assembly factor COA5 (the human ortholog of yeast Pet191) [47, 52]), which carries a typical $(CX_9C)_2$ motif, can give rise to a mitochondrial cardiomyopathy [53]. Mutations in yet another CIV assembly factor, COA6, which contains an unusual cysteine motif[20, 54], destabilize the newly synthesized mitochondrial DNA-encoded subunit COX2 and lead to neonatal hypertrophic cardiomyopathy [54-58] (**Tables 1 and 2**).

Beyond the evolutionary-conserved substrates implicated in the biogenesis of complex CIV (conserved from yeast to mammals), subunits of complex CI (NDUFB7, NDUFS5, NDUFA8 and NDUFS8) have made their appearance on the list of potential Mia40/CHCHD4 substrates (**Table1**) [39, 49]. Among this group of $(CX_9C)_2$ motif-carrying proteins, NDUFB7 and NDUFA8 are IMS-localized and likely stabilize the assembled CI complex by binding to its surface [59]. NDUFA8 carries a duplicated $(CX_9C)_2$ motif, and the kinetics of its mitochondrial import are controlled by CHCHD4 and ALR [39]. One of the complex core I units NDUFS8, which is one of the longest human CHCHD containing proteins [49], carries a potential mitochondrial localization presequence at its N terminus (MitoProt II-v1.101[60]). Mutations in NDUFS8 can manifest as Leigh syndrome [61] (**Table 2**).

In addition to the above-mentioned potential CHCHD4 substrates, the post-transcriptional biogenesis of several other protein subunits of the respiratory chain complexes CI, CIII and CIV is indirectly regulated by the CHCHD4-dependent import pathway [40, 62]. For instance, in mammalian cells, AIF depletion negatively affects the expression of nuclear-encoded subunits in CI (NDUFA9, NDUFS7, NDUFB6, NDUFB8 and NDUFA13) and CIII (UQCR1,

UQCR2 and UQCRFS1), as well as that of the mitochondrial-encoded CIV subunit, COX2, in a tissue-specific fashion [40, 62]. This consequence of AIF depletion can be reversed by the expression of a CHCHD4 variant whose mitochondrial import does not depend on AIF [40], establishing that CHCHD4 operates downstream of AIF.

Mitochondrial lipid homeostasis

In yeast, the Mia40-regulated import machinery controls mitochondrial phospholipid homeostasis via the Mia40/Erv1-dependent import of the $(CX_9C)_2$ type motif-carrying substrate Mdm35, with wide consequences for membrane ultrastructure, mitochondrial dynamics and activity [46]. Within the IMS, Mdm35 stably interacts with members of the evolutionary-conserved UPS/PRELI-like proteins UPS1 and UPS2 [63-68], which mediate the transfer of phospholipids between the outer and inner mitochondrial membranes. For instance, UPS1 controls the transfer of phosphatidic acid (PA) from the outer to the inner membrane, where PA is channeled into a chain of enzymatic reactions resulting in the production of cardiolipin (CL)[65, 66, 69].

The human orthologue of Mdm35 is TRIAP1 (TP53-regulated inhibitor of apoptosis 1; also called p53CSV for p53-inducible cell-survival factor) [47, 49, 70]. TRIAP1 and its downstream effector PRELI (homologous to yeast Ups1) are necessary for the production of cardiolipin (CL), which affects oxidative phosphorylation and impacts the apoptotic response stimuli [71-74]. In physiological conditions, optimal electron transfer activity of cytochrome *c* requires its CL-mediated electrostatic interaction with the inner membrane. Depletion of TRIAP1 or PRELI reduces the content of CL within the inner membrane, reduces the attachment of cytochrome *c* to the inner membrane and favors its mitochondrial release, thus sensitizing cells to apoptosis induction (MOMP)[3, 18, 74]. Thus, the supply of exogenous

phosphatidylglycerol (PG), one of the intermediates of CL biosynthesis, partially restores the susceptibility of TRIAP1-deficient cells to lethal stimuli [71, 74]. Of note, TRIAP1 was initially identified as a TP53-responsive anti-apoptotic protein that was induced in conditions of sub-lethal genotoxic stress [70, 75] as well as a TP53 antagonist at the level of cell cycle regulation [76]. TRIAP1 overexpression was reported for multiple myeloma [77] and breast cancer [78] (**Table 2**). It will be important to define whether the putative function of TRIAP1 as an oncogene is linked to its functional interaction with TP53 or rather to its impact on mitochondrial physiology.

