

1 **A dual function of TMEM70 in OXPHOS: assembly of complexes I and V**

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16 **Abstract**

17 Protein complexes from the oxidative phosphorylation (OXPHOS) system are assembled with the help of
18 proteins called assembly factors. We here delineate the function of the inner mitochondrial membrane protein
19 TMEM70, in which mutations have been linked to OXPHOS deficiencies, using a combination of BioID,
20 complexome profiling and coevolution analyses. TMEM70 interacts with complex I and V and for both
21 complexes the loss of TMEM70 results in the accumulation of an assembly intermediate followed by a reduction
22 of the next assembly intermediate in the pathway. This indicates that TMEM70 has a role in the stability of
23 membrane-bound subassemblies or in the membrane recruitment of subunits into the forming complex.
24 Independent evidence for a role of TMEM70 in OXPHOS assembly comes from evolutionary analyses. The
25 TMEM70/TMEM186/TMEM223 protein family, of which we show that TMEM186 and TMEM223 are
26 mitochondrial in human as well, only occurs in species with OXPHOS complexes. Our results validate the use of
27 combining complexomics with BioID and evolutionary analyses in elucidating congenital defects in protein
28 complex assembly.

29

30 **Introduction**

31 The oxidative phosphorylation (OXPHOS) system, situated in the mitochondrial inner membrane, is composed
32 of five enzyme complexes (I–V) and two electron carriers, coenzyme Q and cytochrome *c*¹. The first four
33 complexes form the electron transport chain (ETC) that couples the transfer of electrons from NADH and
34 Succinate to oxygen, to the transfer of protons across the membrane. The thus generated proton gradient is used
35 by complex V (CV) or ATP synthase to generate adenosine triphosphate (ATP)². From the five enzymes of the
36 OXPHOS system, the first and the last, i.e., complex I (CI) or NADH:ubiquinone oxidoreductase and CV are
37 particularly relevant to this study. Mammalian CI is a 45 subunit L-shaped complex, seven subunits of which are
38 mitochondrially encoded, (MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5 and MT-ND6) with a
39 lipophilic arm integrated into the inner mitochondrial membrane and a hydrophilic peripheral arm jutting out into
40 the mitochondrial matrix³. The enzyme can be subdivided into three different functional modules⁴ that are
41 assembled separately^{5,6}: the P-module (proton translocation) that represents the lipophilic membrane arm, the N-
42 module (NADH dehydrogenase) and the Q-module (coenzyme Q reduction). Mammalian CV is an enzyme
43 complex composed of 18 subunits (including the regulatory protein IF₁), two of which are mitochondrially
44 encoded (MT-ATP6 and MT-ATP8)⁷. It comprises two modules: the globular F₁ domain (subunits α , β , γ , δ and
45 ϵ) that contains the catalytic region, and the membrane-embedded F₀ domain, that contains the rotary motor. The
46 F₁ module is connected to F₀ (subunits DAPIT, 6.8PL, a, A6L, e, f, g and c) by two stalks, one central, with
47 subunits γ , δ , ϵ and one peripheral that consists of the oligomycin sensitivity conferral protein (OSCP) and
48 subunits b, d, and F₆. The central stalk and the c-subunits octamer establish the rotor of the fully assembled
49 enzyme⁸.

50 For the optimal assembly of the OXPHOS complexes extra proteins are needed: the so-called assembly factors,
51 which assist in the assembly of a complex but are not part of the final complex. Assembly factors have been
52 discovered by genetic and experimental approaches, often in combination with bioinformatic analyses⁹. Those
53 analyses have exploited that the assembly factors are often part of large protein families¹⁰. They show
54 conservation of molecular function and co-evolve with CI itself¹¹. This is e.g. the case for the CI assembly factor
55 IND1, that was predicted to play a role in the assembly of Fe-S clusters in CI via the role of its cytoplasmic
56 homologs in the assembly of other Fe-S proteins and via its co-evolution with CI proteins¹². However, the exact
57 molecular function of many assembly factors has not been elucidated.

58 From all the complexes that are part of the OXPHOS system, the one with the lowest number of known
59 assembly factors is CV. Its known assembly factors are ATPAF1 and ATPAF2¹³, which both interact with the F₁
60 module, and TMEM70¹⁴. TMEM70 is a transmembrane protein that localizes in the inner membrane of the

61 mitochondria^{15,16}. It contains two transmembrane regions that form a hairpin structure of which the N- and C-
62 termini are located in the mitochondrial matrix¹⁷. Mutations in *TMEM70* have been reported to severely diminish
63 the content of CV in a large cohort of patients^{14,18-26}, and of all nuclear encoded proteins affecting CV, *TMEM70*
64 is the most commonly mutated in disease²⁷. This observation led to the hypothesis that *TMEM70* is a CV
65 assembly factor. However, there is no evidence of its direct interaction with CV proteins, and its specific role in
66 CV assembly remains unclear^{16,26}.

67 Interestingly, defects in *TMEM70* have not exclusively reported deleterious effects on CV but also that
68 combined with less severe CI deficiency²², a combined OXPHOS deficiency²⁸ or even an isolated CI
69 deficiency²⁰. Furthermore, *TMEM70* has been shown to co-migrate with an assembly intermediate that forms
70 part of *Cl*⁶, suggesting it might form part of its assembly process.

71 The present study aims to elucidate the full role of *TMEM70* in the OXPHOS system using standard biochemical
72 techniques combined with two novel techniques: complexome profiling and the BioID proximity-dependent
73 labelling assay, together with an in-silico approach to detect *TMEM70* homologs and reconstruct the co-
74 evolution of *TMEM70* with other mitochondrial proteins.

75

76 **Results**

77

78 **BirA* tagged *TMEM70* biotinylates complex I, complex V and the small subunit of the ribosome**

79 To study whether there is a direct interaction of *TMEM70* with components of CI and CV and thus link its
80 absence to both OXPHOS deficiencies, or whether it interacts with any other mitochondrial protein complex, we
81 used HEK293 Flp-In T-Rex293 cell lines engineered to express BirA*- tagged *TMEM70* in a doxycycline-
82 inducible manner (BioID). In this experiment, designed to biotinylate proteins that occur in close proximity to
83 our protein of interest, we detected 538 proteins (Supplementary Table S1). From those, we obtained a list of 135
84 putative interaction partners of *TMEM70* based on proteins showing a significant increase in biotin positive –
85 doxycycline positive conditions compared to biotin positive – doxycycline negative conditions and biotin
86 negative – doxycycline positive conditions (Supplementary Fig. S1, Supplementary Table S2). The
87 mitochondrial proteins biotinylated by BirA*-*TMEM70* (n = 102) were significantly enriched for proteins
88 involved in CI – including assembly factors *NDUFAF1*, *NDUFAF2*, *NDUFAF4* and *NDUFAF5* – and CV, as
89 well as the small subunit of the mitochondrial ribosome (analysis done with DAVID²⁹, Table 1, Supplementary
90 Table S3).

91

Category	Term	GO	Cluster	FDR
GOTERM_BP_DIRECT	mitochondrial respiratory chain complex I assembly	GO:0032981	1	6.53e-11
GOTERM_BP_DIRECT	mitochondrial electron transport, NADH to ubiquinone	GO:0006120	1	4.73e-6
GOTERM_CC_DIRECT	mitochondrial respiratory chain complex I	GO:0005747	1	8.03e-5
GOTERM_MF_DIRECT	NADH dehydrogenase (ubiquinone) activity	GO:0008137	1	9.56e-5
GOTERM_BP_DIRECT	mitochondrial translational elongation	GO:0070125	2	4.02e-4
GOTERM_CC_DIRECT	Mitochondrial small ribosomal subunit	GO:0005763	2	1.15e-3
GOTERM_BP_DIRECT	Mitochondrial translation termination	GO:0070126	2	6.17e-3
GOTERM_CC_DIRECT	Mitochondrial proton-transporting ATP synthase complex	GO:0005753	3	1.85e-2
GOTERM_CC_DIRECT	Mitochondrial ATP synthesis coupled proton transport	GO:0042776	3	2.60e-2

92

93 Table 1. Enriched clusters obtained from potential mitochondrial interactors of TMEM70

94

95

96 **A comprehensive assessment of the *TMEM70* knockout effect on the OXPHOS system**

97 An interaction of *TMEM70* with CI and CV, as we observed in the BioID results, might explain why patients
98 harbouring *TMEM70* mutations have CI and CV deficiencies^{20,22,28}. To obtain a detailed view on how the
99 absence of *TMEM70* affects those mitochondrial protein complexes, we performed complexome profiling on a
100 *TMEM70* knockout cell line and on controls. We compared two HAP1 wild-type cell lines with one mutant cell
101 line that had a 32bp deletion in exon 1 of the *TMEM70* gene, and performed a biological replicate by again
102 enriching mitochondria from these cell lines and repeating the complexome profiling. The deletion in exon 1 of
103 *TMEM70* causes a frameshift and a premature stop codon in the cDNA, with no detectable presence of the
104 protein by MS/MS (Table 2). Complexome profiling, which consists of a BN-PAGE followed by mass
105 spectrometry, allows us to see the majority of the proteins belonging to each of the native complexes and their
106 distribution over the various assembly intermediates^{30,31}. In the six complexome profiles, four controls and two
107 *TMEM70* knockouts, we detected in total 3766 proteins, of which 814 are annotated as mitochondrial in
108 MitoCarta 2.0³². The profiles were normalized such that the sum of the intensities of the mitochondrial proteins

109 between the samples were equal, and the profiles were aligned with COPAL³³ to allow comparison between the
 110 replicates (see Supplementary Table S4 for the complete results).
 111 In order to assess the overall effects caused by the lack of TMEM70 on OXPHOS, and to confirm its interactions
 112 with complexes I and V suggested by our BioID results (Table 1) as well as by previous studies^{6,22}, we measured
 113 the presence of the subunits belonging to the fully formed OXPHOS complexes (see Methods). We observed a
 114 significant decrease in the abundance of the subunits belonging to CI and CV and small, non-significant
 115 increases in the other three OXPHOS complexes (Fig. 1a; *p* values: CI – 1.4e-12 (median ~60% compared to
 116 controls), CII – 0.125, CIII – 0.322, CIV – 0.204, CV – 1.5e-5 (median ~30% compared to controls), Wilcoxon
 117 signed-rank test, Supplementary Fig. S2). We also tested their enzymatic activities by spectrophotometric
 118 analysis and, accordingly, results showed a 30% decrease in CI activity (*p* = 0.156) and a 70% decrease and CV
 119 activity (*p* = 0.009), Fig. 1b), whereas the other OXPHOS complexes showed no apparent change in activity.
 120 Furthermore, we also observed a reduction in fully formed complexes I and V in BN-PAGE/immunoblotting
 121 analysis in KO conditions compared to controls (Supplementary Fig. S3).

