Assembly factors for the membrane arm of human complex I

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Mitochondrial respiratory complex I is a product of both the nuclear and mitochondrial genomes. The integration of seven subunits encoded in mitochondrial DNA into the inner membrane, their association with 14 nuclear-encoded membrane subunits, the construction of the extrinsic arm from 23 additional nuclear-encoded proteins, iron–sulfur clusters, and flavin mononucleotide cofactor require the participation of assembly factors. Some are intrinsic to the complex, whereas others participate transiently. The suppression of the expression of the NDUFA11 subunit of complex I disrupted the assembly of the complex, and subcomplexes with masses of 550 and 815 kDa accumulated. Eight of the known extrinsic assembly factors plus a hydrophobic protein, C3orf1, were associated with the subcomplexes. The characteristics of C3orf1, of another assembly factor, TMEM126B, and of NDUFA11 suggest that they all participate in constructing the membrane arm of complex I.

Significance

Mammalian complex I, the largest and most complicated enzyme of the mitochondrial respiratory chain, is an L-shaped assembly of 44 proteins with one arm in the mitochondrial matrix and the orthogonal arm buried in the inner membrane. It is put together from preassembled subcomplexes. This investigation concerns the little studied process of the assembly of the membrane arm module from proteins emanating from both nuclear and mitochondrial genomes. We have identified two membrane protein assembly factors C3orf1 and TMEM126B, not found in the mature complex, that help this process by putting together two membrane arm subcomplexes. Defects in the assembly of complex I are increasingly being associated with human pathologies.
pression rates, or degradation (Dataset S1), any changes in the protein levels relative to control cells, by mass spectrometry. As acids in cell culture (SILAC) so as to quantify differences in stably was subjected to stable isotope labeling of amino Complex I.

Association of C3orf1 and TMEM126B with Incompletely Assembled Complex I. A 143B cell line in which NDUFA11 had been sup-

Ablation of C3orf1, TMEM126B, ATP5SL, and DNAJC11 and Assembly of Complex I. The suppression of expression of C3orf1 or TMEM126B reduced both the cellular oxygen consumption, and the level of intact complex I (Fig. 4), and subcomplexes of 315 and 370 kDa accumulated. They were detected with antibodies against the peripheral arm and the membrane arm component NDUFB8, respectively. The accumulation of the 370-kDa subcomplex was concomitant with a reduction in a 550-kDa subcomplex (Fig. 4), presumably the same subcomplex accompanying suppression of expression of NDUFA11 (Fig. 2). Also, when TMEM126B was suppressed, the levels of both complexes III and IV increased (Fig. 4). As the analyses were performed in the presence of n-dodecyl-

Fig. 1. Oxygen consumption of 143B cells with ablated NDUFA11. Oxygen consumption rate (OCR) 96 h after transfection with two siRNAs (NDUFA11-1 and -2, each 30 nM) against NDUFA11 (black and dark gray) compared with control cells (light gray). (A) Complex-I-dependent OCR of 143B cells determined by subtraction of rotenone-inhibited OCR values from initial values. (B) Addition of rotenone and duroquinol and OCR derived from activities of complexes III and IV. Error bars are SD.

Fig. 2. Effect of transient suppression of the expression of NDUFA11 on assembly of complex I. Analysis by BN-PAGE of mitoplasts from 143B cells following suppression of expression of NDUFA11 with siRNA NDUFA11-1 for 96 h. (A–E) Protein complexes detected with antibodies against subunits of the hydrophilic arm of complex I (NDUF5), the hydrophobic arm of complex I (NDUF8), and complexes III, IV, and II, respectively.

Copurification of C3orf1 with the 315-kDa Subcomplex. Attempts were made to introduce versions of C3orf1 and TMEM126B with C-terminal StreptII and FLAG tags into HeLa Flp-In T-Rex cells, but only the expression of C3orf1 with tags (C3orf1-FL) succeeded.