Mitochondrial morphology, remodeling and dynamics

Mitochondria constantly adapt their ultrastructure to meet the oscillating bioenergetic demands of the cell [79]. While some membrane-anchored proteins such as mitofusin type transmembrane GTPases (MFN1 and MFN2) and dynamin-related GTPases (DRP1 and OPA1) orchestrate the fusion and division processes that control the number and the shape of the organelle, others are required for the appropriate folding of the inner membrane and its contact with the outer membrane [79]. This latter set of proteins controls the creation of inner membrane microdomains, impact the number and shape of cristae and the formation of cristae junctions. Among these are the evolutionary-conserved architectural proteins that participate to the inner membrane associated complex, MICOS, which couples mitochondrial import to the bioenergetic status of the organelle [79, 80]. The redox-regulated Mia40-dependent import machinery is connected to the MICOS complex by regulating the import and oxidation of its protein subunits [80-84].

The evolutionary-conserved MIC19 (alias CHCHD3) (**Tables 1**) is a myristoylated inner

membrane-bound protein that interacts with the peripheral surface of the MICOS complex, facing the IMS, where its presence is necessary for cristae formation and communication with the outer mitochondrial membrane [80, 85-92]. While, the implication of the $(CX_9C)_2$ motif carrying protein MIC19 in the MICOS complex is well documented, the molecular basis for the cell-specific regulation of its import and/or mitochondrial accumulation by the CHCHD4-dependent import machinery needs further characterization [40, 81, 93]. A search for rare copy number variations has identified the human *MIC19* locus as a candidate risk factor for attention deficit hyperactivity disorder (ADHD)[94] (**Table 2**). Moreover, abnormal expression of MIC19 protein has been associated with pathological metabolism conditions [95, 96] (**Table 2**). MIC25 (known also as CHCHD6) is the metazoan homolog of yeast Mic19, which was also identified as a $(CX_9C)_2$ motif-carrying subunit of the MICOS complex [49, 80, 88, 97] (**Table 1**). While MIC19 was established as a crucial component of MICOS, MIC25 seems to fulfill a more peripheral function, and its role in cristae formation still needs to be established [88, 91, 97].

In human cells, the activity of the soluble IMS-localized $(CX_9C)_2$ motif-carrying protein CHCHD10 (**Table 1**), which is enriched in cristae junctions, impacts mitochondrial ultrastructure through hitherto poorly understood mechanisms [98]. A missense mutation (S59L) in CHCHD10 that is associated with frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) [98, 99] causes respiratory deficiencies accompanied by mitochondrial dysgenesis and fragmentation in patient fibroblasts [98] (**Table 2**). CHCHD10 and its paralogue CHCHD2, which are encoded by genes localized on chromosomes 22q11.23 and 7p11.2, respectively, have emerged as a result of gene duplication during the evolution from yeast to man [49]. Human CHCHD2 is essential for optimal respiratory activity [100-102], in particular that of CIV [100, 102]. CHCHD2 depletion reduces the expression of the mtDNA-encoded COX2 subunit of CIV [100]. Indeed, bioinformatic analyses of natural variations in

human gene expression suggest that CHCHD2 is implicated in the mitochondrial protein translation [103]. Missense mutations in CHCHD2 are associated with autosomal dominant late-onset Parkinson's disease [104] (**Table 2**). The disease-associated mutations in CHCHD2 and CHCHD10 do not affect their C-terminal $(CX_9C)_2$ motifs and are rather clustered in an internal evolutionary-conserved domain that they share with their yeast ancestor Mix17 (previously named Mic17; new nomenclature [80]) (**Figure 2).** Secondary structure analyses (http://phobius.sbc.su.se, http://cho-fas.sourceforge.net) predict that internal hydrophobic segments of CHCHD2 and CHCHD10 form helical structures **(Figure 2C)** and mediate membrane binding. Of note, the pathogenic C176T mutation (S59L amino acid substitution) in CHCHD10 might alter the secondary structure of this hydrophobic domain (**Figure 2C**) and thus could perturb membrane binding. Nonetheless, it is conceivable that the above-mentioned domain could mediate interactions with other proteins that then would relay the functional impact of pathogenic mutations. Yeast Mix17, the precursor of human CHCHD2 and CHCHD10, which is imported into the IMS in a Mia40/Erv1-dependent fashion, carries at its N terminus a potential mitochondrial localization signal that is, however, not cleaved during its import [46] **(Figure 2).** So far, there is no experimental evidence for the functionality of this evolutionary-conserved N-terminal presequence in CHCHD10 or CHCHD2 (**Figure 2**). Moreover, the implication of CHCHD4 in the import of CHCHD2 and CHCHD10, possibly through oxidation of the C-terminal $(CX_9C)_2$ conserved motif (**Figure 2**), remains to be formally demonstrated. However, the physical interaction between CHCHD4 and CHCHD2, as well as the inhibition of CHCHD2 expression by an ALR / ERV1 inhibitor (MitoBloCK-6) [102, 105], strongly suggest that the import of CHCHD2 depends on the CHCHD4/ALR system.

