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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 (CX₉C) 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 (CX₉C) 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 (CX₉C) 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

1 **Mitochondrial protein import: Historical background and its discovery**

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

7
8 A hypothetical reconstruction of cellular evolution postulates that some two billion years ago
9 the organelle appeared in the eukaryotic cell by the engulfment of an endosymbiotic
10 prokaryotic organism. This endosymbiotic relationship was consolidated by the progressive
11 incorporation of originally bacterial genes into the nuclear genome [9, 10], thereby improving
12 the integration of mitochondria in various aspects of cellular metabolism [1, 2, 6, 9-12].
13 Modern cells control mitochondrial function at several levels by regulating (i) the
14 transcription of nuclear genes, (ii) the cytoplasmic translation of mRNAs, as well as (iii) the
15 import of the proteins into one of the four subcompartments, namely the outer and inner
16 membranes, the matrix and the intermembrane space [5, 13, 14].

17
18 The mitochondrial genome of human cells encodes only 13 proteins, while the function of the
19 organelle requires the import of >1000 nuclear-encoded proteins [13, 14]. Studies in yeast
20 revealed that most of the nuclear-encoded mitochondrial proteins pass into the organelle
21 through one general entry gate at the outer membrane, the outer membrane translocase (TOM),
22 which contains the protein Tom40 as its central protein-conducting channel. However, after
23 engaging with the TOM translocon, mitochondrial proteins follow different sorting routes,
24 dictated by specific targeting codes in their sequence or overall structure that guide them to
25 their final destination. Additional translocase machineries like the sorting and assembly

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

37

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

46

47 **Import into the mitochondrial intermembrane space**

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

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

55

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

69

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

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

76 MISS) of the incoming precursor proteins via hydrophobic interactions. In the subsequent
77 'docking step', Mia40 binds covalently via an intermolecular disulfide bond engaging the
78 active site CPC tripeptide of the Mia40 to one cysteine of the substrate [28]. Thus, Mia40 acts
79 as a chaperone that induces the folding of the substrate in the first 'sliding' step, followed by
80 a disulfide donor function in the second 'docking' step. The end result of this process is the
81 catalyzed oxidative folding and entrapment of the substrate in the IMS [16, 22, 29, 30]. At the
82 end of its catalytic cycle, after donating a disulfide to the substrate, the Mia40 CPC motif is
83 reduced. The reoxidation of the CPC motif is ensured by the FAD-linked sulfhydryl oxidase
84 Erv1 (human equivalent: ALR) [31-33]. This step is mediated specifically by the natively
85 disordered N-terminal segment of Erv1 [34] that contains a CX₂C motif. The pair of electrons
86 that are released during the formation of the disulfide bond are subsequently transferred in a
87 cascade of reactions from the N-terminal CX₂C motif of Erv1 (C30/C33) to its FAD-proximal
88 CX₂C pair (C130/C133), onto the flavin moiety, and then to cytochrome *c*, cytochrome
89 oxidase (COX) and finally molecular oxygen. Alternatively, Erv1-released electrons can be
90 absorbed by cytochrome C peroxidase (Ccp1) [35]. Although it is known that this pathway
91 can operate under anaerobic conditions, the final electron acceptor in these situations is still
92 elusive.

93
94 Even though the principal catalytic and structural features of Mia40, in particular within the
95 segment that contains the redox active cysteine-proline-cysteine (CPC) and the (CX₉C)₂
96 motifs, have been conserved throughout eukaryotic evolution [27, 36-40], there are important
97 differences between yeast and mammals. In contrast to yeast Mia40 (~40 kDa), human
98 CHCHD4 does not possess an N-terminal presequence and hence lacks a membrane anchor
99 [27, 36-38, 40]. Thus, CHCHD4 is a relatively small (16 kDa) soluble protein that is no
100 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

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

127

128 **C-X_n-C domain-containing proteins in health and disease**

129 Mia40 substrates that carry (CX₅C)₂ motifs participate to a large array of activities that are
130 related to the biogenesis of respiratory chain complexes, lipid homeostasis or mitochondrial
131 dynamics [16, 46-48] (**Figure 1, Key Figure**). The doubling of the number of substrates
132 during evolution from yeast to man [46, 47, 49] and the discovery of human disease-
133 associated mutations affecting members of this family (**Table 2**) reflect the
134 pathophysiological relevance of the MIA import pathway as they offer challenging
135 opportunities to decipher the molecular activity of each of them.