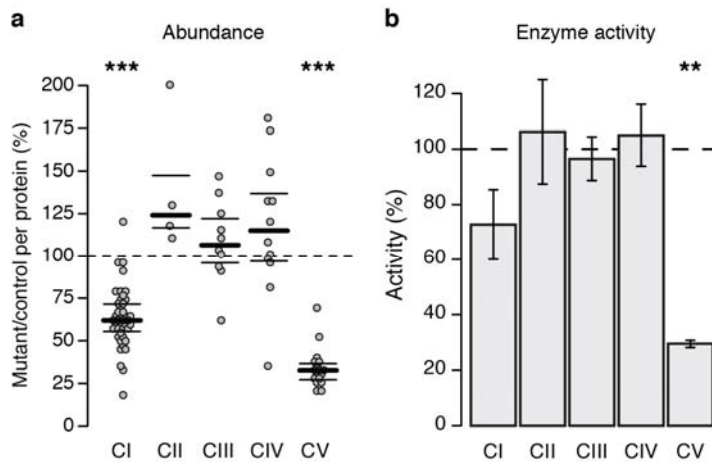
By MS/MS

Sequence	P1_1	P1_2	P631_1	P631_2	KO_1	KO_2
AITYNAMIAETSTVFNQNDVK	10	10	17	8	0	0
HVFTTFYAK	18	2	20	2	0	0
IYTGNMAR	19	5	19	1	0	0
IYHEATTDYK	22	5	16	0	0	0
SIIVNPVIFPNR	10	4	12	4	0	0
Total	79	26	84	15	0	0

By matching

Sequence	P1_1	P1_2	P631_1	P631_2	KO_1	KO_2
AITYNAMIAETSTVFNQNDVK	34	7	35	2	0	0
HVFTTFYAK	8	21	10	13	0	0
IYTGNMAR	29	40	41	21	1	1
IYHEATTDYK	13	22	40	21	0	2
SIIVNPVIFPNR	8	12	23	4	2	0
Total	93	102	149	61	3	3

122 Table 2. Peptides assigned to TMEM70 obtained in all 6 samples by MS/MS and by matching
123
124



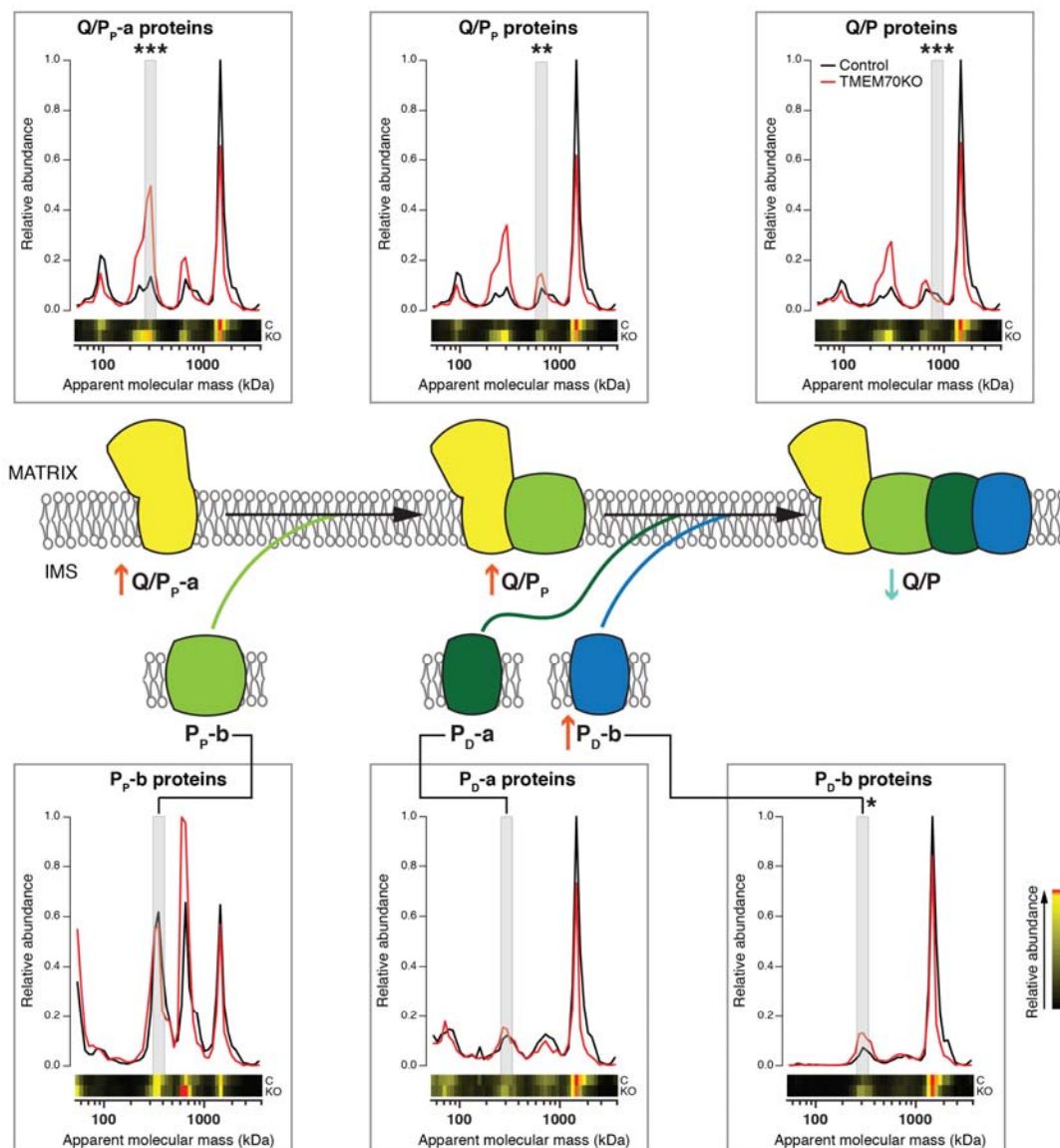
125
126 Figure 1. Lack of TMEM70 has an effect in CI and CV. **a** Ratios between mutants and controls measured per
127 subunit and grouped by complex. CI and CV show an apparent reduction of ~40% and 70% respectively. **b**
128 Enzymatic activities shown by TMEM70 KO with respect to the parental control cell line. Complexes I and V
129 show a decrease, although statistically significant only for the latter ($p = 0.156, 0.8, 0.51, 0.577$ and 0.009 , for
130 complexes I, II, III, IV and V, respectively; $n = 3$). Error bars represent the standard deviation.

131

132 Impaired assembly of complex I

133 We first focused on the differences in the assembly process of CI between the *TMEM70* wild-type and in
134 *TMEM70* knockout HAP1 cells. As mentioned above, there was a reduction of fully formed CI in cells lacking
135 *TMEM70* (Fig. 1a), suggesting a possible CI assembly defect. CI assembly proceeds via pre-assembled
136 intermediates that later on interact, until the fully formed and active enzyme is made⁵. When analysing the
137 assembly steps of CI under *TMEM70* KO conditions in the complexomics results, we observed a clear and
138 significant accumulation of the Q/P_p-a intermediate (Fig. 2 and Supplementary Fig. S4; median ratio = 4.14; $p =$
139 4.89×10^{-4}) relative to controls, consistent with the accumulation observed in two-dimensional BN-PAGE/SDS-
140 PAGE for NDUFS3 intermediates (Supplementary Fig. S5). At this stage, the Q-module formed by NDUFS2,
141 NDUFS3, NDUFS7, NDUFS8 and NDUF5A and the assembly factors NDUF3 and NDUF4 is anchored
142 to the membrane with proteins belonging to the proximal P-module, TIMMDC1 and MT-ND1⁶. Following the
143 formation of this intermediate, and based on one of the previously described assembly pathways of complex I

144 (Fig. 2), the remaining subunits of the proximal module (P_P -b), which do not show any significant changes while
145 forming part of this intermediate (Fig. 2 and Supplementary Fig. S4; median ratio = 0.94; $p = 0.102$), are added
146 to form the intermediate Q/P_P that, in the TMEM70 depleted cells, also accumulates relative to the controls (Fig.
147 2 and Supplementary Fig. S4; median ratio = 2.41; $p = 2.6e-3$). The most distal part of the enzyme, the P_D
148 intermediate, is composed of two different intermediates: P_{D-a} and P_{D-b} . TMEM70 has been observed to co-
149 migrate with this distal membrane part (P_D) of complex I, and within this distal part, with the intermediate P_{D-a} ,
150 that is just next to the proximal membrane part of the complex⁶. With respect to the P_D part of the enzyme, the
151 intermediate P_{D-b} showed a minor but significant accumulation (Fig. 2 and Supplementary Fig. S4; median ratio
152 = 2.18; $p = 0.016$), while no accumulation was observed for P_{D-a} (median ratio = 1.04; $p = 0.383$). These results
153 are as well compatible with the alternative assembly pathway of complex I (Supplementary Fig. S6), which also
154 shows a significant decrease of the intermediates that follow after the incorporation of P_{D-a} (P_P -b/ P_{D-a} ,
155 Supplementary Fig. S6 and Supplementary Fig. S4; median ratio = 0.64; $p = 0.004$). Finally, both assembly
156 pathways converge with the formation of intermediate Q/P , which is also reduced in the absence of TMEM70
157 (Fig. 2, Supplementary Fig. S6 and Supplementary Fig. S4; median ratio = 0.70; $p = 1.9e-7$).



158

159 Figure 2. The assembly of complex I is impaired in the absence of TMEM70. Individual plots depict migration
 160 profiles of the average of the iBAQ values of the proteins that belong to the stated assembly intermediate (see
 161 Methods) in parental HAP1 cells (black line) and TMEM70 KO HAP1 cells (red line). The significant
 162 accumulation (red arrow) shown by intermediates Q/P_p and P_D-b together with the significant depletion (blue
 163 arrow) of the subsequent product (Q/P) in the TMEM70 knockout cells suggests an impairment in the
 164 incorporation of the first two to form the latter. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$ based on results depicted
 165 in Supplementary Figure S4

