A spontaneous mitonuclear epistasis converging on Rieske Fe-S protein exacerbates complex III deficiency in mice

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We previously observed an unexpected fivefold (35 vs. 200 days) difference in the survival of respiratory chain complex III (CIII) deficient Bcs1lp.S78G mice between two congenic backgrounds. Here, we identify a spontaneous homoplasmic mtDNA variant (m.G14904A, mt-Cybp.D254N), affecting the CIII subunit cytochrome b (MT-CYB), in the background with short survival. We utilize maternal inheritance of mtDNA to confirm this as the causative variant and show that it further decreases the low CIII activity in Bcs1lp.S78G tissues to below survival threshold by 35 days of age. Molecular dynamics simulations predict D254N to restrict the flexibility of MT-CYB ef loop, potentially affecting RISP dynamics. In Rhodobacter cytochrome bc1 complex the equivalent substitution causes a kinetics defect with longer occupancy of RISP head domain towards the quinol oxidation site. These findings represent a unique case of spontaneous mitonuclear epistasis and highlight the role of mtDNA variation as modifier of mitochondrial disease phenotypes.
Mitochondrial respiratory chain complex III (cytochrome bc complex, CIII) oxidizes coenzyme Q, reduces cytochrome c, and translocates protons to generate membrane potential for ATP synthesis\(^1\). The mitochondrial inner membrane AAA-family translocase BCS1L, frequently mutated in CIII deficiency\(^2-4\), is required for the topogenesis of the electron-transferring RISP (Rieske iron-sulphur protein, UQCRFS1) subunit and its assembly into CIII\(^5-7\). Homozygous Bcs1lp.S78G (Bcs1lp.S78G) knock-in mice\(^8\), carrying a GRACILE syndrome\(^9,10\) patient mutation\(^1\), recapitulate many manifestations of human CIII deficiency. They display post-weaning growth failure, hepatopathy, renal tubulopathy, and, in a C57BL/6J BomTac-derived genetic background (Lund colony), deterioration due to metabolic crisis with extreme hypoglycemia by 35 days of age (P35)\(^11\). Intriguingly, the variant affects the mtDNA-encoded mt-Cybp.D254N\(^1\) protein, UQCRFS1) subunit and its assembly into CIII\(^5\). We utilized maternal inheritance of mtDNA and crossbred Bcs1lp.S78G heterozygotes from the two inbred colonies to obtain F1 progeny carrying otherwise equalized nDNA background (heterozygous for all initially homozygous alleles differing between the colonies) and either WT or variant mitochondria (Fig. 1b). The homozygous Bcs1lp.S78G progeny of females from the Lund colony (C57BL/6j BomTac) had median survival of 38 days while that of females from the Helsinki colony (C57BL/6j Crl) 150 days (Fig. 1c). As WGS showed no other mtDNA differences between the strains, the crossbreeding unequivocally confirmed that the maternally inherited mt-Cybp.D254N variant was the major determinant of the survival of Bcs1lp.S78G mice. We have previously described the early-onset disease manifestations (growth restriction, hypoglycemic, hepatopathy, and kidney tubulopathy) of Bcs1lp.S78G mice in both the short- and long-living strains separately\(^8,13,15-18\). Here we reassessed some of the main manifestations in the genetically comparable F1 mice. The presence of mt-Cybp.D254N aggravated the growth restriction of the mutant mice (Fig. 1d). Remarkably, mt-Cybp.D254N alone also slightly decreased the weight of the F1 juvenile Bcs1l WT progeny. All Bcs1lp.S78G mice had abnormally low blood glucose, but in those carrying mt-Cybp.D254N, the blood glucose was below quantification limit in several individuals, indicating severe hypoglycemia expected to lead to coma and death (Fig. 1e). Histopathological analysis revealed hepatopathy with glycogen depletion and incipient fibrosis that was similar in both mutant genotypes up to age P29–P33 (Supplementary Fig. 2). However, in the very end-stage (>P34) livers, diffuse hepatocyte death was present, indicating rapid deterioration to fulminant hepatic failure in the mt-Cybp.D254N-carrying mutants (Supplementary Fig. 3). Staining for cleaved caspase 3, a standard marker of apoptosis, confirmed massive hepatic cell death in the end-stage mt-Cybp.D254N-carrying mutants (Fig. 1g, h, Supplementary Fig. 3). This time point coincided with near-complete exhaustion of hepatic ATP (Fig. 1f). In contrast, only few dying hepatocytes were present in comparable liver sections from Bcs1lp.S78G mice with WT mitochondria (Fig. 1g, h). Instead of rapid ATP depletion, these mice showed a trend toward normalization after P34 (Fig. 1f). These findings are in line with our previous studies\(^14,15\), showing that the hepatopathy of Bcs1lp.S78G mice in the long-living colony does not progress to liver failure even at end stage (P150–P200).

The kidneys of all Bcs1lp.S78G mice were atrophic but luminal casts suggesting proteinuria were obvious only when these mice carried mt-Cybp.D254N, indicating more advanced kidney disease (Supplementary Fig. 4).

