The clinically relevant triple mutation in the mtND1 gene inactivates Escherichia coli complex I
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Energy-converting NADH:ubiquinone oxidoreductase, respiratory complex I, is the main entry point for electrons from NADH into many respiratory chains. Complex I couples the transfer of two electrons from NADH to a quinone (Q) with the translocation of four protons across the membrane [1–6]. It has a two-part structure consisting of a peripheral arm catalysing electron transfer and a membrane arm executing proton translocation [6–10]. The mitochondrial complex of mammals consists of 45 subunits including the 14 conserved core subunits that are found in all species containing an energy-converting NADH:Q oxidoreductase [1–6,11]. Seven of the 14 core subunits are encoded by mitochondrial DNA (mtDNA) in humans [1,11]. Dysfunctions of complex I lead to clinically highly diverse presentations including early-onset disorders such as mitochondrial encephalomyopathy and Leigh syndrome as well as lactic acidosis and cardiomyopathy [12–14]. Mutations in patients are found in the mitochondrial and the nuclear genome due to the dual genetic origin of the complex. Mutations in mtDNA are further influenced by the degree of heteroplasmy that varies greatly between patient’s cells and tissues [15–18]. Sequence data from patients suffering from mitochondrial diseases are easy to obtain nowadays, which constantly increases the number of mutations detected. However, their effect on the assembly, stability and activity of the complex is usually not traceable, as only small amounts of material are obtained from both biopsies and cell lines [19,20].

This problem can be circumvented by using bacterial model systems such as Escherichia coli that have been developed to identify the effect of specific mutations on biochemical properties of the mutated complex.
Without question, eukaryotic model systems such as *Yarrowia lipolytica* are evolutionarily much closer related to the human enzyme, but the bacterial systems have the invaluable advantage that mutations, even multiple mutations, found in mtDNA can be integrated into their genome without technical problems. The three-dimensional structure of the core subunits of complex I is conserved from bacteria to mammals, so that the structural minimal form of *E. coli* complex I can be used as a simplified model for the human one [7–10,26,27]. It should, however, be kept in mind that the human and the bacterial complex are situated in different cellular environments. The human complex is part of a specialized organelle and is often found in supercomplexes [28], which is not the case with *E. coli* complex I.

The 13 different subunits of the *E. coli* complex I are named NuoA to NuoN, with two of the 14 core subunits being fused to the single subunit NuoCD [29]. They are encoded by the *nuo*-genes and add up to a molecular mass of approximately 530 kDa [30]. We work with a chromosomal *nuo* null strain, in which all *nuo*-genes are expressed from one plasmid, thus facilitating their mutation in our experimental approach [25]. An efficient and straightforward purification strategy was developed that allows a detailed analysis of the enzyme variants with respect to their composition, stability and catalytic activity [25].

Several patients have been described, who carry an inversion of 7 bps in the mitochondrial encoded ND1 gene (m.3902–3908inv7) [31–33]. This leads to the triple mutation D199G/L200K/A201Vmt-ND1 with D199 as the only conserved amino acid residue. The patients suffer from various symptoms such as exercise intolerance, myalgia, nephropathy, deafness and diabetes mellitus [31–33]. So far, we characterized the mutation of the conserved position D199 using our established *E. coli* system [25]. It turned out that the single variant is stably assembled and showed a decreased electron transfer activity that was fully coupled to proton translocation. Remarkably, this variant exhibits significant activation kinetics, which was interpreted as a disturbed Q chemistry [2,25,34–36]. Various amino acid residues are found in positions L200 (e.g. Phe, Gln and Asn) and A201 (e.g. Thr, Gln and Pro), respectively (Fig. S1). However, the triple mutation is located in a highly conserved region discussed to link Q reduction with proton translocation [1–4,25,35,37] (Fig. 1, Fig. S1). Therefore, we introduced the triple mutation into NuoH, the *E. coli* homologue of ND1, and characterized the variant. It turned out that the triple variant is stably assembled but almost completely inactive, which explains its deleterious effect in humans.