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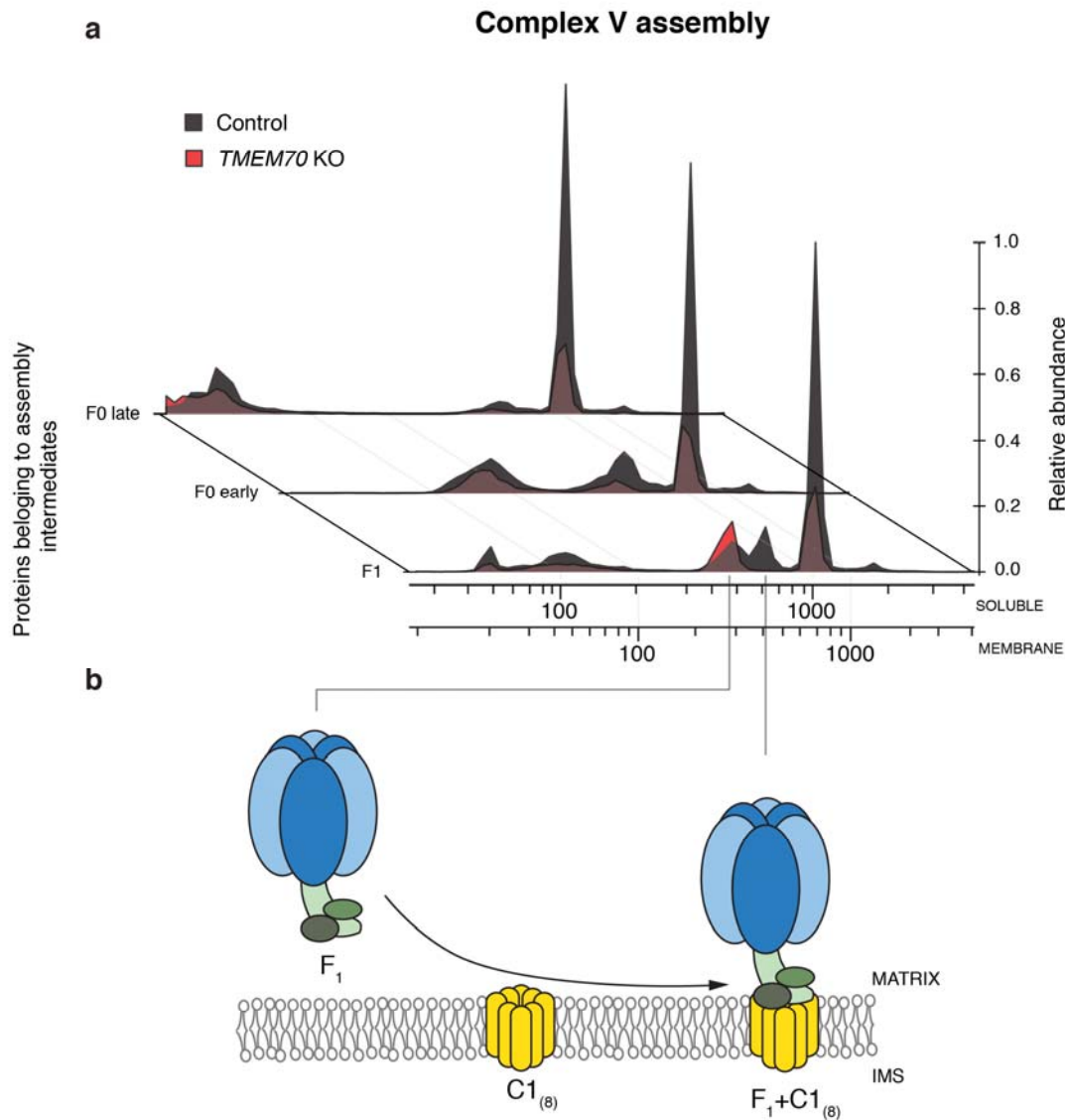
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169 **Loss of the interaction between the c-ring and F₁ module in the *TMEM70* knockout**

170 In order to understand the observed decrease in assembled CV in the absence of *TMEM70*, we also examined its
171 assembly process in the complexomics data (Supplementary Table S4). We could identify all subunits of CV
172 with two exceptions: the subunit ϵ (in all samples) and the components of the c-ring octamer (C1 subunits) (in
173 the replicate experiment of the *TMEM70* KO cell line). Based on these data, the subunits of CV were distributed
174 over multiple intermediates in the control: the F₁ soluble and F₁ anchored intermediates, with subunits α , β , γ and
175 δ in the former, and those plus C1 in the latter (Supplementary Fig. S7a), the F_O late assembly intermediate with
176 subunits ATP8, ATP6, DAPIT, 6.8PL, d, F6 and OSCP (Supplementary Fig. S7b) and the F_O early assembly
177 intermediate with subunits b, e, f and g (Supplementary Fig. S7c) as well as the fully formed complex V.
178 Subunits belonging to F₁ show a migration pattern matching previously suggested states³⁴, namely, F₁ soluble
179 (α - β hexamer together with γ , δ and ϵ monomers), F₁ attached to the membrane bound c-ring, and F₁ forming
180 part of the fully assembled complex (Fig. 3a Control, Fig. 3b). With respect to the F_O module, our results show a
181 co-migration pattern that hints towards the formation of two intermediates before the fully assembled complex:
182 one formed in an early stage composed of subunits b, e, f and g and one containing these subunits plus the ATP8,
183 ATP6, DAPIT, 6.8PL, d, F6 and OSCP subunits (Supplementary Fig. S7).

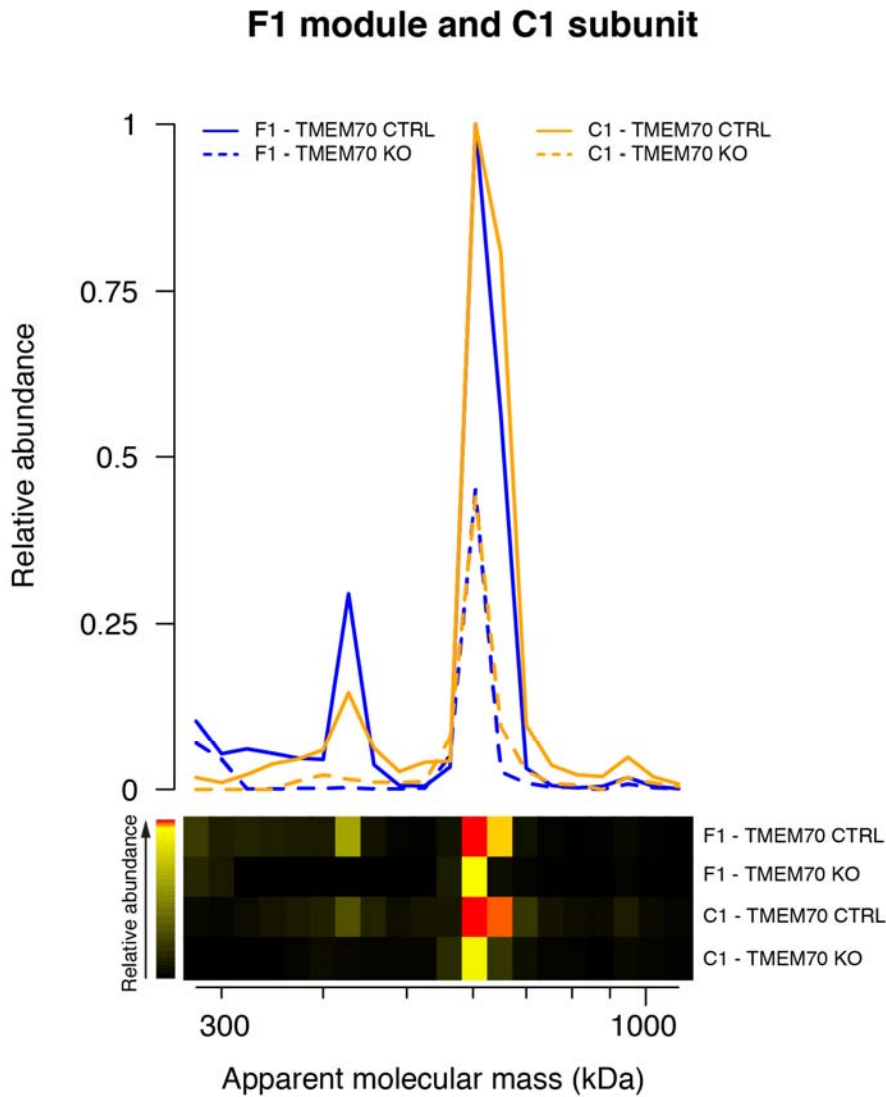
184 When we explored the assembly of CV in the *TMEM70* knockout cell line in detail, we found that the above-
185 mentioned association of the C1 subunit octamer with the α - β hexamer and the γ , δ and ϵ subunits that form the
186 F₁ module was missing in the absence of *TMEM70* (median ratio = 0.04, the small number of subunits does not
187 allow obtaining significant values using non-parametric statistics). Interestingly, the canonical soluble F₁
188 intermediate showed a slight accumulation compared to the control (median ratio = 1.27) (Fig. 3a). Consistently,
189 this intermediate was found to be absent in the one-dimensional BN-PAGE immunoblot (Supplementary Fig.
190 S8). With respect to the c-ring components in the complexome analysis by mass spectrometry, we reasoned that
191 the tryptic in-gel digestion may not generate enough detectable peptides of proteins that are small and highly
192 hydrophobic, such as the C1 subunit. To address this issue, we repeated the complexome profiling, but now
193 digesting the samples with chymotrypsin instead of trypsin (Supplementary Table S5). Results revealed that the
194 intermediate in the wild type with an apparent mass of ~420 kDa indeed represents the F₁ module associated
195 with the C1 subunit octamer (Fig. 4). Furthermore, the absence of this intermediate in *TMEM70* devoid cells
196 was accompanied by the absence of C1 subunit at the same apparent mass, thus confirming the interaction of the
197 soluble F₁ with the membrane subunit C1 (Fig. 4).

198 When focusing on the subunits belonging to the F_0 early assembly intermediate and/or the F_0 late assembly
199 intermediate, we observed that the differences between the *TMEM70* knockout and the control are based on an
200 overall reduction (Fig. 3a). Together, these results suggest a role of *TMEM70* in the membrane stability of the
201 $C1$ subunit octamer or its interaction with the F_1 module.