**Results**

**Short-lived Bcs1lp.S78G mice carry a novel mtDNA variant.** WGS (n = 3 for Lund C57BL/6j BomTac and n = 2 for Helsinki C57BL/6j Crl) revealed 844 homoyzgous single-nucleotide polymorphisms and 3655 small insertion/deletions between the strains, only 8 of which were in coding regions of genes (Supplementary Table 1). One of these was an mtDNA variant (m. G14904A) not present in any Mus musculus sequence in GenBank. Genotyping of approximately 80 mice throughout past generations using archived genomic DNA from ear biopsies revealed that the variant was introduced from wild-type (WT) C57BL/6j BomTac females repeatedly after 2008, when con-generation of the Bcs1lp.A232G knock-in allele was started (Supplementary Fig. 1a). Inspection of the pedigrees of the mt-Cybp genotyped mice showed that two early-generation females had given birth to both WT and variant-carrying progeny, suggesting initial heteroplasmacy. However, sequencing of 346 bacterial clones of mt-Cybp PCR amlicon from the liver, kidney, heart, and skeletal muscle DNA showed no sign of heteroplasmacy in somatic tissues in later generations (Supplementary Fig. 1b). The fact that the variant was homoplasmic in an apparently healthy WT mouse colony suggested that it must be non-pathogenic. Indeed, analysis of mtDNA sequences deposited to GenBank showed that the three-toed sloth species (Bradypus) of South America, known for their very low metabolic rate\(^16,17\), naturally carry this variant (Fig. 1a). Intriguingly, the variant affects the mtDNA-encoded CIII subunit cytochrome b, changing a conserved aspartate 254 to asparagine (mt-Cybp.D254F). A negatively charged amino acid in this position is highly conserved across eukaryotes and aerobic prokaryotes (Fig. 1a), with limited conservancy in archaea. Therefore, as mt-Cybp.D254N potentially directly affects CIII function, it appeared as a most likely genetic modifier of the survival of Bcs1lp.S78G mice.

**mt-Cybp.D254N dictates the short survival of Bcs1lp.S78G mice.** After the crossbreeding experiment showed unequivocally that mt-Cybp.D254N was the major determinant of the survival of Bcs1lp.S78G mice, we set out to investigate its effect on CIII activity. We have previously shown\(^8\) that, in the short-living background, the progressive CIII dysfunction is severest in the liver, the activity being normal at birth but thereafter rapidly decreasing to as low as 10% of WT activity near end stage (P33–P35). The first symptoms appear soon (P24) after weaning at approximately 50% residual activity. Below 25% residual activity, the mice become severely hypoglycemic and die within a few days\(^8,13\). We measured CIII activity in the key affected (liver, kidney) and histologically non-affected (skeletal muscle, heart) tissues in the F1 mice and found that the low CIII activity was further decreased by mt-Cybp.D254N in all four tissues (Fig. 2). The mean hepatic CIII activity at P33 was 9% of WT values in mt-Cybp.D254N; Bcs1lp.S78G mice, well below the survival threshold.
**Fig. 1** Identification of a spontaneous mtDNA variant as a modifier of Bcs1l mutant phenotype. 

**a** Alignment of amino acids 236–275 (numbering for *Mus musculus* sequence) of MT-CYB protein of various organisms. Asterisk (*), Lund in-house C57BL/6JBomTac-derived mouse colony. 

**b** Cartoon of the crosses to assess the effect of the maternally inherited mtDNA on the survival of Bcs1l<sup>S78G</sup> mice. 

**c** Survival analysis of Bcs1l<sup>S78G</sup> mice with either the C57BL/6JBomTac* (*n* = 8) or C57BL/6JCrl (Helsinki colony) maternal parent (*n* = 6). Light blue- and red-shaded areas illustrate 95% CI. 

**d** Weight and 

**e** Blood glucose plotted against mouse age. 

**f** Hepatic ATP concentration of Bcs1l mutant mice with and without the mt-Cyb<sup>p.D254N</sup> variant plotted against mouse age. 

**g** The degree of hepatic apoptosis, cleaved caspase-3-positive cross-sectional area, plotted against age. 

**h** Representative micrographs of immunostained liver sections for cleaved caspase 3 from 34- to 35-day-old mice. Scale bar represents 100 µm. 

**Statistics:** 

- **c**: log-rank (Mantel–Cox) test, 
- **d**: one-way ANOVA followed by planned comparisons, 
- **e**: Kruskal–Wallis followed by Mann–Whitney U tests. Error bars represent 95% CI of the mean in all figures.
ANOVA followed by planned comparisons. Error bars represent 95% CI of activity, a value observed in the liver of manifested in homogenates were normalized to relative respiratory chain content as to mitochondrial protein amount. The data from muscle and heart activity data from isolated liver and kidney mitochondria were normalized of shown in our earlier studies. Furthermore, the mean CIII activity 4 of WT CIII activity (Fig. 2a). The mean hepatic CIII activity remained at 25% of WT in Bcslp.S78G homozygotes with WT mt-Cyb (Fig. 2a, c), as also shown in our earlier studies. Furthermore, the mean CIII activity of Bcslp.S78G homozygotes with WT mtDNA did not decrease <50% in the kidney and heart, whereas in mt-Cybp.D254N carriers it did (Fig. 2c, f). Further measurements from a panel of mice backcrossed several times to the C57BL/6Jcr background confirmed the synergistic effect of Bcslp.S78G and mt-Cybp.D254N on CIII activity (Fig. 2c, d, g, h and Supplementary Fig. 5). Interestingly, mt-Cybp.D254N alone consistently decreased cardiac CIII activity in both sample panels (Fig. 2f, g, Supplementary Fig. 5), whereas this trend was not as obvious in other tissues (Fig. 2). For comparison, we analyzed the enzymatic activities of other respiratory chain complexes in isolated liver and kidney mitochondria from the F1 mice (Supplementary Fig. 6). CI and CIV activities were similar in all groups. CII activity was slightly lower in the Bcslp.S78G,mt-Cybp.D254N liver as compared to the other genotypes, while in the kidney, CII activity was increased by sole mt-Cybp.D254N or Bcslp.S78G but not by their combination. In sum, mt-Cybp.D254N further compromised CIII activity in Bcslp.S78G mice, decreasing it below survival threshold.