Overexpression of CHCHD2 results from increased gene transcription [106, 107] or from the coamplification of *CHCHD2* with *EGFR* in non-small cell lung cancer (NSCLC) [101] (**Table 2**). CHCHD2 is a survival protein, and its depletion sensitizes cancer cells to genotoxic chemotherapeutics and tyrosine kinase inhibitors [107]. In addition, CHCHD2 may increase cellular migration [101, 108]. However, it remains to be understood through which precise mechanisms CHCHD2 may contribute to malignancy. It should be noted that the overexpression of CHCHD4 also correlates with increased severity of tumor grade and reduced patient survival, a finding that has been related to the CHCHD4-mediated stabilization of hypoxia-inducible factor 1α (HIF1α) [109] (**Table 2**). However, it is unknown how CHCHD4 achieves this effect and whether CHCHD2 may affect HIF1α as well.

Mitochondrial translation

Cells depleted of CHCHD4 (or of its interactor AIF) exhibit reduced expression of the mtDNA-encoded CIV subunit COX2 [40, 62]. COX2 does not require mitochondrial import because it is synthesized within the organelle, thus the mechanism through which CHCHD4 or AIF depletion could reduce COX2 expression was unclear. As a possibility, the expression of COX2 could be low due to the decreased stability of CIV that results from nuclear DNA-encoded CIV subunits not being imported into the mitochondria. Alternatively, or in addition, CHCHD4 might affect translation of COX2 via an indirect effect, through the regulation of the import of its potential substrate CHCHD1 (**Table 1**), which is a component of the small subunit of the mitochondrial ribosome [47, 49, 110]. The RNA-binding and $(CX_9C)_2$ motif-carrying protein CHCHD1 is the mammalian homolog of yeast Mrp10 [47, 49, 111]. The import of Mrp10 into yeast mitochondria depends on an evolutionary-conserved N-terminal presequence. However, the Mia40-catalyzed oxidation of cysteines in the $(CX_9C)_2$ motif was not required for the import or for the activity of Mrp10 and rather stabilized the protein within

298 the matrix [112]. It will be important to investigate whether similar mechanisms apply to
299 human CHCHD1.
300
301 **Concluding remarks**

302 The redox-regulated protein import machinery, composed of CHCHD4, AIF and ALR/ERV1,
303 is now established as a key player that couples the mitochondrial IMS to the cytoplasm and
304 other cellular compartments by catalyzing the oxidative import and/or folding of specific sets
305 of cysteine-rich substrates. Among these substrates, those that carry $(CX_9C)_2$ type motifs are
306 emerging as crucial factors implicated in a variety of mitochondrial functions.
307
308 In mice, hypomorphic mutation of *AIF* (located on the X chromosome) or its organ-specific
309 knockout in liver or muscle causes resistance against high-fat diet-induced diabetes and
310 obesity, likely through subclinical defects in oxidative phosphorylation [113]. Heterozygous
311 knockout of *CHCHD4* causes a similar phenotype, namely resistance to obesity [114],
312 suggesting that AIF and CHCHD4 are epistatic to each other. While full knockout of *AIF* or
313 *CHCHD4* are embryonic lethal [40, 115], distinct AIF point mutations induce an array of
314 human diseases ranging from severe X-linked mitochondrial encephalomyopathy, Cowchock
315 syndrome (X-linked Charcot-Marie-Tooth disease with axonal sensorimotor neuropathy,
316 deafness and cognitive impairment) to infantile motor neuron disease or auditory neuropathy
317 spectrum disorder [116-119]. The fact that at least one of these pathogenic AIF mutations
318 (G308E, which gives rise to a severe phenotype) reduces AIF binding to CHCHD4 [40],
319 underscores the physiological importance of the AIF-CHCHD4 interaction. In this context, it
320 appears intriguing that many of the mutations in other CHCHD proteins (several of which are
321 CHCHD4 substrates) have been linked to phenotypes that are also affected by AIF, such as
322 propensity to diabetes and obesity, as well as a range of mitochondriopathies (**Table 2**).