202
203 Figure 3. The assembly of complex V misses a F_1 intermediate in the absence of *TMEM70*. **a** Migration profiles
204 of complex V intermediates in control HAP1 cells (dark grey) and *TMEM70* KO HAP1 cells (red).
205 Intermediates are represented in the z-axis by the average of the iBAQ values of all the subunits detected by
206 mass spectrometry that belong to that intermediate. In absence of *TMEM70*, F_1 intermediate accumulation is
207 followed by F_1 -c intermediate depletion whereas the other intermediates do not show specific effects besides an

208 overall reduction of their presence. **b** The F₁ soluble intermediate is anchored to the inner mitochondrial
209 membrane binding the c-ring octamer
210



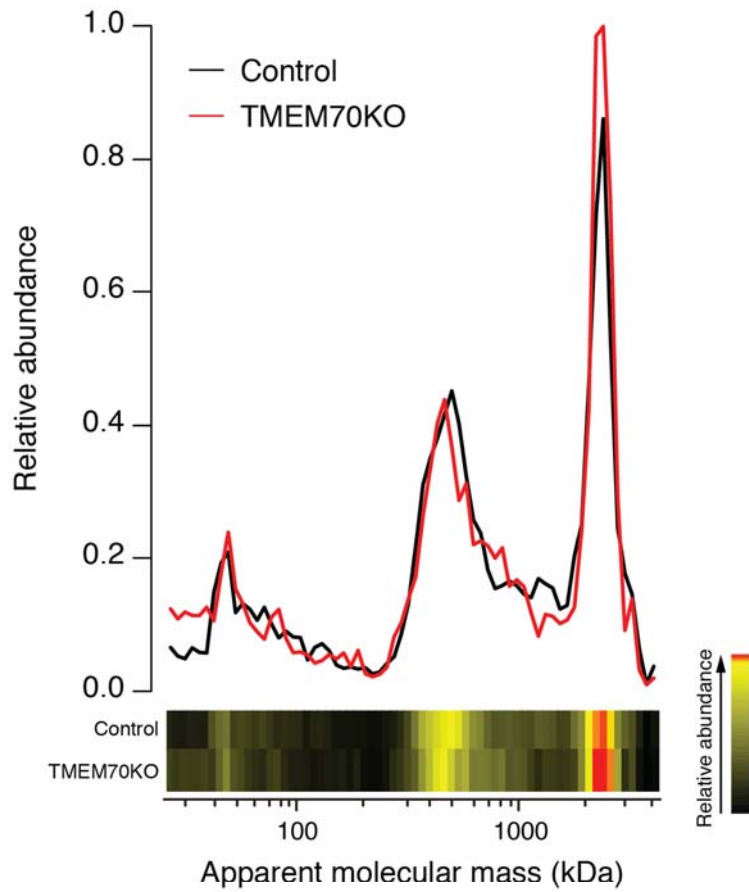
211
212 Figure 4. ATP synthase F₀ complex subunit C1 interaction with F₁ is missing in the absence of TMEM70.
213 Complexome profiling results after chymotrypsin digestion showing the average of detected F₁ subunits and C1
214 protein in control and TMEM70 KO HAP1 cells show a missing intermediate in the latter formed by both groups
215 of proteins (F₁ proteins and an octamer of C1 subunits).
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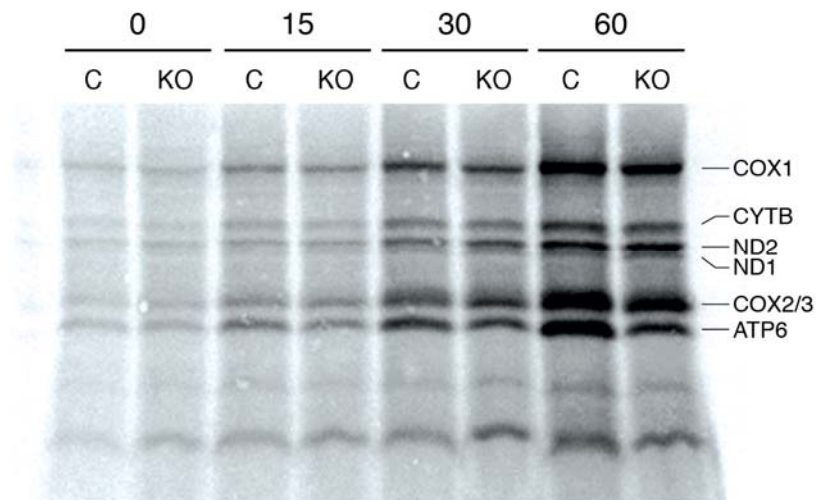
219 **Mitochondrial translation is not disturbed by the lack of TMEM70**

220 After confirming the involvement of TMEM70 in complex I and V that was indicated by the BioID results
221 (Table 1), we proceeded with the third complex from the BioID analysis, the mitochondrial ribosome, to
222 determine the possible effect of TMEM70 depletion on the assembly of the small subunit of the mitochondrial
223 ribosomes and/or in mitochondrial translation. To do so, we first assessed the assembly of the small subunit of
224 the mitochondrial ribosomes with our complexomics data in both the presence and absence of TMEM70
225 (Supplementary Table S4). We did not observe any discrepancies in the formation of intermediates throughout
226 the assembly of the mitochondrial ribosome when comparing both conditions (Fig. 5a), except for a slight but
227 significant increase of the final product ($p = 7.5e-3$, Supplementary Fig. S9) in the absence of TMEM70. In order
228 to assess whether such a difference would affect mitochondrial translation, we labelled newly translated
229 mitochondrial products with ^{35}S -methionine 35 comparing newly synthesized products between control and
230 *TMEM70* KO conditions. We collected cells 5 minutes, 15 minutes, 30 minutes and 60 minutes after 1h of
231 permanently inhibiting cytoplasmic translation (Fig. 5b). Results showed no differences between the two
232 conditions affecting the mitochondrially translated proteins of complexes III and IV. However, we did observe a
233 reduction in ATP6 that becomes more pronounced over time, which is consistent with the strong reduction in
234 fully assembled CV.
235 The lack of differences in both the assembly process of the ribosomes and the translation efficiency of those
236 suggest no significant role of TMEM70 in their assembly or their normal functioning.

a
Mitochondrial ribosome: small subunit



b



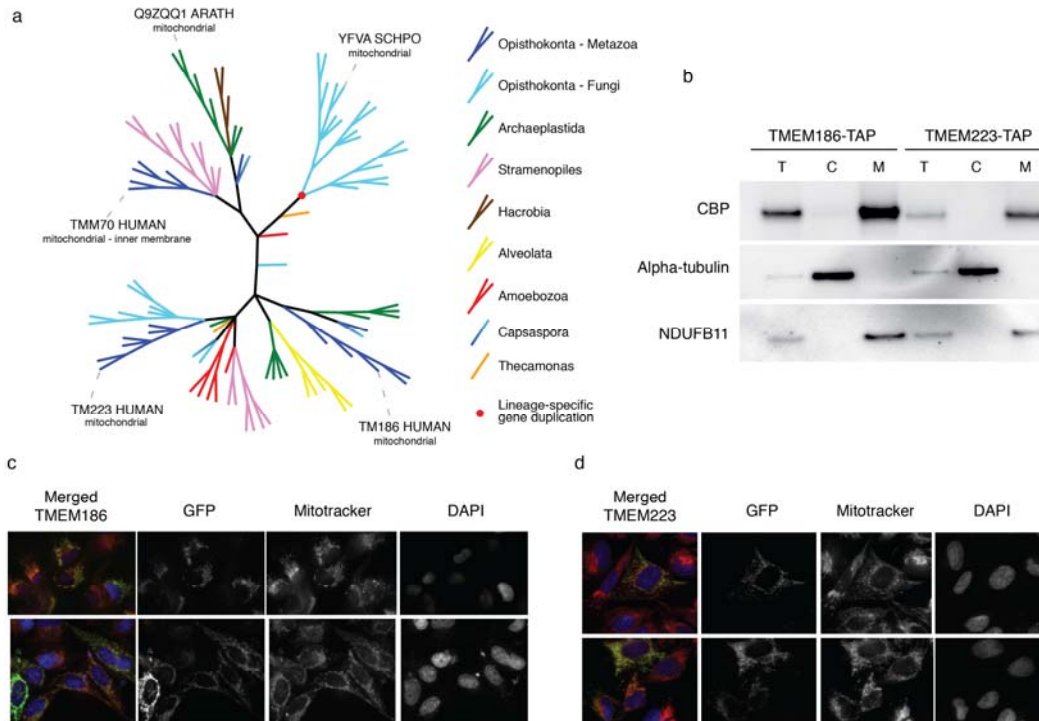
238 Figure 5. The assembly of the small subunit of the mitochondrial ribosome and the translation of mitochondrial
239 proteins are not affected in the absence of TMEM70 even though there is an increase of the final product. **a**
240 Complexome profile of proteins belonging to the small subunit of the mitochondrial ribosome. Despite being
241 enriched in the BioID dataset, its assembly does not appear to be disturbed in the absence of TMEM70. **b** Pulse
242 labelling of mitochondrial encoded products after 5 min, 15 min, 30 min and 1 h. Newly synthesised products do
243 not seem to show any differences in abundance, with the exception of ATP6 after 15 or more minutes.

244

245 **The mitochondrial protein family TMEM223/TMEM186/TMEM70 co-evolves with OXPHOS**

246 In order to examine whether TMEM70 has co-evolved with OXPHOS proteins we determined its phylogenetic
247 distribution. Sequence searches for TMEM70 using sensitive Hidden Markov Models (Methods) revealed that it
248 has two paralogs in human: TMEM223 ($E=2.9e-8$) and TMEM186 ($E=1.2e-28$) (Fig. 6a). Results obtained from
249 cellular fractionation (Fig. 6b) and microscopy (Fig. 6c and 6d) indicate a mitochondrial localization of both
250 paralogs. Although the level of sequence identity between the three family members is low (e.g. in human
251 TMEM70-TMEM186: 14%, TMEM70-TMEM223: 9%, TMEM186-TMEM223: 15%), *in silico* predictions
252 indicate a similar asymmetric hairpin topology for all three proteins; a short N-terminal sequence located in the
253 mitochondrial matrix followed by an in/out and an out/in transmembrane helix and a longer C-terminal sequence
254 (Supplementary Fig. S10), consistent with the experimental results for TMEM70¹⁷. Furthermore, also the
255 *Saccharomyces cerevisiae* protein Mrx15, that is homologous to the TMEM70/186/223 family and within this
256 family most similar to TMEM223 ($E=8e-28$, 10% identity), has this asymmetric hairpin topology³⁶. All three
257 members of the TMEM70/186/223 family are phylogenetically widespread (Fig. 6a), and appear to have been
258 present in the last eukaryotic common ancestor (LECA). We then asked whether any other mitochondrial
259 proteins have a similar phylogenetic distribution as members of the TMEM70/186/223 family. Given the low
260 sequence identity between orthologs in this protein family and between their potential interactors, we used a two-
261 tier approach. First, we employed an in-house orthology database based on pairwise sequence comparisons and
262 containing 52 diverse eukaryotic species to derive phylogenetic profiles of the human proteome, using
263 differential Dollo parsimony that measures the number of independent loss events along an evolutionary tree as
264 an evolutionary distance measure. For TMEM70, the protein with the highest co-evolution signal (NDUF1) and
265 four of the ten most co-evolving proteins were CI proteins (Supplementary Table S6). Second, we manually
266 analysed, using the top hits of the first approach and using more sensitive, profile-based analyses (Methods), the
267 co-evolution of TMEM70/186/223 with CI, CV and TMEM14. The latter is, based on the first step of the

268 analysis, a protein family that co-evolves with TMEM70/186/223, and is also located in the inner mitochondrial
 269 membrane. Overall, we observe that members of the TMEM70/186/223 protein family only occur in species
 270 with OXPHOS, and are absent from species without CI and or CV. Nevertheless, the reverse does not apply as
 271 there are taxa, like the Kinetoplastida, that do have CI and CV but do not have detectable homologs of the
 272 TMEM70/186/223 family (Supplementary Table S7).