Aspartate 254 is located in the MT-CYB of loop involved in RISP binding20,21, which raised the question whether its substitution to aspartagine might exacerbate the RISP assembly defect caused by the Bcs1l mutation. To quantify RISP assembled into CIII, we subjected digitonin-solubilized liver (Fig. 3a) and kidney (Fig. 3b) mitochondrial fractions to blue native gel electrophoresis. The steady-state level of RISP in CIII dimer and supercomplexes was markedly affected by the Bcs1l genotype, but with no consistent further impairment by mt-Cybp.D254N in these tissues.

Fig. 2 Synergistic effect of Bcslp.S78G and mt-Cybp.D254N on CIII activity. a-h CIII activity in liver (a, b) and kidney mitochondrial fractions (c, d) and heart (e, f) and skeletal muscle homogenates (g) from F1 hybrid progeny (a, c, e, g) and from mice backcrossed to C57BL/6Jcr (b, d, f, h). CIII activity data from isolated liver and kidney mitochondria were normalized to mitochondrial protein amount. The data from muscle and heart homogenates were normalized to relative respiratory chain content as assessed by measuring cyanide-sensitive cytochrome c oxidation. Cardiac and skeletal muscle homogenate CIII activity relative to tissue protein are shown in Supplementary Fig. 5. The orange line represents 50% residual activity, a threshold for appearance of hypoglycemic and hepatic manifestations in Bcs1l mutant mice. The red line represents 25% residual activity, a value observed in the liver of Bcs1l mutant mice in the colony with short survival at the onset of the terminal stage. Statistics: one-way ANOVA followed by planned comparisons. Error bars represent 95% CI of the mean.

mt-Cybp.D254N modifies mitochondrial bioenergetics. We have previously shown that CI&II-linked oxidative phosphorylation (OXPHOS) is compromised in Bcs1l.S78G mice in both the short- and long-living colonies8,15,18. To assess how mt-Cybp.D254N might affect respiratory electron transfer and OXPHOS, we subjected liver and kidney mitochondria from the F1 hybrid mice (Supplementary Fig. 7) and a panel of mice backcrossed to C57BL/6Jcr (Fig. 4, Supplementary Figs. 8 and 9) to high-resolution respirometry. The CIII defect of Bcs1l mutant mice with or without mt-Cybp.D254N did not compromise OXPHOS driven by sole NADH-producing substrates (CI-linked respiration) (Supplementary Figs. 7 and 8) as we have previously reported15,18 and in line with known high threshold for CIII inhibition22. Therefore, we proceeded to assess maximal OXPHOS capacity via convergent electron flow to the coenzyme Q pool via CI and CII by subsequent addition of succinate (CI&II-linked OXPHOS). This parameter strongly correlated with residual CIII activity (Supplementary Fig. 10) and revealed the CIII dysfunction in mice carrying Bcs1l.S78G and a further exacerbation by mt-Cybp.D254N (Fig. 4a and Supplementary Figs. 7, 9).

After measuring CI&II-linked phosphorylating respiration, we inhibited ATP synthesize with oligomycin to induce leak respiration, a high membrane potential state in which limited respiration occurs due to proton leakage. Similar to CI&II-linked phosphorylating respiration, the CI&II-linked leak respiration was also low in Bcs1l.S78G and even lower in Bcs1l.S78G,mt-Cybp.D254N tissues (Fig. 4b and Supplementary Fig. 7). Interestingly, mt-Cybp.D254N alone decreased CI&II-linked leak respiration in kidney mitochondria (Fig. 4b and Supplementary Fig. 7b). We did not observe this in liver mitochondria from the F1 hybrids (Supplementary Fig. 7a), but in liver mitochondria from mice backcrossed to C57BL/6Jcr (Fig. 4b) the trend was significant and similar to that in the kidney.

In some tissues, the phosphorylating system (adenine nucleotide translocase, phosphate transporters, and the ATP synthesize) limits the electron-transferring proton translocases and subsequent respiration. Therefore, we also measured maximal capacity of the electron transfer by dissipating the membrane potential
with the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). The rate of uncoupled CI\&CII-linked respiration revealed that the respiration in BcsII mutant mitochondria with or without mt-Cyb\(^{D254N}\) was limited by both the CIII defect and by the phosphorylating system (Fig. 4c and Supplementary Fig. 7). mt-Cyb\(^{D254N}\) alone did not limit CI\&CII-linked maximal electron transfer capacity. However, as the leak respiration was decreased, the mt-Cyb\(^{D254N}\) mice showed elevated electron transfer coupling efficiency (Fig. 4d, e, Supplementary Fig 7), which is a ratio calculated from leak respiration and maximal electron transfer capacity. The decreased leak respiration and increased electron transfer coupling efficiency suggest that mt-Cyb\(^{D254N}\) decreases proton leakage at CIII, or indirectly elsewhere, or it affects a rate-limiting step in CIII enzymatic cycle when it is working against high membrane potential.

Finally, as a measure of CII and CIII-derived reactive oxygen species production, we measured H\(_2\)O\(_2\) emission under succinate-driven phosphorylating respiration with reverse electron flow to CI blocked by rotenone (Supplementary Fig. 11). Total H\(_2\)O\(_2\) emission (per mitochondrial protein) was not significantly different between the groups. However, relative to oxygen consumption it was increased in the BcsI\(_{p,S78G}\) mitochondria from the liver (median: 1.5% vs 0.8%, Mann–Whitney U test \(p<0.0001\)) and kidney (0.8% vs 0.5%, \(p = 0.003\)) as compared to BcsII WTs.