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**Materials and methods**

**Strains, plasmids and oligonucleotides**

A derivative of *E. coli* strain BW25113 [38] chromosomally lacking the gene *ndh* was used as host to overproduce complex I [39]. The chromosomal *nuo* operon of this strain was replaced by a resistance cartridge (*nptII*). *E. coli* strain DH5αΔ*nuo* was used for site-directed mutagenesis [39]. Oligonucleotides were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Table S1). Restriction enzymes were obtained from Fermentas. Plasmid pBADnuoH [40] was used to introduce the triple mutation on *nuoH* by site-directed mutagenesis according to the Quik-Change protocol (Stratagene, La Jolla, CA, USA). A silent
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mutated was introduced generating a new restriction site to identify positive clones by restriction analysis. The primer pair nuoH_triple (Table S1) was used to generate the plasmid pBADnuso_HnuoF triple[1]. PCR was performed using the KOD Hot Start DNA Polymerase (Novagen, EMD Millipore Corp., Burlington, MA, USA). Mutations were confirmed by DNA sequencing (GATC Eurofins, Konstanz, Germany).

Cell growth and preparation of cytoplasmic membranes

To determine an effect of the mutation on complex I, strains were grown aerobically in minimal medium with 25 mM acetate as sole carbon source [25] at 37 °C while agitating at 180 rpm. Expression of the nuo operon was induced by an addition of 0.02% (w/v) L-arabinose. For protein preparation, cells were grown in a rich autoinduction medium containing 34 µg mL⁻¹ chloramphenicol as described [25]. Cells were harvested by centrifugation, suspended in a fivefold volume buffer A (50 mM MES/NaOH, 50 mM NaCl, pH 6.0) containing 0.1 mM PMSF and a few grains of DNaseI and disrupted by three passages through an HPL-6 (Maximator) to a membrane suspension in buffer A. Membranes were extracted by an addition of 1% (w/v) LMNG (2,2-didecylpropane-1,3-bis-(2-hydroxypropyl)methylphosphonate; Anatrace) to a membrane suspension in buffer A (buffer A with 5 mM MgCl₂) containing 0.1 mM PMSF. After centrifugation, the supernatant was loaded onto 24 mL Amicon Ultra-15 centrifugal filter (Millipore, Merck Millipore Ltd., Cork, Ireland) and applied onto a Superose 6 size exclusion chromatography column (300 µL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated in buffer A* with 10% (v/v) glycerol and 0.005% (w/v) LMNG. The fractions with highest NADH/ferricyanide oxidoreductase activity were used for further studies.

Activity assays

Activity assays were performed at 30 °C. The NADH oxidase activity of cytoplasmic membranes was determined by a Clark-type oxygen electrode (DW1, Hansatech) as described [25]. The electrode was calibrated by an addition of a few grains of sodium dithionite to air saturated buffer [41]. The NADH/ferricyanide oxidoreductase activity was determined as decrease in the absorbance of ferricyanide at 410 nm [25] with a diode-array spectrometer (QS cuvette, d = 1 cm, Hellma; TIDAS II, J&M Aalen, J&M Analytik AG, Esslingen, Germany) using an ε of 1 mM⁻¹ cm⁻¹ [42]. The NADH:decyl-Q oxidoreductase activity was measured as decrease of the NADH concentration at 340 nm using an ε of 6.3 mM⁻¹ cm⁻¹ (QS cuvette, d = 1 cm, Hellma; TIDAS II, J&M Aalen). The assay contained 60 µm decyl-Q, 2 µg complex I and a tenfold molar excess (5 µg) E. coli cytochrome bo₃ oxidase in buffer A*LMNG. The reaction was started by an addition of 150 µM NADH [25].