136

137 *Biogenesis of Respiratory chain complexes*

138 Beyond the aforementioned connection between Mia40/CHCHD4 and Erv1/ALR (which
139 establishes electron flow from Mia40/CHCHD4 to the respiratory chain) (**see BOX 1**), Mia40
140 has been linked to small (CX₅C)₂ motif-bearing proteins such as Cmc1, Cmc2, Cox6B, Coa4,
141 Coa5, Coa6, Cox17, Cox 19 and Cox23 that are implicated in the biogenesis and assembly of
142 respiratory chain complex CIV (cytochrome C oxidase, COX) [16, 46-50] (**Table 1**).
143 Similarly, in mammals, the abundance of CHCHD4 (or that of its partner ALR) correlated
144 with respiratory chain activity and the oxidation kinetics of subunits CMC1, COA4 (also
145 called CMC3) and COX19 [39]. In human cells, the expression levels of CHCHD4 (or that of
146 its binding partner AIF) and the CIV complex copper chaperone COX17 correlate [36, 40].
147 Indeed, NMR analyses indicate that human COX17 interacts with CHCHD4 via an
148 intermolecular disulfide bond [29, 48]. Missense mutations in several of these CHCHD4
149 substrates are implicated in human diseases (**Table 2**). For instance, a missense mutation in

150 the human homolog of the yeast Cox12 (COX6B1), which carries an imperfect (CX₉C)₂
151 cysteine motif, is associated with a severe infantile encephalomyopathy [49, 51](**Tables 1**
152 **and 2**). Mutations in the CIV assembly factor COA5 (the human ortholog of yeast Pet191)
153 [47, 52]), which carries a typical (CX₉C)₂ motif, can give rise to a mitochondrial
154 cardiomyopathy [53]. Mutations in yet another CIV assembly factor, COA6, which contains
155 an unusual cysteine motif[20, 54], destabilize the newly synthesized mitochondrial DNA-
156 encoded subunit COX2 and lead to neonatal hypertrophic cardiomyopathy [54-58] (**Tables 1**
157 **and 2**).

158

159 Beyond the evolutionary-conserved substrates implicated in the biogenesis of complex CIV
160 (conserved from yeast to mammals), subunits of complex CI (NDUFB7, NDUFS5, NDUFA8
161 and NDUFS8) have made their appearance on the list of potential Mia40/CHCHD4 substrates
162 (**Table1**) [39, 49]. Among this group of (CX₉C)₂ motif-carrying proteins, NDUFB7 and
163 NDUFA8 are IMS-localized and likely stabilize the assembled CI complex by binding to its
164 surface [59]. NDUFA8 carries a duplicated (CX₉C)₂ motif, and the kinetics of its
165 mitochondrial import are controlled by CHCHD4 and ALR [39]. One of the complex core I
166 units NDUFS8, which is one of the longest human CHCHD containing proteins [49], carries a
167 potential mitochondrial localization presequence at its N terminus (MitoProt II-v1.101[60]).
168 Mutations in NDUFS8 can manifest as Leigh syndrome [61] (**Table 2**).