**mt-Cyb\(^{D254N}\)** restricts RISP head domain movement. As mt-Cyb\(^{D254N}\) did not seem to have a consistent effect on CIII assembly, which is compromised by the BcsII mutation, a more subtle mechanism must underlie their synergistic effect on CIII activity in mice. We inspected published crystal structures of euarkyotic CIII to model structural changes that the D254N amino acid change might cause in MT-CYB or in the complex. These showed that the RISP head domain (RISP-HD) brushes the ef loop segment of MT-CYB and docks to both the MT-CYB and CYTC1 subunits in two different conformations to promote electron transfer (Fig. 5a–c). Thus an amino acid substitution in the ef loop segment may lead to compromised RISP dynamics\(^{23,24}\).

In order to obtain further microscopic insight, we performed fully atomistic classical molecular dynamics (MD) simulations of cytochrome bc\(_1\) in membrane-solvent environment. These showed that D254N likely restricts the conformational flexibility in the surrounding region (Fig. 5d–g), displayed as the root mean square fluctuation of the segment T250 to V270 in the ef loop segment of MT-CYB (Fig. 5d), which strongly interacts with RISP. Accordingly, we observed a simultaneous reduction in mobility for the segment P270 to W290 in the ef loop residues with the QH2 head group. In simulations without Q molecules (Supplementary Fig. 12). This is in part due to the brushing of ef loop residues with the QH\(_2\) head group. In WT simulations, residues P270 and Y298 contacted the QH\(_2\) head group (≤3.5 Å criteria) for ca. 70% and 90% of the simulation time, respectively. Similarly, in mutant simulations, P270 and Y298 interacted with QH\(_2\) for 100 and 18% of total simulation time, with an additional interaction observed between QH\(_2\) and E271 (48%). These interactions are likely to reduce the ef loop dynamics in tight coupling with substrate binding and dynamics. Nevertheless, a subtle difference in mobility was observed for the ef loop segment in agreement with the current experimental and earlier biochemical data\(^{26}\).
MT-CYB

Generated with a substrate cocktail comprising malate, pyruvate, and glutamate. Succinate served as a direct substrate for CII. Mitochondria subjected to sensitive structural and functional measurements. Succinate-dependent photosynthetic growth (Supplementary Fig. 13, Supplementary Table 2). To measure RISP movement, predicted by the MD simulations to be affected by the stiffened mutant loop, we monitored spectroscopically the enhancement of phase relaxation of RISP [2Fe-2S] cluster by oxidized heme $b_5$. This method allows monitoring of changes in the distribution of RISP-HD positions between the $Q_o$ position and cytochrome $c_1$ position. The enhancement is strongest when the motion of RISP-HD is restricted predominantly toward positions at the $Q_o$ site, such as in the $+2$Ala mutant (Fig. 5h, i) that was used as one reference point. Unrestricted motion in the native enzyme resulted in a much broader distribution of RISP-HD positions and a relatively weak enhancement (Fig. 5h, i), providing us with another reference point. The D278N mutant displayed an increased enhancement compared to native enzyme but not as large as in the $+2$Ala. This indicates that the D278N mutation caused a subtle but remarkable effect on the motion, shifting the average position of RISP-HD toward the $Q_o$ position and confirming the predictions from the MD simulations of the eukaryotic complex.

**mt-Cybp.D254N** modifies whole-body metabolism. To assess the metabolic effect of **mt-Cybp.D254N** at whole-body level, we...
subjected the mice to indirect calorimetry (Fig. 6). Bcs1lp.S78G homozygotes with WT mitochondria did not significantly differ from WT mice during daytime measurements, whereas Bcs1lp.S78G homozygotes carrying mt-Cybp.D254N variant clearly did, as indicated by low respiratory exchange ratio (RER) and energy expenditure (Fig. 6a–c). Both parameters suggested a profound starvation-like metabolic state in these mice. The decreased energy expenditure remained highly significant also after treating body weight as a covariate. Mice are nocturnal animals and nighttime whole-body metabolism data showed an even more distinct separation of Bcs1lp.S78G;mt-Cybp.D254N mice from all other genotypes (Fig. 6a–c). Measurements during nighttime distinguished also Bcs1lp.S78G mice with WT mitochondria and, surprisingly, those carrying mt-Cybp.D254N alone from WT mice. In fact, mt-Cybp.D254N variant and the Bcs1lp.S78G mutation caused a similar decrease in RER and energy expenditure. Differences in physical activity did not explain these differences (Supplementary Fig. 14).
Average 12-h energy expenditure estimated by indirect calorimetry for daytime and nighttime. The plotted values represent weight-normalized energy expenditure from ANCOVA model with genotype as a fixed factor and weight as a covariate.

System (CLAMS) apparatus was used to measure O2 consumption and CO2 production by the four different mouse groups (backcrossed to C57BL/6JCrl) at age P28. Strikingly, at age P28, statistics: one-way ANOVA, another circulating marker of mitochondrial stress-inducible endocrine factor, Fgf21, a mainly hepatocyte-derived endocrine factor linked to mitochondrial stress and stressed energy metabolism, and Gdf15, a macrophage-secreted marker of mitochondrial dysfunction. Statistics: one-way ANOVA (b, d, e) or ANCOVA (c) followed by planned comparisons with Welch’s t statistics.

Fig. 6 Whole-body metabolism in wild-type and Bcs1lP.S78G mice with and without mt-Cybp.D254N. Comprehensive Laboratory Animal Monitoring System (CLAMS) apparatus was used to measure O2 consumption and CO2 production by the four different mouse groups (backcrossed to C57BL/6JCrl) at age P28-29. a Circadian rhythm of respiratory exchange ratio. b Average 12-h respiratory exchange ratio of daytime and nighttime measurements. c Average 12-h energy expenditure estimated by indirect calorimetry for daytime and nighttime. The plotted values represent weight-normalized energy expenditure from ANCOVA model with genotype as a fixed factor and weight as a covariate. d Hepatic gene expression of a hepatocyte-derived mitochondrial stress-inducible endocrine factor, fgl2. e Hepatic gene expression of Gdf15, a macrophage-secreted marker of mitochondrial dysfunction. Statistics: one-way ANOVA (b, d, e) or ANCOVA (c) followed by planned comparisons with Welch’s t statistics.