Liposomes were prepared from E. coli polar lipids (25 mg·mL⁻¹ in CHCl₃; Avanti) as described [41]. They were extruded by 21 passes through an extruder (0.1 µm polycarbonate membrane, Mini Extruder; Avanti). For reconstitution, 0.5 mg protein were mixed with reconstitution buffer (1 : 3; v/v) (10 mM Bis-tris-propane/MES, 100 mM KCl, 73 mM sucrose, 2.5 mM MgSO₄, 0.05% (w/v) L-α-phosphatidylycholine, 1.1% (w/v) n-octylglucoside, 0.6% (w/v) sodium deoxycholate, 0.6% (w/v) sodium cholate, pH 7.5) and incubated for 5 min on ice. 250 µL liposomes were mixed with 8 µL sodium cholate (20%, w/v), the liposomes and the protein in reconstitution buffer were mixed and incubated for 20 min at room temperature. Liposomes were formed using a size exclusion column (PD-10 Desalting Column, 8.3 mL, Sephadex G-25, GE Healthcare) equilibrated in lipid buffer to remove excess detergent. The eluate was centrifuged (4 °C, 200 000 g, 30 min; Rotor, A-100, Airfuge, Beckman) and sedimented proteoliposomes were gently re-suspended in 500 µL lipid buffer.

The generation of ΔpH was determined by monitoring the fluorescence quenching of the pH-sensitive dye 9-amino-6-chloro-2-methoxyacridine (ACMA, Sigma) [25]. The assay contained 100 µM decyl-Q (Sigma), 0.2 µM ACMA and 50 µL proteoliposomes in ACMA buffer (10 mM Bis-tris-propane/MES, pH 6.75, 100 mM KCl and 2 mM MgCl₂). Fluorescence was detected with a LS 55 Fluorescence Spectrometer (Perkin Elmer, Llantrisant, UK) using excitation and emission wavelengths of 430 and 480 nm, respectively.
An addition of 1 µg gramicidan (Sigma) was used to dissipate the ΔΨ [25].

The generation of ΔΨ was determined by monitoring the changes in absorption of the potential-sensitive dye oxonol VI (Sigma) [25]. The assay contained 0.5 µM oxonol VI, 50 µM Q₆ and proteoliposomes in oxonol buffer (10 mM MES/KOH, pH 6.75, 2 mM MgSO₄, 100 mM KCl, 10 mM NH₄Cl). The absorbance at 588–625 nm was measured with a diode-array spectrometer (QS cuvette, d = 1 cm, Hellma; TIDAS II, J&M Aalen). An addition of 1 µg gramicidan was used to dissipate ΔΨ [25].

Other analytical methods

Protein concentration was determined according to the biuret method using BSA as a standard [43]. The concentration of purified complex I was determined by UV/vis-spectroscopy (QS cuvette, d = 1 cm, Hellma; TIDAS II, J&M Aalen) using an ε of 781 mM⁻¹cm⁻¹ as derived from the amino acid sequence [44]. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed with a 10% separating gel and a 3.9% stacking gel [45]. Multiple sequence alignments were performed using CLUSTALX 2.1 [46].

Results

The triple mutant D199G/L200K/A201V in human complex I corresponds to the triple mutation D213G/Q214K/P215V in human complex I (Fig. S1). This mutation, called tripleH for simplicity hereafter, was introduced into the pBAD nuo expression plasmid that encodes the entire E. coli nuo operon under the control of the inducible pBAD arabinose promoter [40]. The expression strain BW25113Δndh nup: pII_FRT was transformed with plasmids either encoding the parental genes or the ones with the triple mutation. Due to the lack of the alternative NADH dehydrogenase (ndh) [39] and the chromosomally encoded complex I, all NADH-induced activities of membranes from this strain exclusively reflect activities of complex I encoded on the plasmid.

The transformed host strain was grown in minimal medium with acetate as non-fermentable carbon source. Here, an intact complex I is required for fast growth to high OD values, because complex I maintains a low NADH/NAD⁺ ratio and contributes to the proton motive force [25]. The strain producing wild-type complex I grew the fastest, while the control strain that does not contain the plasmid grew the slowest (Fig. 2). The strain with the plasmid coding the tripleH variant showed an intermediate growth. Induction of gene expression by an addition of arabinose led to a significant increase of the growth rate of the strain containing the wild-type complex; however, the addition of arabinose did not alter the growth rate of the strain producing the tripleH variant (Fig. 2). Furthermore, after about 7 h of growth, the mutant strain grew as slowly as the control strain, which lacks any membrane-bound NADH dehydrogenase. Since the strain with the tripleH mutation grew as if no complex I were present, either the assembly or the function of the complex must be significantly disturbed by the mutation.