The protein interactions of C3orf1 were examined by SILAC labeling of cells expressing C3orf1-FL. The mass spectrometric analysis of affinity purified C3orf1-FL and associated proteins demonstrated that 17 subunits of complex I, and five known assembly factors, copurified with C3orf1-FL (Fig. 5 and Dataset S2); ATP5SL and DNAJC11 were not detected. The most enriched and closely clustered proteins in the analysis were complex I subunits MT-ND1, NDUFA5, NDUF52, NDUFS3, NDUFS7, and NDUFS8, components of the 315-kDa assembly intermediate (16), plus NDUF3, NDUFA8, and NDUFA13, components of subcomplex Ia. Therefore, NDUFA3, NDUFA8, and NDUFA13 also are parts of the same assembly intermediate. They were accompanied by assembly factors NDUFAF3 and NDUFAF4, both involved in formation of the 315-kDa assembly intermediate (30). The complex I assembly factors ACAD9, ECSIT, and NDUFA11 are associated with the 370-kDa assembly intermediate of complex I (31, 32), and have been

Fig. 5 and Dataset S1); NDUFAF3, ACAD9, and NDUFAF2 were associated with subassemblies with masses of 400, 460, and 830 kDa, respectively (15). Based on the reestimated masses of subcomplexes (Fig. S2) they probably corre-

In contrast, suppression of expression of either ATP5SL or DNAJC11, or both proteins together, had no effect on the as-

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growth and apoptosis (36); and NDUFA2 has a thioredoxin fold dehydrogenases (35); NDUFA13 is reported to regulate cell is an acyl carrier protein (34); NDUFA9 is related to steroid their functions are mostly unknown, one subunit, NDUFAB1, 14 are hydrophobic membrane arm components (3). Although pernumerary subunits are associated with the peripheral arm and no known role in the assembly of complex I; 18936 | horizontal axes represent 1:1 ratios, respectively, where there are no changes in protein abundance between NDUFA11 depleted cells and control cells; ○, proteins with a relative ratio unchanged between the NDUFA11 ablated material and the control; ▲, known assembly factors; ◆, enriched proteins with no known role in the assembly of complex I; ▲, complex I subunits that are diminished significantly; ●, proteins diminished by the removal of NDUFA11; X, exogenous contaminants.

reported to comigrate with TMEM126B in native gel profiles (27). Here, isoforms 1 and 5 of TMEM126B were found with relative abundances similar to these extrinsic assembly factor proteins. Mass spectrometric evidence for the isoforms is presented in Fig. S6. There was no evidence for isoforms 2, 3, and 4. Other proteins enriched significantly are constituents of subcomplex III, the distal region of the membrane arm of complex I. The decreasing SILAC protein ratios correspond to membrane protein subunits of increasing distance from the quinone-binding site (Fig. 5). The SILAC ratios of some subunits of complex I were unchanged, indicating that they were not enriched in the complex purified with C3orf1-FL (Dataset S2), and that therefore they are not associated with the 315-kDa subcomplex. They include NDUFA9, which has been reported previously to be a component of the 315-kDa subcomplex (33), although this observation was not corroborated (27). Three other proteins, TMEM126A, HSPA9, and GHTIM, also accumulated with C3orf1-FL, but to a much lesser extent than the complex-I related proteins listed above (Dataset S2).

To demonstrate directly that C3orf1-FL is associated with the 315-kDa subcomplex, the proteins that were immunocaptured with C3orf1-FL were fractionated by blue native (BN)-PAGE, and two protein complexes containing C3orf1-FL were detected by Western blotting, with molecular masses of 315 and 370 kDa, respectively, for the major and minor subcomplexes, respectively (Fig. 5).

Discussion

Supernumerary Subunits and Assembly of Complex I. Sixteen supernumerary subunits are associated with the peripheral arm and 14 are hydrophobic membrane arm components (3). Although their functions are mostly unknown, one subunit, NDUFAB1, is an acyl carrier protein (34); NDUFA9 is related to steroid dehydrogenases (35); NDUFA13 is reported to regulate cell growth and apoptosis (36); and NDUFA2 has a thioredoxin fold (37). As pathological mutations are associated with some supernumerary subunits, those ones probably help to assemble and/or maintain the integrity of the complex. NDUFA11 is an example. It contains two to four predicted transmembrane α-helices

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**Fig. 3.** Exogenous proteins associated with subcomplexes of complex I. Comparison of protein abundance in immunocaptured complex I from control cells and cells with suppressed NDUFA11. Each data point represents a specific protein, and its position corresponds to the abundance ratios from the two complementary heavy and light SILAC experiments. Vertical and horizontal axes represent 1:1 ratios, respectively, where there are no changes in protein abundance between NDUFA11 depleted cells and control cells; ○, proteins with a relative ratio unchanged between the NDUFA11 ablated material and the control; ▲, known assembly factors; ◆, enriched proteins with no known role in the assembly of complex I; ▲, complex I subunits that are diminished significantly; ●, proteins diminished by the removal of NDUFA11; X, exogenous contaminants.