However, at this point it is not clear whether the massive dysfunction of respiratory chain complexes caused by reduced AIF and CHCHD4 expression can be explained by the downregulation of $(CX_9C)_2$-carrying CHCHD4 substrates or whether it is secondary to the loss of other mitochondrial import proteins including the TIMM protein (such as the $(CX_3C)_2$ motif-containing protein DDPI).

The functional exploration of individual $(CX_9C)_2$ containing proteins will broaden our understanding of cellular metabolism and of a range of inherited human diseases. Moreover, the elucidation of the exact rules that govern the interactions among CHCHD4, AIF, ALR, and each of the multiple CHCHD4 substrates (see outstanding questions) will be crucial for the comprehension of this crucial molecular pathway. At present, it remains an enigma how pathogenic mutations in AIF or CHCHD family proteins can give rise to highly organ-specific phenotypes such as deafness or neurodegenerative disease. Hence, cell type and organ-restricted specificities in the CHCHD-dependent import pathways should be actively sought out.

BOX 1: Mia40/CHCHD4 substrates

$(CX_3C)_2$ motif carrying Mia40 substrates: One of the earliest and best studied groups of $(Cx_3C)_2$-containing Mia40 substrates in yeast corresponds to the family of small Tim proteins [45], which are IMS-localized chaperone proteins with an average size of 10 kDa (Tim8, Tim9, Tim10, Tim12 and Tim13). Tim proteins are organized in hetero-oligomeric complexes and facilitate the mitochondrial import of hydrophobic membrane proteins by ushering them from the outer membrane translocase to their final destination in the inner or outer membranes [45]. All small Tim proteins (called TIMM in human cells) are correctly folded and matured by two intramolecular disulfide bonds established between the cysteines of their twin CX_3C

motifs. Defective disulfide bond formation provokes their degradation by the mitochondrial ATP-dependent protease Yme1 [120]. In mammalian cells, the loss of AIF disturbs the mitochondrial import of CHCHD4 and consequently impacts the biogenesis of TIMM8A (also known as DDP1) [40], which is one member of the highly conserved small TIMM family [121]. In human cells, deletion or loss-of-function mutations in the gene encoding DDP1/TIMM8A is responsible for a rare X-linked recessive neurological disorder called deafness-dystonia or Mohr-Tranebjaerg syndrome [122]. One such loss-of-function mutation causes the replacement of a cysteine by a tryptophan (C66W) within the CX_3C motif, preventing the correct folding of DDP1 and consequently its ability to participate in mitochondrial import reactions [123].

$(CX_9C)_2$ motif carrying Mia40 substrates: See section "C-X_9-C domain-containing proteins in health and disease" in the main text.

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Other Mia40 substrates: Recent studies have revealed that the interaction of Mia40 with substrate proteins is not limited to CX_nC-containing precursors but also includes proteins with alternate cysteine motifs that are not organized structurally in a CHCH domain and are larger in size. One example is the 27 kDa copper chaperone Ccs1, which contains a $CX_2CX_6CX_{36}CX_nC$ motif [124]. Other examples are Erv1 itself, which contains a $CX_{15}C$ motif [125], and the Fe/S cluster protein Dre2 (human orthologue: Anamorsin) [126]. Several novel Mia40 substrates were recently shown to rely on the chaperone activity of Mia40 rather than on its redox activity. Although the proteolytic activity of the mitochondrial inner membrane protease Atp23 requires formation of five disulfide bonds, its import can occur independently of oxidation [127]. In this case, the hydrophobic cleft of Mia40, rather than the redox active

372 CPC tripeptide, is critical for Atp23 import. Mia40/CHCHD4 also chaperones the Tim22
373 protein, a polytopic protein that integrates into the IM through multiple transmembrane
374 segments and functions as a membrane insertase and the key component of the TIM22
375 complex [128]; the matrix-targeted protein Mrp10 [112], which is a subunit of the
376 mitochondrial ribosome; and APE1, a protein involved in the repair of damaged nuclear and
377 mtDNA [129].
378 Yet another type of Mia40/CHCHD4 substrate is represented by MICU1, a mitochondrial
379 regulator of the Ca^{2+} uniporter (MCU)[130]. In human cells, the import of MICU1 in the IMS
380 requires an N-terminal targeting sequence while its interaction with CHCHD4 and cysteine
381 oxidation are secondary to its membrane potential-dependent import [130]. This latter
382 example underscores the versatile implication of the Mia40/CHCHD4 system in multiple
383 distinct import pathways.