The amount of the complex and the tripleH variant in cytoplasmic membranes was determined by measuring the NADH/ferricyanide oxidoreductase activity. Unexpectedly, the NADH/ferricyanide oxidoreductase activity of the mutant membranes did not significantly differ from that of the parental strain (Table 1) indicating a similar amount of the complex in both membranes. The physiological activity of complex I was determined by measuring NADH oxidase activity. Here, the tripleH mutant strain showed a drastically diminished activity of about 6% of that of the parental strain (Table 1). The addition of 20 µM piericidan A, a specific complex I inhibitor, had no effect on the residual activity indicating that it does not originate from complex I. The NADH oxidase activity of complex I was inhibited to more than 95% by the inhibitor. Thus, the tripleH mutation completely abolished electron transfer activity.
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Table 1. NADH oxidase and NADH/ferricyanide oxidoreductase activity of cytoplasmic membranes from E. coli wild-type and triple^H mutant strain. Data were obtained from three measurements of two biological replicates.

|        | NADH oxidase activity | NADH/ferricyanide oxidoreductase activity |
|--------|-----------------------|------------------------------------------|
| U mg⁻¹ | %                     | U mg⁻¹                                   |
| WT     | 0.257 ± 0.018         | 100 ± 7                                  |
|        | 1.56 ± 0.31           | 100 ± 20                                 |
| Triple^H | 0.017 ± 0.001       | 6 ± 6                                    |
|        | 1.69 ± 0.33           | 108 ± 19                                 |

To determine, whether the lack of activity was due to incomplete or unstable assembly of the complex, proteins were extracted from cytoplasmic membranes with the detergent LMNG and separated by sucrose gradient density centrifugation (Fig. 3). The position of the complex and the variant in the gradient was determined by its NADH/ferricyanide oxidoreductase activity. As expected, the parental complex sedimented around fraction 16. The variant sedimented at the same position of the gradient with approximately the same total activity (Fig. 3) in agreement with its NADH/ferricyanide oxidoreductase activity in the membrane (Table 1).

To determine the activity of the stably assembled variant, both proteins were prepared to homogeneity by affinity and size-exclusion chromatography (Fig. S2). From 50 g cells 7–9 mg protein were obtained with the parental protein, while the preparations of the triple^H variant reproducibly yielded only 4–5 mg possibly indicating a decreased long-term stability. However, the elution profiles and the SDS-gel of the preparations of complex I and the variant showed no significant differences (Fig. S2).

The preparations of the triple^H variant had an insignificantly higher specific NADH/ferricyanide oxidoreductase compared to that of the complex (Table 2). In contrast, the variant showed only 7% of the specific NADH:Q oxidoreductase activity of the parental complex (Table 2). In accordance with the NADH oxidase activity of mutant membranes, the residual activity was not inhibited by an addition of 20 µM piericidin A indicating that the variant is fully inactive. The activity of the preparation of complex I was inhibited to more than 95% by piericidin A. However, we also cannot rule out the possibility that the mutation affects sensitivity to piericidin A, although this is unlikely as we used a high inhibitor concentration in the assays.

To determine whether the residual electron transfer activity is coupled to proton translocation, both preparations were reconstituted into pre-formed liposomes as described [47]. The orientation of the enzymes in the liposomes was determined by measuring the NADH/ferricyanide oxidoreductase activity of intact and dissolved proteoliposomes [25]. The share of the accessible NADH binding sites was 35% with both proteoliposome preparations. The following experiments were performed as technical triplicates from two biological replicates, each.