To shed further light on the metabolic effects of mt-Cybp.D254N, we measured hepatic gene expression of Fgl2, a mainly hepatocyte-derived endocrine factor linked to mitochondrial stress and stressed energy metabolism, and Gdf15, another circulating marker of mitochondrial dysfunction. We have previously shown that both are induced in Bcs1l mutant mice at later age14,15. Also here Fgl2 and Gdf15 expression were upregulated by the Bcs1l mutation (Fig. 6d, e), in line with expected metabolic stress and energy deprivation. Strikingly, mt-Cybp,D254N alone induced the expression of Fgl2 to similar level as the pathogenic Bcs1l mutation (Fig. 6d), showing together with the respirometric and indirect calorimetric data that mt-Cybp,D254N causes distinct metabolic alterations despite being an apparently non-pathogenic variant.

Discussion

We discovered an extreme genetic background effect in the survival of CIII deficient mice and tracked the cause to a spontaneous variant in the mtDNA-encoded CIII subunit MT-CYB. This overtly silent mtDNA variant likely arose >10 years ago in an isolated colony of C57BL/6JBomTac mice and became homoplasmic by random drift in the germline, and finally expanded to the whole colony from a single common matrilinear ancestor. Incidentally, Bcs1l mutant mice with defective assembly of RISP subunit into CIII were generated and conegenized in this facility8. This resulted in transfer of the mtDNA variant-carrying mitochondria into the Bcs1lP.S78G mice and a hidden genetic interaction, exacerbating their CIII deficiency and causing a lethal metabolic crisis by 5–6 weeks of age. We discovered this only after transferring the mice to another facility and crossing them to a commercial C57BL/6JCrl strain, in which the homozygotes lived 4–5 times longer. We identified a candidate mtDNA variant, mt-Cybp,D254N, using WGS and utilized maternal inheritance of mtDNA to explicitly show that this variant was the main determinant of the survival.

In addition to BCS1L mutations, also MT-CYB mutations are a rare cause of CIII deficiency in humans. For example, mutations affecting the e loop and Qo site cause CIII deficiency manifesting as cardiomyopathy and exercise intolerance30. A heteroplasmic in-frame deletion of 8 amino acids spanning D254, the amino acid affected in our mice, was reported in a patient with exercise intolerance, a relatively benign phenotype31. Aspartate 254 is located in
the RISPbinding of loop of MT-CYB, and patient and artificial experimental mutations in this region are known to affect either quinol oxidation and subsequent electron transfer or RISP assembly. In our mice, mt-Cyb.D254N did not significantly affect the steady-state level of RISP in CII. Instead, MD simulations predicted that a neutral asparagine in this position stiffens the loop and restricts RISP-HD motion between the Qα and cytochrome c1 sites. Pulse electron paramagnetic resonance (EPR) measurements in isolated Rhodobacter bec complex confirmed this. In another bacterial system, Rhodobacter sphaeroides, the equivalent experimental substitution also caused a subtle kinetics defect. We propose that, in the 5 weeks of age, while BsClP.D254N mice with only one active Qo site, the subtle RISP kinetics defect due to D254N becomes limiting, synergistically decreasing CII activity. Subsequently, CII activity drops below the survival threshold, causing lethal metabolic crisis by subtle RISP kinetics defect due to D254N, likely leading to heterodimers with only one active Qo site, the acid oxidation (low RER) and induce hepatic chrome c1 sites. Pulse electron paramagnetic resonance (EPR) of mice from a C57BL/6JBomTac-derived colony (n = 2) were sequenced on Illumina HiSeqX platform (National Genomics Infrastructure, SciLifeLab, Sweden) with 30× coverage. Analysis of the sequence data was performed using FastQC, Trimmomatic, BWA, GATK, Bcftools, and Annovar Ensembl software at Bioinformatics Infrastructure for Life Sciences, Sweden. Mice were genotyped for the mt-Cyb variant by Sanger sequencing of a PCR fragment (primers: forward 5′-ACCTCTCTTCTCCTCCAC-GAA-3′ and reverse 5′-AGCTTAGAGTGCGGATTG-3′). Heteroplasmy was evaluated by cloning the mt-Cyb amplicon from different tissues into pBlueScript plasmid and sequencing bacterial clones.

**Animal experiments and ethics.** *Bsc1P.D254N* knock-in mice in congenic C57BL/6J (Harlan stock 000664) or C57BL/6J(BomTac)-derived background were maintained in the animal facilities of University of Helsinki, Finland with 12-h light/ dark cycle at 22–23 °C. Manual behavioral scoring was used to evaluate the health of homoygous mice as described previously and when the score reached 7/12 the animals were considered to be at end stage of the disease and were euthanized to minimize spontaneous deaths. Genomic DNA was isolated from ear clippings and the mice were genotyped for the *Bsc1P.D254N* mutation as described. The animal experiments were authorized by the national Animal Experiment Board, Finland (ELLA) under the ethical permits ESAV1/6365/04.10.07.2017 and and carried out in accordance with the Federation of Laboratory Animal Science Associations (FELASA) guidelines.

**Sample collection.** The experimental F1 cohort and the mice backcrossed at least thrice to C57BL/6J were fasted for 2 h during the light cycle of the animals before sample collection. The mice were euthanized by carbon dioxide inhalation followed by physical dislocation. Blood glucose was measured using Eute PLC Lite (Abbott, UK) meter. Tissues were immediately collected for histology, mitochondrial isolation, and/or snap-frozen in liquid nitrogen for storage at −80 °C.