273
 274 Figure 6. TMEM70 has two paralogs, TMEM186 and TMEM223 that are both located in the mitochondria.
 275 Furthermore, for TMEM70 orthologs in *Arabidopsis thaliana* and *Schizosaccharomyces pombe*, there is
 276 experimental evidence that localizes them in the mitochondria^{58,59}, while also a *S. cerevisiae* homolog of the
 277 family, YNR040W/Mrx15 that due to extreme sequence divergence could not be put into the tree, is located in
 278 mitochondria³⁶. **a** Phylogenetic distribution of TMEM70 homologs. **b** Cellular fractionation of cells expressing
 279 TMEM186-TAP and TMEM223-TAP. Immunodetection against NDUFB11, as a marker of the mitochondrial
 280 enriched fraction (M), Alpha-tubulin as a marker of the cytoplasm (C), and CBP. (T) stands for total fraction. **c**
 281 and **d** Fluorescence microscopy images of cells expressing TMEM186-GFP (**c**) and TMEM223-GFP (**d**) stained
 282 with Mitotracker (mitochondrial network) and DAPI (nucleus).

283

284

285 **Discussion**

286 Our results describe a role of TMEM70 in the assembly of CI and CV of the OXPHOS system. This finding is in
287 line with previous studies of patients harbouring mutations in *TMEM70*, that did not always have an isolated CV
288 deficiency but rather combined OXPHOS deficiencies or even an isolated CI deficiency^{18,20,22,28}. The conclusions
289 are supported by the results from BioID, complexome profiling analyses and enzyme measurements of the
290 OXPHOS complexes. Although the assembly of both CI and CV are significantly affected in the absence of
291 TMEM70, the effect on CV is more pronounced than on CI, which might explain the preponderance of CV
292 phenotypes relative to CI phenotypes reported for patients with mutations in *TMEM70*.

293 In order to obtain a comprehensive list of potential interacting candidates that could hint at the function of
294 TMEM70, we began by performing a biotin ligase proximity-dependent assay (BioID) of BirA* tagged
295 TMEM70, which indicated that the protein is in close contact with CI, CV and the small subunit of the
296 mitochondrial ribosome. To determine a potential role of TMEM70 in the assembly of CI and CV in detail, we
297 studied all the proteins of both complexes meticulously by complexome profiling^{30,31}. Thus, we were able to
298 detect the majority of the proteins of each complex and of their assembly intermediates. This allowed us to
299 determine the effects that the lack of TMEM70 has on CI and CV assembly. For CI, the Q/P_{P-a} and Q/P_P
300 intermediates accumulate in the absence of TMEM70, suggesting that the impairment in the pathway comes
301 right after their assembly. In the case of Q/P_{P-a}, and following one of the alternative assembly pathways
302 (Supplementary Fig. S6), its accumulation could be caused by a reduction of forming the intermediate P_{P-b}/P_{D-a}.
303 If we follow the other alternative assembly pathway (Fig. 2), intermediate Q/P_P accumulation followed by Q/P
304 depletion, suggests a problem with the incorporation of P_{D-a} and P_{D-b} intermediates, the latter of which also
305 accumulates in the TMEM70 KO. The interaction between CI and TMEM70 is in line with a previous study by
306 Guarini *et al.*³⁷, who showed TMEM70 as a significant interactor of ECSIT and NDUFS5, both proteins
307 belonging to the P_{P-b}/P_{D-a} intermediate and, in the case of ECSIT, also part of the Q/P_P intermediate. The
308 interaction of TMEM70 with proteins of the P_P module³⁷, the comigration with the distal part (P_D) of CI⁶, and the
309 accumulation of Q/P_{P-a}, Q/P_P and P_{D-b} intermediates together with the depletion of P_{P-b}/P_{D-a} and Q/P
310 intermediates, suggests that TMEM70 is involved in the assembly of the membrane arm (P-module) of CI by
311 assisting in assembly or the stabilization of the P_{D-a} intermediate. A role in the assembly or the stability of a
312 membrane intermediate is consistent with the membrane architecture of the protein, consisting of two
313 transmembrane helices followed by a long C-terminal tail²².

314 With respect to CV, we delineate a specific role of TMEM70 in its assembly. In the complexomics data we show
315 how, upon lack of TMEM70, the intermediate where F₁ is bound to the membrane-embedded c-subunits

316 octamer³⁴ is completely absent. Despite the absence of the F₁-c intermediate, there was still some CV being
317 assembled, although at only 30% compared to the control. One possibility is that in TMEM70 depleted cells the
318 assembly of the complex follows an alternative path that was already described by He *et al*³⁸, where F₁ instead of
319 being attached to the c-ring octamer first, instead attaches to the F₀ and the peripheral stalk before combining
320 with the c-ring. Nevertheless, in our complexome profiling data we do not detect such an F₁F₀ intermediate that
321 would support this alternative path.

322 With respect to the small subunit of the mitochondrial ribosome, although we observed a slight but significant
323 increment of mitochondrial ribosomes upon *TMEM70* knockout, we did not observe any detectable differences
324 in its assembly nor in the efficiency of the translation of the mitochondrial products. Thus, as the synthesis of
325 mitochondrial encoded subunits was not altered in *TMEM70* devoid cells, we find no evidence for the
326 hypothesis of nucleoid disruption³⁹. The interactions detected by the BioID experiment could be due to the
327 structure of *TMEM70* and the addition of the BirA domain to its C-terminus. Such terminus protrudes into the
328 mitochondrial matrix after the two transmembrane domains. This, together with the possibility of mitochondrial
329 ribosomes being in close proximity to the inner membrane during the translation, could be the reason they were
330 tagged.

331 Through our homology analyses and phylogenetic reconstruction of the co-evolution of *TMEM70/186/223*
332 family with CI and CV we could not detect a *TMEM70* ortholog in *S. cerevisiae*, which is surprising as *S.*
333 *cerevisiae* does have CV. Interestingly, it has been described how in this species, F₁ does not undergo the same
334 assembly steps as in human and does not interact with the c-ring⁴⁰. Furthermore, *S. cerevisiae* lacks CI, fitting
335 our hypothesis of a double role of the protein in human, that is absent from *S. cerevisiae* because one of the
336 target complexes has been lost in evolution (CI), and the manner in which the other target complex is assembled
337 is different than in human. Sensitive homology detection does however reveal the presence of a *TMEM70*
338 homolog in *S. cerevisiae*: *TMEM223*. *TMEM70*, *TMEM223* and the third member of this family that we could
339 detect, *TMEM186*, appear widespread in eukaryotic evolution and only occur in species with OXPHOS
340 complexes. We performed confocal microscopy for analysis of GFP-tagged *TMEM186* and *TMEM223*. The
341 analysis showed the predominant localization of both proteins in the mitochondria, something that we could
342 confirm by cell fractionation and isolation of mitochondria. Like *TMEM70*, *TMEM186* was also identified to
343 co-migrate with CI assembly intermediates following dynamic complexome profiling⁶. Little is known about
344 this protein; however, it was recently identified as an interaction partner of ECSIT³⁷, suggesting a role in CI
345 assembly. Additional studies are required to determine if this protein functions in CI assembly.

346 It is interesting and unusual that a single protein TMEM70 would be involved in the assembly of two different
347 OXPHOS complexes, CI and CV. A common denominator based on our results is that, in both cases, it appears
348 to recruit proteins to membrane intermediates during the assembly of the enzymes. Such a combination of
349 hydrophobic and hydrophilic interactions would be consistent with the topology of the protein that contains two
350 well conserved transmembrane helices together with a long (~100 amino acids) tail that protrudes into the
351 matrix. Mrx15, the yeast homolog of TMEM70 and the ortholog of TMEM223, appears to tether the
352 mitochondrial ribosome to the membrane during translation, using the hydrophilic C terminus to interact with the
353 large subunit of the mitochondrial ribosome while the N terminus forms a hairpin in the inner mitochondrial
354 membrane. We hypothesize that TMEM70 has a similar tethering role to in the assembly of complexes I and V.

355

356 **Materials and methods**

357

358 **Enzyme Measurements**

359 Respiratory chain enzyme analysis in HAP1 were performed as described before⁴¹. Values are expressed relative
360 to the mitochondrial reference enzyme citrate synthase⁴².

361

362 **Cell culture**

363 HAP1 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) in the presence of 10% fetal calf
364 serum and penicillin/ streptomycin.

365 HEK293 cells were cultured in DMEM (Biowhitaker) supplemented with 10% fetal calf serum (FCS) (v/v) and
366 1% penicillin/streptomycin (GIBCO). Inducible cell lines were selected on 5 µg/ml blasticidin (Invitrogen) and
367 200 µg/ml hygromycin (Calbiochem), and for expression of the transgene, 1 µg/ml doxycycline (Sigma Aldrich)
368 was added for 24 hr.

369

370 **Knockout**

371 *TMEM70* knockout (HZGHC003615c010) was ordered at Horizon (Austria). A near-haploid human cell line
372 (HAP1) was edited using CRISPR/Cas (Guide RNA sequence: CGGCTGGAGTACGGGGCCTT) resulting in a
373 frameshift mutation of 32bp in exon 1²⁶. This results in a 100% knockout. In Table 1 an overview of the
374 knockout cell line is shown.

375

376 **Blue-Native, SDS-PAGE Analysis and complex I In Gel Activity Assay**

377 One-dimensional 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 4%–12%
378 blue-native (BN)-PAGE was performed as described previously⁴³. Lanes were loaded with 40 (SDS analysis) or
379 80 (BN analysis) μ g of solubilized mitochondrial protein. For the 1D and 2D BN SDS-PAGE followed by
380 immunoblotting, mitoplasts were solubilized with n-dodecyl- β -D-maltoside, whereas for complexome profiling
381 the solubilization was done with digitonin to preserve supercomplexes. After electrophoresis, gels were
382 processed further for in-gel complex I activity assay, in-gel fluorescence detection, immunoblotting, or two-
383 dimensional 10% SDS-PAGE or blotting, proteins were transferred to a PROTAN nitrocellulose membrane
384 (Schleicher & Schuell).

385

386 **Antibodies and ECL detection**

387 Immunodetection was performed by the use of the following primary antibodies: CI-NDUFS3, CII-SDHA, CIII-
388 core 2, CIV-COX4, CV-ATPase- α/β (gift from the Molecular Bioenergetics Group, Medical School, Goethe-
389 University Frankfurt⁴⁴), C3ORF60 (NDUFAF3) (Eurogentec) and V5 (Invitrogen). Goat-anti-rabbit and goat-
390 anti-mouse IRDye CW 680 or IRDye CW 800 were used as secondary antibodies, to detect the proteins using the
391 Odyssey system from LI-COR Biosciences. Secondary detection was performed using peroxidase-conjugated
392 anti-mouse or anti-rabbit IgGs (Life Technologies). Immunoreactive bands were visualized using the enhanced
393 chemiluminescence kit (Thermo Scientific) and detected using the Chemidoc XRS+ system (Biorad).

394

395 **Pulse labelling of mitochondrial translation products**

396 In vitro labelling of mitochondrial translation products was performed as described previously³⁵. First, cells were
397 incubated for 30 minutes with methionine, cysteine and glutamine free DMEM (Gibco) supplemented with
398 glutamax (1Xconc), 10% dialyzed FBS and 1.1mg/l sodium pyruvate. Afterwards, emetine was added to a final
399 concentration of 100 μ g/ml, after 5 minutes 200 μ Ci/ml ³⁵S methionine label was added during 1 hour. Cells were
400 refreshed with 10% FBS DMEM media and harvested after 1h and after 5h of labelling and treated for SDS-
401 PAGE or BN-PAGE.