Proton pumping was measured by following fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA). An addition of NADH led to a decrease of the fluorescence signal; however, the rate and the extent of quenching obtained with proteoliposomes containing the triple^H variant was only 13% (± 1.3%) of those containing complex I (Fig. 4). Incubating the liposomes with piericidin A or adding the inhibitor to the assay had no effect (Fig. S3). Thus, the addition of NADH does not seem to induce proton translocation, although it cannot be ruled out that the mutation hampered inhibitor binding. The membrane potential was determined by following the decrease in the absorbance of the dye oxonol VI (Fig. 4). In these measurements, hardly any signal could

Table 2. NADH:deyl-Q and NADH/ferricyanide oxidoreductase activity of the preparations of complex I and the triple^H variant. Data were obtained from three measurements of two biological replicates.

|        | NADH:Q oxidoreductase activity | NADH/ferricyanide oxidoreductase activity |
|--------|--------------------------------|------------------------------------------|
|        | U mg⁻¹ | %         | U mg⁻¹ | %        |
| WT     | 27.99 ± 1.75 | 100 ± 6 | 100.7 ± 3.3 | 100 ± 7 |
| Triple^H | 1.90 ± 0.31 | 6.8 ± 16 | 110.6 ± 11 | 110 ± 10 |

Fig. 3. Sucrose gradient of detergent-solubilized membranes from wild-type (black) and the triple^H (red) mutant strain. The NADH/ferricyanide oxidoreductase activity of each fraction is shown; the activities are normalized to 20 mg membrane protein extract applied on each gradient.
be observed with the proteoliposomes containing the variant protein. The signals obtained with the proteoliposomes containing complex I were sensitive to the addition of gramicidin and piericidin A (Fig. S3).

Discussion

The complex I mutation described here was previously found to be clinically relevant [31–33]. The inversion of 7 bps in ND1 leads to a triple mutation while maintaining the reading frame. The mutation is associated with variable neurological manifestations depending on the type of tissue containing this inversion and on the degree of heteroplasmy [15–18]. Of the residues affected by the mutation, only D199MT-ND1 (human numbering) is conserved (Fig. S1). In E. coli, the corresponding D213GH variant was stably assembled, and it exhibits approximately half of the electron transfer activity of complex I. This activity is fully coupled to proton translocation [25]. Consequently, it has been suggested that this mutation has little effect in humans because cellular energy supply is only slightly affected and the NADH/NAD⁺ ratio is kept in balance. Remarkably, the D213GH and the D213EH variant show a lag-phase in activity [22,25] that was interpreted as a disturbed Q chemistry. At least two Q binding positions have been reported in complex I by electron cryo-microscopy (cryo-EM) and molecular dynamics simulations [2–4,7,35,37,48,49]. D213H is located close to one of these positions that might connect a conformational change in the Q binding site with proton translocation (Fig. 1) [50]. It was proposed that the reduced and protonated Q is converted to its anionic form by donating a proton to a cluster of titratable amino acid residues including D213H [1–4,25,35,37]. According to this hypothesis, the quinol anion moves further in its binding cavity to reach a site where it is fully protonated. The proton required for the re-protonation of the quinol anion might be derived from the membrane arm of the complex after proton translocation is completed [2,25,34,35].

In contrast, the tripleH mutation completely abolished the activity of the complex (Tables 1 and 2). Thus, the two other mutations contained within the tripleH mutation added other effects to that of the single D213GH mutation. This is not unexpected, since D213H is located in a region with a high degree of sequence conservation (Fig. S1). A change of three amino acid residues instead of only one could severely disrupt the local structure (Fig. 1). The tripleH mutation has apparently led to structural changes that profoundly impaired Q chemistry as the NADH/ferricyanide oxidoreductase did not change and the EPR spectra of the variant showed no differences from that of the parental protein (Fig. S4). Thus, the triple mutation on NuoH obscured the effect of the D213G mutant single mutation on quinone reduction. It is also possible that the impairment is due to the change of the overall negative charge of the short protein segment to a positive charge. Thus, the tripleH mutation could disturb the finely tuned pKₐ balance in the region of charged amino acid residues, thereby blocking the postulated de-protonation of the quinol or proton translocation as described above.

The triple mutant showed no electron transfer and proton translocation activity; thus, it is not capable of maintaining a balanced NADH/NAD⁺ ratio and

![Fig. 4. Generation of a membrane potential by E. coli complex I (black) and the tripleH variant (red) reconstituted into liposomes. (A) Generation of a ΔpH measured as quench of the ACMA fluorescence. (B) Generation of a ΔΨ measured as the decrease of the oxonol VI absorbance difference (588–625 nm). The reactions were started by an addition of 130 µM NADH, each. Addition of gramicidin dissipated the proton gradient and the membrane potential.](image-url)
contributing to ATP production. The severe effects of the E. coli complex I triple mutation described here may have contributed to the early death in infancy of a patient, who carried these mutations in heart, muscle, and liver tissue [32]. Other patients carrying this mutation exclusively in muscles had a significantly longer life expectancy [31].