**Fig. 4.** Ablation of C3orf1 or TMEM126B and the integrity of complex I. The 143B cells were treated with two siRNAs for 96 h, targeted against C3orf1 (50 nM; A and C) and TMEM126B (100 nM, B and D). (A and B) OCR of siRNA-treated 143B cells. The values were corrected by subtraction of a rotenone-treated oxygen consumption value. Error bars represent the SD. (C and D) Analysis by BN-PAGE of mitoplasts from siRNA-treated 143B cells. The protein subunits of increasing distance from the quinone-binding site (Fig. 5).
and is a component of the membrane region of subcomplex Iα. Human mutations in it result in leukodystrophy (17), and as shown here, it is required for the assembly and/or possibly the structural integrity of complex I. In its absence, subcomplexes of complex I of 550 and 815 kDa accumulate. It has the characteristics of an intrinsic assembly factor.

**Extrinsic Assembly Factors.** The 37 nuclear-encoded subunits of complex I are imported into the organelle and then sorted, folded, and assembled into the peripheral and membrane arms. The 7 remaining subunits MT-ND1–6 and MT-ND4L are hydrophobic proteins translated from mitochondrial DNA. During sorting, folding, and assembly, they become associated with the 14 other membrane arm subunits encoded in the nucleus. The mitochondrially and nuclear-encoded subunits contribute about 60 and 19 transmembrane α-helices, respectively, to the membrane domain of complex I (6). A further 23 nuclear-encoded subunits are assembled into the peripheral arm of the enzyme, and in the process eight iron–sulfur clusters are incorporated into five core subunits in the extrinsic arm (NDUFS1, NDUFS7, NDUFS8, NDUFS10, and NDUFS2), and a FMN molecule is inserted noncovalently into NDUFS1. This complicated process requires the participation of other proteins not present in the complete complex. Known as extrinsic assembly factors, they are required for producing the complete enzyme. Assembly factors (NDUFAF2–4 and FOXRED1) have been identified via human mutations that impair function (29, 30, 38), by homology with other known assembly factors (ECSIT and ACDAD9) (31, 32). The BN-PAGE comigration pattern of TMEM126B with NDUFAF1, ECSIT, and ACDAD9 led to its characterization as a complex I assembly factor (27). How these assembly factors function is not known, but they may stabilize the subcomplexes and help to join them to other subcomplexes to build the complete enzyme. Two additional proteins involved in the assembly of complex I, C20orf7 (NDUFAF5) and MsdA (NDUFAF7), are predicted to be protein methyltransferases and NDUFAF7 methylates subunit NDUFS2 (39, 40). Ind1 is involved in the assembly of iron–sulfur clusters in complex I and C8orf38 (NDUFAF6) stabilizes subunit MT-ND1 (41, 42).

When the expression of supernumerary subunit NDUFA11 was suppressed, all of the assembly factors found previously in association with subcomplexes of complex I were found, plus C3orf1, ATP5SL, and DNAJC11, but the last two were eliminated as possible assembly factors. C3orf1, and also TMEM126B are targeted to mitochondria, and their influence on the assembly of complex I was confirmed by suppressing their expression. The role of C3orf1 as a complex I assembly factor was supported by the copurification of six known assembly factors with subcomplexes containing C3orf1.