402

403 **Generation of inducible cell lines**

404 *TMEM70* was cloned into pDONR201 as described⁴⁵. The complete open reading frame (without stop codon)
405 was created by PCR according to the manufacturer's protocol and cloned into pDONR201 by the Gateway BP

406 Clonase II Enzyme Mix. The pDONR201-TMEM70 was recombined with the pDEST5-BirA*-FLAG-C-ter
407 using the LR Clonase II Enzyme Mix (Invitrogen). Flp-In T-Rex HEK293 cells were grown in DMEM
408 supplemented with 10% FBS and 1% pen/strep (100 U/ml). One day before transfection Pen/strep is removed.
409 When reached 60-80% confluence cells were transfected with each of the constructs with pOG44 using
410 Superfect. Cells were refreshed 3h after transfection. After 48h cells were selected by the addition of
411 hygromycin and Blasticidin (Invitrogen). Clones were selected and when reaching 60-80% confluency, protein
412 expression was induced by using 1 µg/ml doxycycline (Sigma Aldrich) for 24h and checked by Western blot
413 analysis.

414

415 **Biotin ligase proximity assay (BioID)**

416 Doxycycline 1 µg/ml (Sigma Aldrich) was added to Flp-In T-Rex HEK293 cells to induce the expression of the
417 construct. After 24h the cells were washed with PBS 1X twice and placed in complete medium for 3h.
418 Afterwards, a final concentration of 50 µM of biotin was added to the cells to start the biotinylation process ⁴⁶.
419 After 24h cells were washed, collected and lysed in lysis buffer (50 mM Tris-HCl pH7.4, 500 mM NaCl, 0.4%
420 SDS, 1 mM DTT and 1x Protease inhibitor) with 2% TX100 and then sonicated twice. Pre-chilled 50 mM Tris-
421 HCl pH7.8 was added before the third sonication and then all samples were collected at 16.500g for 10 minutes
422 at 4 °C. Supernatant was added to the pre-equilibrated dynabeads and it was incubated overnight in the rotator.
423 Next day, beads were collected in the magnetic separation stand and the supernatant was removed. Beads were
424 washed 4 times with 4 different washing buffers (Wash buffer 1: 2% SDS. Wash buffer 2: 0.1% (w/v)
425 deoxycholic acid, 1 % TX100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7,4. Wash buffer 3: 0.5 % (w/v)
426 deoxycholic acid, 0.5 % (w/v) NP-40 (Igepal), 1 mM EDTA, 250 mM LiCl, 10 mM Tris-HCl pH7.8) for 8
427 minutes in the rotator. After the washing 50 µl 50mM ABC / 8M Urea was added to the beads and resuspended
428 gently by pipetting, then the sample was snap frozen in LN2 and stored at -80 degrees.

429 To prepare the beads first we added 1 µl of reduction buffer (10mM DTT) for 30' at RT, then 1 µl alkylation
430 buffer (50 mM chloroacetamide in 50mM ABC) and incubated for 20' at room temperature (light protected).
431 Afterwards 1ug of LysC was added and incubated for at least 3 hours at room temperature. Subsequently the
432 sample was diluted in 50mM ABC and 1ul of trypsin was incorporated for digestion overnight at 37 degrees on a
433 thermomixer on continuous agitation at 700 rpm. The supernatant containing the peptides was transferred to a
434 new Eppendorf tube and TFA was added to a final concentration of 2%. All samples were incorporated into
435 STAGE TIPS that were previously washed, once all sample has pass the filter 50 µl of buffer A (0.1% formic

436 acid in HPLC water) was added and centrifuged. Then 40 μ l of buffer B (80% ACN and 0.1% formic acid in
437 HPLC water) was added and the eluate was collected in 0.5 ml reaction vials. All samples were concentrated and
438 dried. Afterwards, 25 μ l of buffer A was added and sonicated 2' in water bath before using the detergent removal
439 kit. Measurements were performed by nanoLC 1000 (Thermo Scientific) chromatography coupled online to Q
440 Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific). Chromatography was performed
441 with an Acclaim PepMap 0.3 x 5 mm 5 μ m 100 \AA trap column (Thermo scientific) in combination with a 15cm
442 long x 100 μ m ID fused silica electrospray emitter (New Objective, PicoTip Emitter, FS360-100-8-N-5-C15)
443 packed in-house with ReproSil-Pur C18-AQ 3 μ m 140 \AA resin (Dr. Maisch)⁴⁷. Tryptic peptides were loaded and
444 analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously explained. Raw data
445 files provided by MaxQuant (version 1.5.0.25; www.maxquant.org) were further analysed taking the log10 value
446 of their iBAQ and comparing experiment values (with doxycycline and biotin) with control ones (without
447 doxycycline and with biotin and vice versa). Proteins with a significant increase in their iBAQ values (Wilcoxon
448 rank-sum test, FDR 0.05) with respect to their own control runs, were considered as potential interacting
449 candidates.

450

451 **Complexome profiling**

452 Complexome profiling was performed essentially as described previously^{6,31}, except for the chymotrypsin
453 complexomics data, where chymotrypsin was used instead of trypsin and, from the 60 slices, 20 (slices 30 to 50)
454 where further processed.

455 BN- PAGE gel lanes of mitochondrial enriched fraction of *TMEM70* KO and controls were incubated in fixing
456 solution (50% methanol, 10% acetic acid, 10 mM ammonium acetate [pH 3]) for 60 minutes, washed twice for
457 30 minutes with ultrapure water, cut in 60 even slices and transferred into a 96-well plate (Millipore
458 MABVN1250) containing 150 μ l of destaining solution (50% methanol, 50 mM ammonium hydrogen carbonate,
459 AHC). Then all the slices were washed three times for 30 minutes under gentle agitation at room temperature
460 with the same solution (AHC) to remove the excess of dye. Afterwards by centrifugation (600 x g, 3min, RT) the
461 excess of solution was removed and in-gel tryptic digest of the gel slices was performed as described before^{6,31}.
462 Gel slices were incubated with 120 μ l of 5mM dithiothreitol for 60 minutes which afterwards was removed by
463 centrifugation, and then 120 μ l of 15mM chloroacetamide were added to each well and also removed after 45
464 minutes of incubation. After drying the gel pieces for 45 minutes at room temperature the gel slices were
465 rehydrated in 20 μ l of trypsin solution or chymotrypsin (5 ng/ μ L in 50 mM AHC and 1 mM CaCl₂) for 30 min

466 at 4°C. Alternatively, chymotrypsin was used in an independent replicate. After addition of 150µl of 50mM
467 AHC solution the gel pieces were incubated overnight at 37°C. The supernatant, containing the peptides was
468 collected by centrifugation (600 x g, 3min, RT) into a new 96-well plate. The column was washed subsequently
469 with 80% acetonitrile and re-equilibrated with 5% acetonitrile for 5 minutes once by adding elution solution
470 (30% acetonitrile, 3% formic acid) for 20 minutes and transferred to a sterile (unfiltered) 96-well plate. The
471 supernatant was dried using a SpeedVac concentrator, remaining peptides were additionally extracted in 20 µl of
472 5% acetonitrile/0.5% formic acid.

473 Tryptic peptides were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in a Q-
474 Exactive Orbitrap Mass Spectrometry System equipped with a nano-flow high-performance liquid
475 chromatography system EASY-nLC 1000 at the front end and the Thermo Scientific Xcalibur 2.2 SP1 Software
476 Package. Peptide separation was performed on a PicoTip emitter column filled with 3 mm C18 beads (Dr Maisch
477 GmbH, Germany) using 30 min linear gradients of 5 to 35% acetonitrile with 0.1% formic acid. The mass
478 spectrometer was operated in a Top 20 dependent, positive ion mode switching automatically between MS and
479 MS/MS. Full scan MS mode (400 to 1400 m/z) was operated at a resolution of 70 000 with automatic gain
480 control (AGC) target of 1×10^6 ions and a maximum ion transfer of 20 ms. Selected ions for MS/MS were
481 analysed using the following parameters: resolution 17500; AGC target of 1×10^5 ; maximum ion transfer of 50
482 ms; 4.0 m/z isolation window; for CID a normalized collision energy 30% was used; and dynamic exclusion of
483 30.0 s. A lock mass ion (m/z=445.12) was used for internal calibration⁴⁸.

484 All raw files were analyzed by MaxQuant software (version 1.5.0.25; www.maxquant.org). Spectra were
485 searched against the *H. sapiens* NCBI RefSeq database with additional sequences of known contaminants and
486 reverse decoy with a strict FDR of 0.01. Database searches were done with 20 ppm and 0.5 Da mass tolerances
487 for precursor ions and fragmented ions, respectively. Trypsin was selected as the protease with two missed
488 cleavages allowed. Dynamic modifications included N-terminal acetylation and oxidation of methionine.
489 Cysteine carbamidomethylation was set as fixed modification. For protein quantification, unique plus razor
490 peptides were considered.

491

492 **Normalization and alignment of complexome profiles**

493 After analysis in Maxquant, the complexome profiles were normalised to correct for varying intensities. Profiles
494 were corrected so that the sum of intensities of proteins annotated as mitochondrial in MitoCarta 2.0³² are equal
495 between samples. After normalisation, the profiles were aligned in silico with COPAL³³ to correct for technical

496 variation caused by shifts in protein migration across the gel. Gaps introduced in the alignment were filled by
497 linear interpolation based on its adjacent values.