169

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

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

179

180 *Mitochondrial lipid homeostasis*

181 In yeast, the Mia40-regulated import machinery controls mitochondrial phospholipid
182 homeostasis via the Mia40/Erv1-dependent import of the (CX₉C)₂ type motif-carrying
183 substrate Mdm35, with wide consequences for membrane ultrastructure, mitochondrial
184 dynamics and activity [46]. Within the IMS, Mdm35 stably interacts with members of the
185 evolutionary-conserved UPS/PRELI-like proteins UPS1 and UPS2 [63-68], which mediate
186 the transfer of phospholipids between the outer and inner mitochondrial membranes. For
187 instance, UPS1 controls the transfer of phosphatidic acid (PA) from the outer to the inner
188 membrane, where PA is channeled into a chain of enzymatic reactions resulting in the
189 production of cardiolipin (CL)[65, 66, 69].

190

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

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

208

209 *Mitochondrial morphology, remodeling and dynamics*

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

222

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

224 membrane-bound protein that interacts with the peripheral surface of the MICOS complex,
225 facing the IMS, where its presence is necessary for cristae formation and communication with
226 the outer mitochondrial membrane [80, 85-92]. While, the implication of the $(CX_9C)_2$ motif
227 carrying protein MIC19 in the MICOS complex is well documented, the molecular basis for
228 the cell-specific regulation of its import and/or mitochondrial accumulation by the CHCHD4-
229 dependent import machinery needs further characterization [40, 81, 93]. A search for rare
230 copy number variations has identified the human *MIC19* locus as a candidate risk factor for
231 attention deficit hyperactivity disorder (ADHD)[94] (**Table 2**). Moreover, abnormal
232 expression of MIC19 protein has been associated with pathological metabolism conditions
233 [95, 96] (**Table 2**). MIC25 (known also as CHCHD6) is the metazoan homolog of yeast
234 Mic19, which was also identified as a $(CX_9C)_2$ motif-carrying subunit of the MICOS complex
235 [49, 80, 88, 97] (**Table 1**). While MIC19 was established as a crucial component of MICOS,
236 MIC25 seems to fulfill a more peripheral function, and its role in cristae formation still needs
237 to be established [88, 91, 97].

238 In human cells, the activity of the soluble IMS-localized $(CX_9C)_2$ motif-carrying protein
239 CHCHD10 (**Table 1**), which is enriched in cristae junctions, impacts mitochondrial
240 ultrastructure through hitherto poorly understood mechanisms [98]. A missense mutation
241 (S59L) in CHCHD10 that is associated with frontotemporal dementia (FTD) and amyotrophic
242 lateral sclerosis (ALS) [98, 99] causes respiratory deficiencies accompanied by mitochondrial
243 dysgenesis and fragmentation in patient fibroblasts [98] (**Table 2**). CHCHD10 and its
244 paralogue CHCHD2, which are encoded by genes localized on chromosomes 22q11.23 and
245 7p11.2, respectively, have emerged as a result of gene duplication during the evolution from
246 yeast to man [49]. Human CHCHD2 is essential for optimal respiratory activity [100-102], in
247 particular that of CIV [100, 102]. CHCHD2 depletion reduces the expression of the mtDNA-
248 encoded COX2 subunit of CIV [100]. Indeed, bioinformatic analyses of natural variations in

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

272

273 Overexpression of CHCHD2 results from increased gene transcription [106, 107] or from the
274 coamplification of *CHCHD2* with *EGFR* in non-small cell lung cancer (NSCLC) [101]
275 (**Table 2**). CHCHD2 is a survival protein, and its depletion sensitizes cancer cells to
276 genotoxic chemotherapeutics and tyrosine kinase inhibitors [107]. In addition, CHCHD2 may
277 increase cellular migration [101, 108]. However, it remains to be understood through which
278 precise mechanisms CHCHD2 may contribute to malignancy. It should be noted that the
279 overexpression of CHCHD4 also correlates with increased severity of tumor grade and
280 reduced patient survival, a finding that has been related to the CHCHD4-mediated
281 stabilization of hypoxia-inducible factor 1 α (HIF1 α) [109] (**Table 2**). However, it is unknown
282 how CHCHD4 achieves this effect and whether CHCHD2 may affect HIF1 α as well.