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Author contributions

FH and TF designed the study, FH and MW constructed the expression plasmid, purified the proteins, performed the experiments and acquired the data. FH and TF analysed the data. TF wrote the manuscript, performed the experiments and acquired the data, FH

Data accessibility

The data supporting the findings of this article are available from the corresponding author upon reasonable request.

References

1 Hirst J. Mitochondrial complex I. Annu Rev Biochem. 2013;82:551–75.
2 Kaila VRI. Resolving chemical dynamics in biological energy conversion: long-range proton-coupled electron transfer in respiratory complex I. Acc Chem Res. 2021;54:4462–73.
3 Sazanov LA. A giant molecular proton pump: structure and mechanism of respiratory complex I. Nat Rev Mol Cell Biol. 2015;16:375–88.
4 Parey K, Wirth C, Vonck J, Zickermann V. Respiratory complex I – structure, mechanism and evolution. Curr Opin Struct Biol. 2018;63:1–9.
5 Cabrera-Orefice A, Yoga EG, Wirth C, Siegmund K, Zwicker K, Guerrero-Castillo S, et al. Locking loop movement in the ubiquinone pocket of complex I disengages the proton pumps. Nat Commun. 2018;9:4500.
6 Gnandt E, Dörker N, Strampraad MFJ, de Vries S, Friedrich T. The multitude of iron-sulfur clusters in respiratory complex I. Biochim Biophys Acta. 2016;1857:1068–72.
7 Agip A-NA, Blaza JN, Fedor JG, Hirst J. Mammalian respiratory complex I through the lens of cryo-EM. Annu Rev Biophys. 2019;48:165–84.
subunit NuoH (ND1) in the assembly of peripheral subunits with the membrane domain of *Escherichia coli* NDH-1. *J Biol Chem*. 2009;284:9814–23.

24 Sinha PK, Castro-Guerrero N, Patki G, Sato M, Torres-Bacete J, Sinha S, et al. Conserved amino acid residues of the NuoD segment important for structure and function of *Escherichia coli* NDH-1 (complex I). *Biochemistry*. 2015;54:753–64.

25 Nuber F, Schimpf J, di Rago J-P, Tribouillard-Tanvier D, Procaccio V, Martin-Negrier M-L, et al. Biochemical consequences of two clinically relevant ND-gene mutations in *Escherichia coli* respiratory complex I. *Sci Rep*. 2021;11:12641.

26 Efremov RG, Sazanov LA. Structure of the membrane domain of respiratory complex I. *Nature*. 2011;476:414–20.

27 Schimpf J, Oppermann S, Gerasimova T, Santos Seica AF, Hellwig P, Grishkovskaya I, et al. Structure of the peripheral arm of a minimalistic respiratory complex I. *Structure*. 2022;30:80–94.e4.

28 Vercellino I, Sazanov LA. The assembly, regulation and function of the mitochondrial respiratory chain. *Nat Rev Mol Cell Biol*. 2022;23:141–61.

29 Weidner U, Geier S, Ptock A, Friedrich T, Leif H, Weiss H. The Gene locus of the proton-translocating NADH:ubiquinone oxidoreductase in *Escherichia coli*. Organization of the 14 genes and relationship between the derived proteins and subunits of the mitochondrial complex I. *J Mol Biol*. 1993;233:109–22.

30 Leif H, Sled VD, Ohnishi T, Weiss H, Friedrich T. Isolation and characterization of the proton-translocating NADH:ubiquinone oxidoreductase from *Escherichia coli*. *Eur J Biochem*. 1995;230:538–48.

31 Musumeci O, Andreu AL, Hanske A, Bresolin N, Comi GP, Rothstein R, et al. Intragenic inversion of mtDNA: a new type of pathogenic mutation in a patient with mitochondrial myopathy. *Am J Hum Genet*. 2000;66:1900–4.