498

499 **Quantification of fully assembled complexes and intermediates**

500 Normalized iBAQ values were taken from the profiles after aligning them with COPAL³³ (Supplementary Table
501 S4). In the case of fully assembled complexes, subunits belonging to CI (NDUFB3, NDUFC2, NDUFB1,
502 NDUFA5, NDUFA6, NDUFS4, NDUFB10, NDUFB2, NDUFB5, NDUFS1, NDUFS8, NDUFAB1, NDUFA2,
503 NDUFS2, NDUFS3, NDUFB6, NDUFB4, NDUFB8, NDUFA7, NDUFB7, NDUFV1, NDUFA8, NDUFV3,
504 NDUFA3, NDUFC1, NDUFS5, NDUFA1, NDUFS6, NDUFV2, NDUFS7, NDUFA11, NDUFA9, NDUFB11,
505 NDUFA13, NDUFA12, NDUFB9, ND1, ND2, ND3, ND4, ND5, ND6), CII (SDHA, SDHB, SDHC, SDHD),
506 CIII (CYTB, UQCRQ, UQCR11, UQCRH, UQCRB, UQCRC2, UQCRC1, UQCRFS1, CYC1, UQCR10), CIV
507 (COX6C, COX3, COX2, COX5B, COX4I1, COX6B1, COX7C, COX5A, COX7B, COX7A2, COX8A, COX1),
508 CV (ATP8, ATP6, ATP5G1, ATP5H, ATP5L, ATP5J, ATP5D, ATP5O, ATP5J2, C14orf2, ATP5I, ATP5F1,
509 ATP5C1, USMG5, ATPIF1, ATP5A1, ATP5B) were considered. With respect to CI intermediates, subunits
510 belonging to all the previously described intermediates of CI assembly⁶, namely, Q/P_P-a (NDUFA5, NDUFS2,
511 NDUFS3, NDUFS7, NDUFS8, NDUFAF4, NDUFAF3, TIMMDC1, ND1, NDUFA3, NDUFA8, NDUFA13),
512 P_P-b (NDUFA5, NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFAF4, NDUFAF3, TIMMDC1, ND1,
513 NDUFA3, NDUFA8, NDUFA13), P_D-a (NDUFB6, NDUFB5, NDUFB10, NDUFB11, NDUFB1, ND4,
514 FOXRED1, ATP5SL), P_D-b (NDUFAB1, NDUFB7, NDUFB3, NDUFB8, ND5, NDUFB9, NDUFB2), Q/P_P
515 (NDUFA5, NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFAF4, NDUFAF3, TIMMDC1, ND1, NDUFA3,
516 NDUFA8, NDUFA13, NDUFAF2, NDUFA9, NDUFA1), P_P-b/P_D-a (NDUFS5, NDUFB6, NDUFB5,
517 NDUFB10, NDUFB11, NDUFB1, ND4, FOXRED1, ATP5SL, NDUFB4) and Q/P (NDUFA5, NDUFS2,
518 NDUFS3, NDUFS7, NDUFS8, NDUFAF4, NDUFAF3, TIMMDC1, ND1, NDUFA3, NDUFA8, NDUFA13,
519 NDUFAF2, NDUFA9, NDUFA1, NDUFB6, NDUFB5, NDUFB10, NDUFB11, NDUFB1, ND4, FOXRED1,
520 ATP5SL, NDUFAB1, NDUFB7, NDUFB3, NDUFB8, ND5, NDUFB9, NDUFB2, NDUFS5) were considered.
521 Regarding CV intermediates, subunits belonging to intermediates described in Supplementary Fig. S7 were
522 considered. Those are F₁ and F₁-c (ATP5A1, ATP5B, ATP5C1, ATP5D), F_O early (ATP5F1, ATP5I, ATP5J2,
523 ATP5L) and F_O late (ATP6, ATP8, C14orf2, USMG5, ATP5H, ATP5J, ATP5O, ATP5F1, ATP5I, ATP5J2,
524 ATP5L). For each subunit, we considered and averaged all three values from the peak matching the mass of the
525 complex and its flanking values. Then, we averaged them per condition, finally obtaining one value per subunit.

526 We compared these values between control and KO conditions in a paired manner per subunit using a non-
527 parametric test (Wilcoxon rank-sum test). For intermediates Q/P_P and P_P-b/P_D-a, given their partial overlap due
528 to their similar mass, we selected subunits that are not shared between them in order to be able to measure their
529 behaviour independently.

530

531 **Microscopy**

532 For confocal imaging, HEK293 cells expressing inducible NDUFAF3-GFP were cultured in a Wilco dish
533 (Intracel, Royston, UK), washed with phosphate-buffered saline (PBS), and incubated with 1 μ M Mitotracker
534 Red (Invitrogen) for 15 min and with 10 μ M Hoechst 3342 (Invitrogen) for 30 min, both at 37°C. Before
535 imaging, the culture medium was replaced by a colorless HEPES-Tris (HT) solution (132 mM NaCl, 4.2 mM
536 KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.5 mM D-glucose, and 10 mM HEPES, pH 7.4) and fluorescence images were
537 taken on a ZEISS LSM510 Meta confocal microscope (Carl Zeiss). Images were acquired at a rate of 10 Hz with
538 the use of a \times 63 oil-immersion objective (N.A. 1.4; Carl Zeiss). Zoom factor 2 and pinhole settings were selected
539 for the attainment of an optical section thickness of $< 1 \mu$ m. Measurements were performed at 20°C in the dark.
540 Confocal images of GFP and MitoTracker Red fluorescence were simultaneously collected with the use of an
541 argon laser (laser power 1%) with a 488 nm dichroic mirror and a 500–530 nm band-pass barrier filter in
542 combination with a helium-neon (HeNe) 1 laser (laser power 43%) with a 543 nm dichroic mirror and a 560 nm
543 long-pass filter. With the multitrack setting used, Hoechst fluorescence was subsequently imaged with the use of
544 a 405 nm diode laser (laser power 10%) and a 420–480 band-pass barrier filter.

545

546 **Phylogenetic reconstruction of TMEM70 family**

547 Protein sequences were manually selected from search results obtained with the jackhmmer tool from HMMER⁴⁹
548 version 3.1b2. We initiated the search with TMEM70, TMEM186 or TMEM223 and searched against the
549 UniProtKB database, iterating until getting back all three human paralogs (6, 6 and 5 iterations, respectively). In
550 none of the cases did we retrieve other human proteins than TMEM70, TMEM186 or TMEM223. In order to
551 obtain as many sequences from different phyla as possible, we repeated the strategy using the PSI-BLAST
552 algorithm from BLAST⁵⁰ searching against the refseq protein database. After selecting the sequences, we aligned
553 them with MAFFT⁵¹ v7.306 using the L-INS-I algorithm with the “Leave gappy regions” and “Mafft-homologs:
554 ON” parameters set. The obtained alignment was manually refined by deleting large gaps caused by apparent
555 insertions in only few aligned sequences. Finally, in order to unravel the orthologous groups, we reconstructed

556 the phylogenetic tree with PhyML⁵² version 20120412 and its automatic model selection SMS⁵³ using Bayesian
557 Information Criterion. The resulting tree was plotted with iTOL⁵⁴ version 3.5.4.

558

559 **Co-evolution screen for TMEM70**

560 We employed an in-house orthology database containing 52 diverse eukaryotic species to derive phylogenetic
561 profiles for all human proteins as previously described by van Dam *et al.*⁵⁵. Shortly, we collected high quality
562 proteomes from genome databases and calculated orthologous groups using OrthoMCL (v2.0)⁵⁶. Phylogenetic
563 profiles were computed for each human protein based on the orthologous group they were mapped to. Proteins
564 mapped to the same orthologous group obtained identical profiles. Each profile is a vector of 0's and 1's
565 reflecting the presence of an orthologous group member in a particular species. These profiles, and a species tree
566 for all 52 species as created before⁵⁵ were used as input for the Perl script to derive the differential Dollo
567 parsimony score as previously used by Kensche *et al.*⁵⁷.

568

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575

576 **Author contributions**

577 L.S.-C. and L.G.J.N. conceived the study and designed the research. L.S.-C., M.B. and F.B. performed the
578 experiments and generated the data with help from S.G.-C. and R.R. J.S. aligned the complexomics data. D.M.E.
579 analysed the data. D.M.E. and M.A.H. performed the phylogenetic reconstruction and T.J.P.D. and M.A.H.,
580 performed the coevolution analysis. L.S.-C., D.M.E., M.A.H. and L.G.J.N. wrote the manuscript and S.G.-C.,
581 J.S., T.J.P.D. R.R. and U.B. discussed the results and helped with the manuscript.

582

583 **Competing interests**

584 The authors declare no competing interests.

585

586 **References**

- 587 1 Alston, C. L., Rocha, M. C., Lax, N. Z., Turnbull, D. M. & Taylor, R. W. The genetics and
588 pathology of mitochondrial disease. *J Pathol* **241**, 236-250, doi:10.1002/path.4809 (2017).
- 589 2 Mitchell, P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-
590 osmotic type of mechanism. *Nature* **191**, 144-148 (1961).
- 591 3 Zhu, J., Vinothkumar, K. R. & Hirst, J. Structure of mammalian respiratory complex I. *Nature*
592 **536**, 354-358, doi:10.1038/nature19095 (2016).
- 593 4 Brandt, U. Energy converting NADH:quinone oxidoreductase (complex I). *Annu Rev Biochem*
594 **75**, 69-92, doi:10.1146/annurev.biochem.75.103004.142539 (2006).
- 595 5 Sanchez-Caballero, L., Guerrero-Castillo, S. & Nijtmans, L. Unraveling the complexity of
596 mitochondrial complex I assembly: A dynamic process. *Biochim Biophys Acta* **1857**, 980-990,
597 doi:10.1016/j.bbabbio.2016.03.031 (2016).
- 598 6 Guerrero-Castillo, S. *et al.* The Assembly Pathway of Mitochondrial Respiratory Chain
599 Complex I. *Cell Metab* **25**, 128-139, doi:10.1016/j.cmet.2016.09.002 (2017).
- 600 7 Pedersen, P. L. & Amzel, L. M. ATP synthases. Structure, reaction center, mechanism, and
601 regulation of one of nature's most unique machines. *J Biol Chem* **268**, 9937-9940 (1993).
- 602 8 Walker, J. E. The ATP synthase: the understood, the uncertain and the unknown. *Biochem*
603 *Soc Trans* **41**, 1-16, doi:10.1042/BST20110773 (2013).
- 604 9 Ghezzi, D. & Zeviani, M. Human diseases associated with defects in assembly of OXPHOS
605 complexes. *Essays Biochem* **62**, 271-286, doi:10.1042/EBC20170099 (2018).
- 606 10 Elurbe, D. M. & Huynen, M. A. The origin of the supernumerary subunits and assembly
607 factors of complex I: A treasure trove of pathway evolution. *Biochim Biophys Acta* **1857**, 971-
608 979, doi:10.1016/j.bbabbio.2016.03.027 (2016).
- 609 11 Huynen, M. A., de Hollander, M. & Szklarczyk, R. Mitochondrial proteome evolution and
610 genetic disease. *Biochim Biophys Acta* **1792**, 1122-1129, doi:10.1016/j.bbadis.2009.03.005
611 (2009).