283

284 *Mitochondrial translation*

285 Cells depleted of CHCHD4 (or of its interactor AIF) exhibit reduced expression of the
286 mtDNA-encoded CIV subunit COX2 [40, 62]. COX2 does not require mitochondrial import
287 because it is synthesized within the organelle, thus the mechanism through which CHCHD4
288 or AIF depletion could reduce COX2 expression was unclear. As a possibility, the expression
289 of COX2 could be low due to the decreased stability of CIV that results from nuclear DNA-
290 encoded CIV subunits not being imported into the mitochondria. Alternatively, or in addition,
291 CHCHD4 might affect translation of COX2 via an indirect effect, through the regulation of
292 the import of its potential substrate CHCHD1 (**Table 1**), which is a component of the small
293 subunit of the mitochondrial ribosome [47, 49, 110]. The RNA-binding and (CX₉C)₂ motif-
294 carrying protein CHCHD1 is the mammalian homolog of yeast Mrp10 [47, 49, 111]. The
295 import of Mrp10 into yeast mitochondria depends on an evolutionary-conserved N-terminal
296 presequence. However, the Mia40-catalyzed oxidation of cysteines in the (CX₉C)₂ motif was
297 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 (CX₉C)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**).

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

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

337 **BOX 1: Mia40/CHCHD4 substrates**

338

339 **$(CX_3C)_2$ motif carrying Mia40 substrates:** One of the earliest and best studied groups of
340 $(CX_3C)_2$ -containing Mia40 substrates in yeast corresponds to the family of small Tim proteins
341 [45], which are IMS-localized chaperone proteins with an average size of 10 kDa (Tim8,
342 Tim9, Tim10, Tim12 and Tim13). Tim proteins are organized in hetero-oligomeric complexes
343 and facilitate the mitochondrial import of hydrophobic membrane proteins by ushering them
344 from the outer membrane translocase to their final destination in the inner or outer membranes
345 [45]. All small Tim proteins (called TIMM in human cells) are correctly folded and matured
346 by two intramolecular disulfide bonds established between the cysteines of their twin CX_3C

347 motifs. Defective disulfide bond formation provokes their degradation by the mitochondrial
348 ATP-dependent protease Yme1 [120]. In mammalian cells, the loss of AIF disturbs the
349 mitochondrial import of CHCHD4 and consequently impacts the biogenesis of TIMM8A
350 (also known as DDP1) [40], which is one member of the highly conserved small TIMM
351 family [121]. In human cells, deletion or loss-of-function mutations in the gene encoding
352 DDP1/TIMM8A is responsible for a rare X-linked recessive neurological disorder called
353 deafness-dystonia or Mohr-Tranebjaerg syndrome [122]. One such loss-of-function mutation
354 causes the replacement of a cysteine by a tryptophan (C66W) within the CX₃C motif,
355 preventing the correct folding of DDP1 and consequently its ability to participate in
356 mitochondrial import reactions [123].

357

358 **(CX₃C)₂ motif carrying Mia40 substrates:** See section “C-X₃-C domain-containing proteins
359 in health and disease” in the main text.

360 .

361

362 **Other Mia40 substrates:** Recent studies have revealed that the interaction of Mia40 with
363 substrate proteins is not limited to CX_nC-containing precursors but also includes proteins with
364 alternate cysteine motifs that are not organized structurally in a CHCH domain and are larger
365 in size. One example is the 27 kDa copper chaperone Ccs1, which contains a
366 CX₂CX₆CX₃₆CX_nC motif [124]. Other examples are Erv1 itself, which contains a CX₁₅C motif
367 [125], and the Fe/S cluster protein Dre2 (human orthologue: Anamorsin) [126]. Several novel
368 Mia40 substrates were recently shown to rely on the chaperone activity of Mia40 rather than
369 on its redox activity. Although the proteolytic activity of the mitochondrial inner membrane
370 protease Atp23 requires formation of five disulfide bonds, its import can occur independently
371 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 Ca²⁺ 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 (CX₉C)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).