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Figure legends

Figure 1, Key figure.

Left panel: Schematic representation of the CHCHD4/MIA40-regulated import pathway in metazoan mitochondria. Nuclear-encoded substrates carrying $(CX_3C)_2$ or $(CX_9C)_2$ motifs carrying or other cysteine-rich proteins are translated by cytoplasmic ribosomes and imported, in their reduced form, into the inter membrane space (IMS), through the outer membrane localized TOM translocase. In the IMS, the oxidase CHCHD4/MIA40 that is bound to the flavoprotein AIF regulates the import and/or the post-import folding of the substrates by catalyzing the formation of disulfide bonds. Electrons (red arrow) generated by the oxidation of the substrate are transferred to the CPC motif of CHCHD4/MIA40, to ALR, to cytochrome C (Cyto C), to complex CIV and oxygen (O_2). A direct transfer of electron from ALR to O_2 is an alternative possibility. Electron flow generated by the activity of the respiratory chain complexes is also depicted (red arrow).

Right panel: The mitochondrion-imported $(CX_9C)_2$ motifs-carrying substrates participate to a vast spectrum of activities that include protein import, lipid homeostasis, mitochondrial ultrastructure and dynamics, Ca^{2+} storage, respiratory chain complexes biogenesis and assembly and mitochondrial protein translation. The correct functioning of the CHCHD4/MIA40 substrates affects the cellular bioenergetics, metabolism and response to stress conditions caused by hypoxia or apoptotic signals.

Figure 2. Protein sequence analyses of human CHCHD10 and CHCHD2.

(A) Phylogenetic analyses of metazoan CHCHD10 and CHCHD2 and yeast Mix17. Multiple alignments of protein sequences available from public databases, using the Clustal Omega program, reveal three protein domains conserved between yeast Mix17 and its metazoan homologs CHCHD10 and CHCHD2. The first N-terminal conserved domain is positively

charged and harbors a potential mitochondrial localization presequence. The second conserved region is central and enriched in hydrophobic residues. The third conserved segment is located in the C terminus of the polypeptides and carries the cysteine-enriched $(CX_9C)_2$ motif. UniProt identifiers for analyzed protein sequences are the following: *H. sapiens* CHCHD10 (Q8WYQ3), *P. troglodyte* CHCHD10 (K7D2L2), *B. Taurus* CHCHD10 (A4IF72), *M. musculus* CHCHD10 (Q7TNL9), *X. tropicalis* CHCHD10 (F7CKM8), *D. rario* CHCHD10 (Q6PBP6), *H. sapiens* CHCHD2 (Q9Y6H1), *P. troglodyte* CHCHD2 (H2QUM2), *B. Taurus* CHCHD2 (Q3ZCI0), *M. musculus* CHCHD2 (Q9D1L0), *X. tropicalis* CHCHD2 (Q68ER0), *D. rario* CHCHD2 (NP_957061.1), and *S. cerevisiae* MIX17 (Q03667).

(B) Identification of disease-associated mutations in the central conserved hydrophobic domain of human CHCHD10 and CHCHD2. Within the central conserved segment of human CHCHD10 and CHCHD2 (identified in figure 1), the positions for each reported mutation and the corresponding amino acid substitution are shown (see also Table 3 for the references). The variant H78N for CHCHD2 corresponds to a natural variant identified in the SNP database (rs11546418; uniprot identifier VAR_048699).

(C) Prediction of secondary structural characteristics for wild type (wt) and mutant (S59L) human CHCHD10. Entire primary amino acid sequences of the wt and S59L variant of human CHCHD10 were analyzed using the GOR IV secondary structure prediction program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html), and only the central conserved hydrophobic segment that includes the wt or mutated (S59L) residue is shown. C (random coil), h (alpha helix), e (extended strand).