**Tissue histology and immunohistochemistry.** Formalin-fixed paraffin-embedded tissues were processed and 5–μm sections stained according to routine procedures for general histology (hematoxylin–eosin (H&E)), glycogen (periodic acid-Schiff (PAS)), and collagen (Sirius Red). Tissue was dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. 5 μm sections were cut and mounted on to glass slides. For antigen retrieval was performed by boiling the deparaffinized sections for 20 min in 10 mM sodium citrate buffer, pH 6.0. Antibody was diluted 1:1000 and the staining was performed using Vectastain ABC alkaline phosphatase reagents (Vector Laboratories Inc.) and naphthol AS-MX phosphate substrate (Vector Laboratories Inc.). Nuclei were counterstained with Nuclear Fast Red (Sigma). Sample processing was carried out using an automated slide stainer (Technicon). Tissue sections were then dehydrated and rehydrated before staining with haematoxylin or eosin. For immunostaining, the slides were incubated in 0.3% hydrogen peroxide for 20 min to quench endogenous peroxidase activity and then blocked with 10% normal goat serum. The slides were incubated with the primary antibody for 1 h at room temperature. Alexa Fluor 488-coupled secondary antibody (cat. no. A21202, Invitrogen) was used for all secondary antibodies. For detection, the slides were incubated for 2 h at room temperature with the appropriate secondary antibody solution. The slides were mounted with 80% glycerol containing 0.1% (w/v) p-phenylenediamine. The distribution of specific proteins was visualized by fluorescence microscopy. The slides were viewed using a Carl Zeiss microscope equipped with both phase contrast and epifluorescence illumination. Images were captured using a CoolSNAP ES+ camera (Photometrics) and evaluated using AxiosVision (Carl Zeiss) software.

**Quantitative PCR (qPCR).** Total RNA was extracted from snap-frozen liver samples using RNazol RT reagent (Sigma-Aldrich). RNA purity and concentration were assessed by reading absorbances at 230, 260, and 280 nm and quality by inspecting ribosomal bands after agarose-gel electrophoresis. cDNA was synthesized with ReverAid H minus reverse transcriptase (Thermo Scientific; #EP0410) and random primers. qPCR reactions comprised Phire Hot-Start II DNA polymerase (Thermo Scientific); EvaGreen DNA-binding dye (Biotum); and buffer, dNTPs, and primers as instructed by the manufacturer of the polymerase. The following primer pairs were used: Fgf21 (forward, 5′-AGATGGACGTCCTATGATTGAGC-3′ and reverse 5′-GGGCTTCAGACTGGTACACAT-3′), Gdf15 (forward 5′-GTTCTC CGGAGGCGGAGGTTG-3′ and reverse 5′-CCAGGCTGACTGATCGTGC-3′), Gak (forward, 5′-CTGGCCACAGGAGGTTG-3′ and reverse 5′-CCATGCTCACTACATATTTCAATGACT-3′), and Rab11a (forward, 5′-AAGGCACAGATATG GGACACA-3′ and reverse 5′-CCCTACTGCTCCAGATATGTC-3′). Gak and Rab11a were served as reference genes, CFX96 instrument and CFX Manager software (Bio-Rad) were utilized to perform the qPCR and data analysis.

**Isolation of mitochondria.** Liver and kidney samples were rapidly excised, rinsed of excess blood, minced with scissors, and homogenized in cold isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, 0.1% bovine serum albumin, pH 7.4 at +4 °C) with glass-teflon Potter-Elvehjem homogenizers. Two-step differential centrifugation (800 × g supernatant and 7800 × g pellet, 5 min each) was used to obtain a crude mitochondrial preparation for respirometry and Amplex Red-peroxidase assay from the F1 cohort. For other analyses, the isolation was continued by a purification on 19% Percoll (11,300 × g, 10 min) followed by washing of the pellet (7800 × g, 5 min) with isolation buffer without albumin. Percoll-purified fractions were also used for respirometry from the panel of mice backcrossed to C57BL/6J.

**Homogenization of tissues for enzyme activity measurements.** Snap-frozen heart and skeletal muscle (quadriceps) biopsies were prehomogenized for 15 s with T8 Ultra-Turrax disperser (IKA®-Werke GmbH & Co. KG, Germany) and thereafter homogenized using PBI-Shredder (Pressure Biosciences Inc., MA, USA). Mitochondria isolation buffer served as a homogenization media for the skeletal muscle and 25 mM potassium phosphate buffer (with 40 mM KCl, 1 mM EGTA, pH 7.5) for the heart. We repeated the measurements from the heart and skeletal muscle using samples homogenized with roughened glass-to-glass tissue grinders.
Assessment of respiratory chain enzymatic activities. CII activity was assessed spectrophotometrically by monitoring antimycin A-sensitive cytochrome c reduction with decylubiquinol as electron donor in the following reaction mixture: 50 mM potassium phosphate pH 7.5, 60 mM cytochrome c, 100 µM decylubiquinol, 2 mM sodium azide, 100 mM EDTA, 0.05% Tween-20 and 0.1 mg/ml bovine serum albumin (BSA). Decylubiquinol was reduced with NaN3 and extracted with 2:1 solution of dichloromethane and isooctane. The mixture was vacuum dried under nitrogen, and dissolved in acidified EtOH (1 mM HCl). CII activity data from isolated mitochondria was normalized against mitochondrial protein amount and the data from homogenates to tissue protein or relative mitochondrial mass as assessed by measuring CIV activity. Rotenone-sensitive CI activity was measured with NaN3 as subunit II blocker, and ubiquinone as intermediate electron acceptor coupled to the reduction of a colorimetric dye 2,6-dichloroindophenol (DCIP)44. S公开课 oxidation was measured polarographically with TMPD (N,N,N‘,N’-tetramethyl-p-phenylenediamine dihydrochloride) and ascorbate as electron donors (liver and kidney) or by monitoring cytochrome-sensitive cytochrome c oxidation spectrophotometrically (quadriceps and heart). The polarographic assay was corrected for oxygen and sample-dependent TMPD auto-oxidation by measuring residual oxygen consumption at different oxygen levels after addition of sodium azide. All assays were performed at 37°C.