- 612 12 Bych, K. *et al.* The iron-sulphur protein Ind1 is required for effective complex I assembly.
613 *EMBO J* **27**, 1736-1746, doi:10.1038/emboj.2008.98 (2008).
- 614 13 Pickova, A., Paul, J., Petruzzella, V. & Houstek, J. Differential expression of ATPAF1 and
615 ATPAF2 genes encoding F(1)-ATPase assembly proteins in mouse tissues. *FEBS Lett* **551**, 42-
616 46 (2003).
- 617 14 Cizkova, A. *et al.* TMEM70 mutations cause isolated ATP synthase deficiency and neonatal
618 mitochondrial encephalomyopathy. *Nat Genet* **40**, 1288-1290, doi:10.1038/ng.246
619 (2008).
- 620 15 Calvo, S. *et al.* Systematic identification of human mitochondrial disease genes through
621 integrative genomics. *Nat Genet* **38**, 576-582, doi:10.1038/ng1776 (2006).
- 622 16 Hejzlarova, K. *et al.* Expression and processing of the TMEM70 protein. *Biochim Biophys Acta*
623 **1807**, 144-149, doi:10.1016/j.bbabi.2010.10.005 (2011).
- 624 17 Kratochvilova, H. *et al.* Mitochondrial membrane assembly of TMEM70 protein.
625 *Mitochondrion* **15**, 1-9, doi:10.1016/j.mito.2014.02.010 (2014).
- 626 18 Wortmann, S. B. *et al.* Biochemical and genetic analysis of 3-methylglutaconic aciduria type
627 IV: a diagnostic strategy. *Brain* **132**, 136-146, doi:10.1093/brain/awn296 (2009).
- 628 19 Catteruccia, M. *et al.* Persistent pulmonary arterial hypertension in the newborn (PPHN): a
629 frequent manifestation of TMEM70 defective patients. *Mol Genet Metab* **111**, 353-359,
630 doi:10.1016/j.ymgme.2014.01.001 (2014).
- 631 20 Diodato, D. *et al.* Common and Novel TMEM70 Mutations in a Cohort of Italian Patients with
632 Mitochondrial Encephalomyopathy. *JIMD Rep* **15**, 71-78, doi:10.1007/8904_2014_300
633 (2015).
- 634 21 Honzik, T. *et al.* Mitochondrial encephalomyopathy with early neonatal onset due to
635 TMEM70 mutation. *Arch Dis Child* **95**, 296-301, doi:10.1136/ad.2009.168096 (2010).

- 636 22 Jonckheere, A. I. *et al.* Restoration of complex V deficiency caused by a novel deletion in the
637 human TMEM70 gene normalizes mitochondrial morphology. *Mitochondrion* **11**, 954-963,
638 doi:10.1016/j.mito.2011.08.012 (2011).
- 639 23 Magner, M. *et al.* TMEM70 deficiency: long-term outcome of 48 patients. *J Inherit Metab Dis*
640 **38**, 417-426, doi:10.1007/s10545-014-9774-8 (2015).
- 641 24 Shchelochkov, O. A. *et al.* Milder clinical course of Type IV 3-methylglutaconic aciduria due to
642 a novel mutation in TMEM70. *Mol Genet Metab* **101**, 282-285,
643 doi:10.1016/j.ymgme.2010.07.012 (2010).
- 644 25 Spiegel, R. *et al.* TMEM70 mutations are a common cause of nuclear encoded ATP synthase
645 assembly defect: further delineation of a new syndrome. *J Med Genet* **48**, 177-182,
646 doi:10.1136/jmg.2010.084608 (2011).
- 647 26 Torraco, A. *et al.* TMEM70: a mutational hot spot in nuclear ATP synthase deficiency with a
648 pivotal role in complex V biogenesis. *Neurogenetics* **13**, 375-386, doi:10.1007/s10048-012-
649 0343-8 (2012).
- 650 27 Houstek, J., Kmoch, S. & Zeman, J. TMEM70 protein - a novel ancillary factor of mammalian
651 ATP synthase. *Biochim Biophys Acta* **1787**, 529-532, doi:10.1016/j.bbabi.2008.11.013
652 (2009).
- 653 28 Braczynski, A. K. *et al.* ATP synthase deficiency due to TMEM70 mutation leads to
654 ultrastructural mitochondrial degeneration and is amenable to treatment. *BioMed research*
655 *international* **2015**, 462592, doi:10.1155/2015/462592 (2015).
- 656 29 Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large
657 gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44-57,
658 doi:10.1038/nprot.2008.211 (2009).
- 659 30 Wessels, H. J. *et al.* LC-MS/MS as an alternative for SDS-PAGE in blue native analysis of
660 protein complexes. *Proteomics* **9**, 4221-4228 (2009).

- 661 31 Heide, H. *et al.* Complexome profiling identifies TMEM126B as a component of the
662 mitochondrial complex I assembly complex. *Cell Metab* **16**, 538-549,
663 doi:10.1016/j.cmet.2012.08.009 (2012).
- 664 32 Calvo, S. E., Clauser, K. R. & Mootha, V. K. MitoCarta2.0: an updated inventory of mammalian
665 mitochondrial proteins. *Nucleic Acids Res* **44**, D1251-1257, doi:10.1093/nar/gkv1003 (2016).
- 666 33 Van Strien, J. *et al.* COmplexome Profiling ALignment (COPAL) reveals remodeling of
667 mitochondrial protein complexes in Barth syndrome. *Bioinformatics*,
668 doi:10.1093/bioinformatics/btz025 (2019).
- 669 34 Nijtmans, L. G., Klement, P., Houstek, J. & van den Bogert, C. Assembly of mitochondrial ATP
670 synthase in cultured human cells: implications for mitochondrial diseases. *Biochim Biophys*
671 *Acta* **1272**, 190-198 (1995).
- 672 35 Boulet, L., Karpati, G. & Shoubridge, E. A. Distribution and threshold expression of the
673 tRNA(Lys) mutation in skeletal muscle of patients with myoclonic epilepsy and ragged-red
674 fibers (MERRF). *Am J Hum Genet* **51**, 1187-1200 (1992).
- 675 36 Moller-Hergt, B. V., Carlstrom, A., Stephan, K., Imhof, A. & Ott, M. The ribosome receptors
676 Mrx15 and Mba1 jointly organize cotranslational insertion and protein biogenesis in
677 mitochondria. *Mol Biol Cell* **29**, 2386-2396, doi:10.1091/mbc.E18-04-0227 (2018).
- 678 37 Guarani, V. *et al.* TIMMDC1/C3orf1 functions as a membrane-embedded mitochondrial
679 complex I assembly factor through association with the MCIA complex. *Molecular and*
680 *cellular biology* **34**, 847-861, doi:10.1128/MCB.01551-13 (2014).
- 681 38 He, J. *et al.* Assembly of the membrane domain of ATP synthase in human mitochondria. *Proc*
682 *Natl Acad Sci U S A* **115**, 2988-2993, doi:10.1073/pnas.1722086115 (2018).
- 683 39 Cameron, J. M. *et al.* Complex V TMEM70 deficiency results in mitochondrial nucleoid
684 disorganization. *Mitochondrion* **11**, 191-199, doi:10.1016/j.mito.2010.09.008 (2011).
- 685 40 Song, J., Pfanner, N. & Becker, T. Assembling the mitochondrial ATP synthase. *Proc Natl Acad*
686 *Sci U S A* **115**, 2850-2852, doi:10.1073/pnas.1801697115 (2018).

- 687 41 Janssen, A. J. *et al.* Spectrophotometric assay for complex I of the respiratory chain in tissue
688 samples and cultured fibroblasts. *Clin Chem* **53**, 729-734, doi:10.1373/clinchem.2006.078873
689 (2007).
- 690 42 Srere, P. A. The citrate cleavage enzyme. I. Distribution and purification. *J Biol Chem* **234**,
691 2544-2547 (1959).
- 692 43 Calvaruso, M. A., Smeitink, J. & Nijtmans, L. Electrophoresis techniques to investigate defects
693 in oxidative phosphorylation. *Methods* **46**, 281-287, doi:10.1016/j.ymeth.2008.09.023
694 (2008).
- 695 44 Wittig, I., Velours, J., Stuart, R. & Schagger, H. Characterization of domain interfaces in
696 monomeric and dimeric ATP synthase. *Mol Cell Proteomics* **7**, 995-1004,
697 doi:10.1074/mcp.M700465-MCP200 (2008).
- 698 45 Vogel, R. O. *et al.* Cytosolic signaling protein Ecsit also localizes to mitochondria where it
699 interacts with chaperone NDUFAF1 and functions in complex I assembly. *Genes Dev* **21**, 615-
700 624, doi:10.1101/gad.408407 (2007).
- 701 46 Firat-Karalar, E. N. & Stearns, T. Probing mammalian centrosome structure using BioID
702 proximity-dependent biotinylation. *Methods Cell Biol* **129**, 153-170,
703 doi:10.1016/bs.mcb.2015.03.016 (2015).
- 704 47 Ishihama, Y., Rappsilber, J., Andersen, J. S. & Mann, M. Microcolumns with self-assembled
705 particle frits for proteomics. *J Chromatogr A* **979**, 233-239 (2002).
- 706 48 Olsen, J. V. *et al.* Parts per million mass accuracy on an Orbitrap mass spectrometer via lock
707 mass injection into a C-trap. *Mol Cell Proteomics* **4**, 2010-2021, doi:10.1074/mcp.T500030-
708 MCP200 (2005).
- 709 49 Finn, R. D. *et al.* HMMER web server: 2015 update. *Nucleic Acids Res* **43**, W30-38,
710 doi:10.1093/nar/gkv397 (2015).
- 711 50 Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database
712 search programs. *Nucleic Acids Res* **25**, 3389-3402 (1997).

- 713 51 Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7:
714 improvements in performance and usability. *Mol Biol Evol* **30**, 772-780,
715 doi:10.1093/molbev/mst010 (2013).
- 716 52 Guindon, S. *et al.* New algorithms and methods to estimate maximum-likelihood
717 phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* **59**, 307-321,
718 doi:10.1093/sysbio/syq010 (2010).
- 719 53 Lefort, V., Longueville, J. E. & Gascuel, O. SMS: Smart Model Selection in PhyML. *Mol Biol Evol*
720 **34**, 2422-2424, doi:10.1093/molbev/msx149 (2017).
- 721 54 Letunic, I. & Bork, P. Interactive tree of life (iTOL) v3: an online tool for the display and
722 annotation of phylogenetic and other trees. *Nucleic Acids Res* **44**, W242-245,
723 doi:10.1093/nar/gkw290 (2016).
- 724 55 van Dam, T. J. *et al.* Evolution of modular intraflagellar transport from a coatomer-like
725 progenitor. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 6943-6948, doi:10.1073/pnas.1221011110
726 (2013).
- 727 56 Li, L., Stoeckert, C. J., Jr. & Roos, D. S. OrthoMCL: identification of ortholog groups for
728 eukaryotic genomes. *Genome Res.* **13**, 2178-2189, doi:10.1101/gr.1224503 (2003).
- 729 57 Kensche, P. R., van Noort, V., Dutilh, B. E. & Huynen, M. A. Practical and theoretical advances
730 in predicting the function of a protein by its phylogenetic distribution. *J R Soc Interface* **5**,
731 151-170, doi:10.1098/rsif.2007.1047 (2008).
- 732 58 Nikolovski, N. *et al.* Putative glycosyltransferases and other plant Golgi apparatus proteins
733 are revealed by LOPIT proteomics. *Plant Physiol* **160**, 1037-1051, doi:10.1104/pp.112.204263
734 (2012).
- 735 59 Matsuyama, A. *et al.* ORFeome cloning and global analysis of protein localization in the
736 fission yeast *Schizosaccharomyces pombe*. *Nat Biotechnol* **24**, 841-847, doi:10.1038/nbt1222
737 (2006).

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