**Roles of C3orf1 and TMEM126B.** A number of features of C3orf1 and TMEM126B suggest that they may be involved in the assembly of the membrane arm of the complex. First, they are both hydrophobic proteins, predicted to have three and four transmembrane α-helices, respectively. In this respect, they differ from all of the other known extrinsic assembly factors for complex I. However, although they are both mitochondrial proteins, currently direct proof of their presence in the inner membrane is lacking. As NDUFA11 is also a membrane protein, and the complex fails to assemble fully in its absence, it is reasonable to classify it also as a complex I assembly factor probably involved in putting together the membrane arm. Second, C3orf1, and also NDUFA11, are members of the TIM17/22/23 family, related in the region of the second of the transmembrane α-helices of C3orf1 and in the following hydrophilic loop (Fig. S3). TIM17 and TIM23 are mitochondrial inner membrane proteins that form the protein translocation pore, and TIM22 mediates the integration of mitochondrial carrier proteins into the inner membrane of the organelle (43). The association of C3orf1 and NDUFA11 with this group of proteins suggests a possible role for both of them in inserting hydrophobic proteins into the membrane arm of complex I. Third, C3orf1 is found with the 315-kDa subcomplex, where the hydrophobic inner membrane subunit ND1 with eight predicted transmembrane α-helices (35) is a significant component, and TMEM126B is associated with a 370-kDa subcomplex, containing at least subunits MT-ND2, MT-ND3, MT-ND6, and MT-ND4L (all hydrophobic and containing a total of about 20 transmembrane α-helices).
**Assembly Pathway.** In current models, the assembly of complex I (15, 16) begins with the formation of a 400-kDa subcomplex (reestimated as 315 kDa), nucleated around core subunits NDUF52 and NDUF53, and anchored to the membrane by MT-ND1. The other subunits attributed to this substructure are NDUFA5, NDUFS7, and NDUFS8. Subunits NDUFA3, NDUFA8, and NDUFA13 can now be added, plus three assembly factors, NDUFA3, NDUFA4, and C3orf1. A 460-kDa subcomplex (now 370 kDa) consisting of subunits of the membrane arm of complex I, arises separately, and is likely to contain TMEM126B. In the modified assembly pathway (Fig. 6), the 315- and 370-kDa subassemblies join together to give a subcomplex of 550 kDa (previously 650 kDa). This step was inhibited by the removal of either C3orf1 or TMEM126B, and the 550-kDa subcomplex accumulated in the absence of NDUFA11. Upon addition of the most distal components of the membrane arm, including MT-ND4 and MT-ND5, an 815-kDa (previously 830 kDa) subassembly is formed, lacking only the region of the enzyme that oxidizes NADH. The final step is the addition of the distal region of the peripheral arm (subunits NDUFA12, NDUFS1, NDUFS4, NDUFS6, NDUVF1, NDUVF2, and NDUVF3), aided by the participation of NDUFA2.

This model of assembly of complex I is incomplete, and the lack of genetic diagnosis of about half the patients with defects in the enzyme (18) suggests that additional extrinsic and/or intrinsic assembly factors remain to be identified.

**Methods**

**Cell Culture.** Human 143B osteosarcoma cells (American Type Culture Collection no. CRL8303) and HeLa cells with integrated plasmids pDNA5/FRT and pFR/FlaZeo, constituting the Flp-In T-REx system (Life Technologies) were grown at 37 °C in high (25 mM) glucose medium (DMEM) supplemented with FBS (10% vol/vol), penicillin (100 units/mL), streptomycin (0.1 mg/mL) under 5% (vol/vol) CO2. Transfections of DNA and siRNA were performed with Lipofectamine 2000 (Life Technologies).

In SILAC experiments, cells were grown in “heavy” DMEM containing arginine and lysine isotopically labeled with 15N and 13C, and in “light” DMEM containing 14N and 12C arginine and lysine (Sigma-Aldrich). These media were supplemented with proline (200 μg/mL) to suppress the conversion of arginine to proline, and with dialyzed FBS (Life Technologies) to prevent dilution of heavy isotopes. To ensure maximal incorporation, the cells were doubled at least seven times in media containing heavy isotopes.

**Transcript Suppression.** Transcripts were suppressed transiently in duplicate in 143B cells with 30–100 nM siRNA, NDUFA11 with FlexiTube siRNAs (Qiagen), C3orf1 and TMEM126B with Silencer Select siRNAs (Life Technologies), and ATP5SL and DNAJC11 with Mission siRNAs (Sigma Aldrich). Control siRNA (Allstars Negative Control siRNA; Qiagen) was used at the same concentration. Reduction of transcripts was assessed on cDNA (Cells-to-CT kit, Life Technologies) by quantitative real-time PCR (Taqman gene expression assays; Life Technologies). Levels of all target transcripts were reduced by >80% (normalized to β-actin). Where suitable antibodies were available, the reduction in the protein was examined by Western blotting. Four days after transfection, the phenotypes of cells were assessed. Transcripts were suppressed stably in 143B cells by cloning target sequences identical to the siRNA into the pSuperior.puro vector (Oligoengine). Stable recombinants were selected with puromycin (2 μg/mL).