Respiration. Mitochondrial oxygen consumption was measured in Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) in Krebs-Henseleit buffer (110 mM sucrose, 60 mM lactobionic acid, 20 mM taurine, 20 mM HEPES, 10 mM KH2PO4, 3 mM MgCl2, 0.5 mM EGTA, and 1 g/l fatty acid-free BSA, pH 7.1). Sample, substrates, inhibitors, and uncoupler were injected in the following order: (1) 1 mM malate, 5 mM pyruvate, and 5 mM glutamate; (2) sample; (3) 1.25 mM ADP; (4) 10 µM cytochrome c; (5) 10 mM succinate; (6) 0.5 µg/ml oligomycin A; (7) 1 mM malate, 5 mM pyruvate, and 5 mM glutamate; (2) sample; (3) 1.25 mM ADP; (4) 10 µM cytochrome c; (5) 10 mM succinate; (6) 0.5 µg/ml oligomycin A; (7) FCCP titration to maximum respiration; (8) 0.25 µM rotenone; (9) 1 µg/ml antimycin A. Different oligomycin preparations and concentrations (0.5 µg/ml, sc-201551, Santa Cruz vs 0.4 µg/ml, Sigma, O4876) were used for the FI and later respirometric panels, respectively. The change in oligomycin preparation, a problem discussed by Ruas et al.46, may explain why we did not observe decreased leak respiration in the liver of mt-CytoD224N mice in the FI data. To normalize any difference in mitochondria mass, we set the maximal capacity of the terminal oxidase, CIV, as the reference state (polarographic CIV activity assay). This assay normalization has proven to provide a robust normalization as the measurement is performed immediately after the actual respirometry experiment using same equipment and exactly identical sample amount47. Supplementary Fig. 9 shows CIV-linked phosphorylating respiration relative to mitochondrial protein.

Measurement of mitochondrial H2O2 production. Amplex Ultra Red peroxidase assay was used to measure mitochondrial H2O2 production simultaneously with respirometry. Oxygraph-2k was equipped with a fluorometer and appropriate filters for measuring Amplex Ultra Red fluorescence (OROBOROS Instruments). The assay was performed in the following concentrations: 15 µM Amplex Ultra Red (Invitrogen), 1 µM horseradish peroxidase, 5 µM/sulphite dismutase, 10 mM succinate, 1.25 mM ADP, and 0.25 µM rotenone. Horseradish peroxidase-independent Amplex Red oxidation by mitochondrial toluene was inhibited by 50 µM 5-iodo-2′-deoxyuridine fluoride. Grossly similar O2 concentrations were maintained for all samples. The assay was calibrated with a 0.2-nmol bolus of H2O2. Stock concentration of H2O2 was 0.2 nmol (bolus of H2O2). All assays were performed at 37°C. Mitochondrial oxygen consumption was measured in Oxygraph-2k apparatus equipped with a SMT-105 sensor (OROBOROS Instruments). The mice were acclimatized in the cages for a minimum of 6 h before the collection of experimental data. This system also recorded movement of the mice.

Indirect calorimetry. In vivo oxygen consumption and carbon dioxide production were measured using Comprehensive Lab Animal Monitoring System (CLAMS (Cranbury Instruments). The mice were acclimatized in the cages for a minimum of 6 h before the collection of experimental data. This system also recorded movement of the mice.

Computational methods. For the crystal structure analyses, structures 1BES47, 110L48, 1ILO49, 1SVQ48, and 2F1U48 were used. For the MD simulations, a model system of WT enzyme was constructed using the high-resolution (1.9 Å) crystal structure of cytochrome bc2 from Saccharomyces cerevisiae (PDB: 3CSK)51. Detergent molecules, cyt c, Q, site inhibitor stigmatellin, and other crystallographic lipid molecules were removed prior to the model setup. Since cytochrome bc2 functions as a homodimer, we incorporated the entire protein structure into a lipid bilayer using CHARMM-GUI52,53. The lipid bilayer consisted of three different types of lipids representing the composition of inner mitochondrial membrane, that is, 50.3% POPC (1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine) 35.0% POPE (1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine), and 14.7% cardiolipin. The membrane–protein system was solvated with water molecules and K+ and Cl− ions to neutralize the system charge as well as to mimic the 150 mM salt concentration. A similar modeling setup was applied to construct the mutant (D254N) system (D255 in S. cerevisiae). The atomic partial charges of metal cofactors as well as bonded and non-bonded parameters were taken from an earlier study48. All redox centers were modeled in their oxidized state. We also performed calculations on different oxidation states of ubiquinone (Q) and ubiquinol (QH2), for which a QH+ molecule at the Q1 site and an oxidized Q molecule at the Q2 site, were modeled by aligning to the positions of stigmatellin (PDB: 3CSX) and quinone (Q6 in PDB: 1EYZ)48, respectively. The force field parameters of Q molecules were taken from an earlier study51. For all components of the system, CHARMM force field was used55,56, and simulations were performed with the GROMACS program57. The entire system was energy minimized (ca. 6000 steps) using Steepest Descent method available in GROMACS, followed by an equilibration (60 ns). The long production runs (1 µs each for WT and mutant systems, totaling 4 µs) were performed at constant temperature (310 K and pressure (1 atm), which were established with the use of Nose–Hoover thermostat57 and Parrinello–Rahman barostat58, respectively. Long-ranged electrostatics was dealt with PME59 implemented in GROMACS, and LINCS algorithm60 was used to achieve 2 fs time step. Alignment of trajectories was performed using the protein backbone atoms on the zero frame. The data were plotted as an average from both monomers and also separately (Supplementary Figs. 15 and 16).