32 Blakely EL, Rennie KJ, Jones L, Elstner M, Chrzanowska-Lightowlers ZM, White CB, et al. Sporadic intragenic inversion of the mitochondrial DNA MTND1 gene causing fatal infantile lactic acidosis. *Pediatr Res*. 2006;59:440–4.

33 Pronicka E, Piekutowska-Abramczuk D, Ciara E, Trubicka J, Rokicki D, Karkuciak J, et al. New perspective in diagnostics of mitochondrial disorders: two years’ experience with whole-exome sequencing at a national paediatric centre. *J Transl Med*. 2016;14:174.

34 Nuber F, Mérone L, Oppermann S, Schimpf J, Wohlwend D, Friedrich T. A quinol anion as catalytic intermediate connecting proton translocation with electron transfer in *E. coli* complex I. *Front Chem*. 2021;9.

35 Kaila VRI. Long-range proton-coupled electron transfer in biological energy conversion: towards mechanistic understanding of respiratory complex I. *J R Soc Interface*. 2018;15:20170916.

36 Röpke M, Riepl D, Saura P, Di Luca A, Mühlbauer ME, Jussupow A, et al. Deactivation blocks proton pathways in the mitochondrial complex I. *Proc Natl Acad Sci USA*. 2021;118:e2019498118.

37 Kampjut D, Sazanov LA. The coupling mechanism of mammalian respiratory complex I. *Science*. 2020;370:eabg4209.

38 Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA*. 2000;97:6640–5.

39 Burschel S, Kreuzer Decovic D, Nuber F, Stiller M, Hofmann M, Zupok A, et al. Iron-sulfur cluster carrier proteins involved in the assembly of *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I). *Mol Microbiol*. 2019;111:31–45.

40 Pohl T, Uhlmann M, Kaufenstein M, Friedrich T. Lambda Red-mediated mutagenesis and efficient large scale affinity purification of the *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I). *Biochemistry*. 2007;46:10694–702.

41 Friedrich T, van Heek P, Leif H, Ohnishi T, Forche E, Kunze B, et al. Two binding sites of inhibitors in NADH:ubiquinone oxidoreductase (complex I). Relationship of one site with the ubiquinone-binding site of bacterial glucose:ubiquinone oxidoreductase. *Eur J Biochem*. 1994;219:691–8.

42 Friedrich T, Hofhaus G, Ise W, Nehls U, Schmitz B, Weiss H. A small isoform of NADH:ubiquinone oxidoreductase (complex I) without mitochondrially encoded subunits is made in chloramphenicol-treated *Neurospora crassa*. *Eur J Biochem*. 1989;180:173–80.

43 Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem*. 1949;177:751–66.

44 Gill SC, von Hippel PH. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem*. 1989;182:319–26.

45 Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem*. 1987;150:17–25.

46 Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007;23:2947–8.

47 Marszalek JR, Hirst J. Cork-in-bottle mechanism of inhibitor binding to mammalian complex I. *Sci Adv*. 2021;7:eabg4000.
complex I – open questions and mechanistic implications. *Front Chem.* 2021;9:672851.

50 Sharma V, Belevich G, Gamiz-Hernandez AP, Róg T, Vattulainen I, Verkhovskaya ML, et al. Redox-induced activation of the proton pump in the respiratory complex I. *Proc Natl Acad Sci USA.* 2015;112:11571–6.

51 Bridges HR, Fedor JG, Blaza JN, Di Luca A, Jussupow A, Jarman OD, et al. Structure of inhibitor-bound mammalian complex I. *Nat Commun.* 2020;11:5261.

52 Gutiérrez-Fernández J, Kaszuba K, Minhas GS, Baradaran R, Tambalo M, Gallagher DT, et al. Key role of quinone in mechanism of respiratory complex I. *Nat Commun.* 2020;11:4135.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Multiple sequence alignment of NuoH.

**Fig. S2.** Preparation of complex I and the tripleH variant.

**Fig. S3.** ACMA measurement in the presence of piericidin A.

**Fig. S4.** EPR spectra of the preparation of the tripleH variant.

**Table S1.** Oligonucleotides used for site-directed mutagenesis.