Studies in bacteria. A mutant strain of R. capsulatus containing the substitution of aspartic acid to asparagine on position 278 in cytochrome b and a Strep tag at its C-terminus was generated with QuikChange-site directed mutagenesis method (Strategen) using the pPET1 plasmid (containing WT petABC operon) as a template and following PCR primers for mutagenesis (the changed nucleotide in underlined triplet is in bold):

| Primer Name | Sequence (5’–3’) |
|-------------|------------------|
| Forward     | CGGGTGGCCGAGG-3’ |
| Reverse     | CGGGTGGCCGAGG-3’ |

The BoxXII/AsuII restriction fragment bearing the introduced mutation was exchanged with its counterpart on a pMT5I WT plasmid vector and introduced into the MT-RBG1 R. capsulatus strain devoid of petABC operon using triparental crossing method59. The presence of the introduced mutation was confirmed by sequencing the inserted fragment on a plasmid isolated from R. capsulatus D278N strain.

R. capsulatus strains expressing WT or D278N cytochrome bc2 complex were cultured semi-aerobically in MPYE medium. Chromatophores were isolated from petABC operon in 3-liter bacterial cultures in 50 mM MOPS buffer pH 7.0, 100 mM KC1, and 1 mM EDTA using French press. For cytochrome bc2, complex purification, chromatophores were solubilized with n-dodecyl-β-D-maltopyranoside detergent (Anatrace) in 50 mM Tris buffer pH 8.0, 100 mM NaCl, and 1 mM EDTA, dialyzed against 0.1 M KCl, and the supernatant was centrifuged, and the supernatant was subjected to gel filtration chromatography columns (IBA)49. X-band CW EPR spectra of the [2Fe-2S] clusters were measured on Bruker Elexsys E580 spectrometer using Bruker SHQ412 resonator equipped with an ESR900 cryostat (Oxford Instruments). Measurement parameters were as follows: microwave frequency 9.38 GHz, microwave power 2 mW, modulation amplitude 1.5 mT, modulation frequency 100 kHz, and temperature 20 K.

Pulse EPR measurements of [2Fe-2S] cluster relaxation. The temperature dependence of phase relaxation rates of the reduced [2Fe-2S] cluster was measured by pulse EPR spectroscopy in a way similar to that established by Sarewicz...
et al. Measurements were carried out on Bruker Elexsy E580 spectrometer using Q-band ER510/D2 resonator inserted into CP935 cryostat. Samples of purified cytochrome bc$_2$ complexes were prepared in bicin buffer pH 8.0 containing 100 mM NaCl, 20% glycerol, and 1 mM sodium ascorbate. Spin echo decay traces were recorded with the use of two pulse sequence ($\pi$/2–$\tau$–$\pi$) in which the pulse separation time ($\tau$) was swept. Starting from shortest available $\tau$ to 160 ns, the separation time was sequentially incremented by 4 ns interval in 1024 steps. Each time point of the trace was obtained by integration of the spin echo signal appearing after 2 $\tau$ time. The length of the $\pi$/2 and $\pi$ pulses was set to 16 and 32 ns, respectively, and the microwave power was adjusted to give maximum spin echo signal. Each spin echo decay trace was registered at $g = 1.9$ transition of the [2Fe–2S] cluster spectrum, which for Q-band frequency of 33.6 GHz corresponds to 1264 mT magnetic field induction. Reduction rates of the reduced [2Fe–2S] cluster were obtained by fitting exponential decay to the electron spin echo traces measured in 12–23 K temperature range.

Statistics. Survival data were analyzed with log-rank (Mantel–Cox) test. One-way analysis of variance (ANOVA) and four planned comparisons (t statistics with Welch’s correction when appropriate) were performed for normally distributed data: (1) WT vs Bcs1p$^{−}$S78G, (2) WT vs mt-Cybp$^{D254N}$, (3) Bcs1p$^{−}$S78G vs Bcs1p$^{+}$S78G, mt-Cybp$^{−}$D254N, and (4) WT and mt-Cybp$^{D254N}$ vs Bcs1p$^{−}$S78G and Bcs1p$^{+}$S78G, mt-Cybp$^{−}$D254N. The normality assumption of ANOVA was assessed with Shapiro–Wilk method and by manually inspecting the distribution of ANOVA residual. Welch’s t test was required. Kruskal–Wallis and Mann–Whitney U tests were used instead. Analysis of covariance with genotype as a fixed factor and body weight as a covariate was used to calculate weight-normalized energy expenditure. All tests were performed as two-sided tests. The experimental unit ($n$) in the analyses was one individual animal. Throughout the figures, the data are presented as mean and 95% confidence interval of the mean if not otherwise stated. The data points in scatter plots are biological independent replicates.

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**Author contributions**

V.F. and J.K. conceived the project; K.T. performed whole-genome sequence data analysis; J.K. and V.S. MT-CYb protein sequence analyses; V.G. and J.K. mt-Cyb genotyping; J.P., V.G. and J.R. mouse behavioral scoring and autopsy; J.P. and V.G. CIII activity assay and respirometry; J.P. Amprex Red-oxidase assay, qPCR, CI, CII and CIV activity assays, BNGE, and statistics; J.P., V.G. and J.R. tissue histology; R.E., R.P. and A.O. Rhodobacter work; and N.A., V.S. and M.W. in silico modeling and simulations. J.P., V.G. and J.K. wrote the manuscript draft and J.P., R.E., N.A., V.S., M.W., A.O., V.F. and J.K. revised it.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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