**Epileptogenic Tagging and Confocal Microscopy.** Human cDNAs encoding C3orf1 (Source BioScience) and TMEM126B (GeneCopoiea) were cloned into pcDNAs/FRT/TO, with sequences for C-terminal FLAG and/or Streptil tags, and incorporated stably into HeLa Flp-In T-Rex cells. Plasmids pOG44 and pcDNAs/FRT/TO containing tagged inserts (1 μg DNA; pOG44:pcDNAs/FRT/TO, 7.1 by weight) were cotransfected. After 24 h, zeocin (50 μg/mL) in the medium was replaced by hygromycin (200 μg/mL). Mitochondria from stable isogenic recombinant clones were labeled with MitoTracker Orange (100 nM; Life Technologies). The cells were fixed with paraformaldehyde (4% wt/vol), permeabilized with Triton X-100 (0.1% vol/vol), and tagged proteins were labeled with mouse M2 anti-FLAG antibody (Sigma-Aldrich) followed by Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies). The fluorescence was detected in a Zeiss 510 LSM confocal microscope.

**Measurement of Respiration.** The oxygen consumption rate (OCR) of 143B cells was measured in an XF24 extracellular flux analyzer (Seahorse Biosoceis) as described in ref. 39.

**Protein Purification.** Extraction of proteins in HeLa Flp-In T-Rex cells was induced for 24 h with doxycycline (20 ng/mL). Cells (15 mg protein) were solubilized in PBS with complete EDTA-free protease inhibitor mixture (Roche) and enriched for mitoplasts with digitonin (500 μg/mL; detergent: protein:1:10 wt:wt). Mitoplasts (1.5 mg protein) were solubilized in 1% n-dodecyl-β-d-maltoside (DDM) in PBS containing protease inhibitor (Roche), glycerol (10% vol/vol), and a mixture of synthetic phospholipids [1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine, 0.09 mg/mL; 1-palmitoyl-2-oleoyl-snglycerol-3-phosphoethanolamine, 0.03 mg/mL; 1-palmitoyl-2-oleyl-sn-glycerol-3-phospho(1-rac-glycerol), 0.03 mg/mL (Avanti)]. Insoluble material was filtered off. Complex I and subcomplexes were purified at 4 °C with immunocapture resin (Mitosciences), and FLAG-tagged proteins with M2 FLAG agaroose (Sigma-Aldrich). Bound proteins were eluted with 0.2 M glycine and DDM (0.05% wt/vol), pH 2.5. Native proteins were eluted in “solubilization buffer,” containing 3× FLAG peptide (125 ng/μL) and DDM (0.05% wt/vol).

**Electrophoresis and Western Blotting.** Protein complexes were fractionated by BN-PAGE in 3–12% acrylamide gradient Bis-Tris gels (Life Technologies). Mitoplasts made with digitonin (2 mg/mL; detergent:protein:1:2.5 wt:wt), were solubilized in 1.67% (wt/vol) DDM (detergent: protein, 6.1, wt:wt). Fractionated proteins were transferred to a polyvinylidene difluoride membrane and probed with antibodies in PBS with milk powder (approximately 5%, wt/vol) and Tween 20 (0.01%, vol/vol). The antibodies were against complex I subunits NDUF8B and NDUF5A, subunit 5a of complex IV (CosVa), the SDHB subunit of complex II, the core 2 subunit of complex III (UQCR2), and β-actin (Sigma-Aldrich). Antibodies against complex I subunits NDUFS1 and NDUFB10 were purchased from Proteintech. A chicken polyclonal anti-peptide antibody was prepared against residues 1–17 of NDUFA11 (Agrisera). The molecular masses of subcomplexes of complex I were estimated from their migration by BN-PAGE. The gels were calibrated with bovine respiratory complexes I and III–V plus standard proteins from a NativeMark kit (Life Technologies).

**Mass Spectrometry.** Each analysis was based on two SILAC experiments (44), one in which the ablated cells were labeled in heavy medium and the controls in light medium, and the second vice versa. Each sample for mass
spectrometric analysis consisted of a 1:1 mixture of complex I (and its sub-
complexes) immunopurified from mitoplasts from control and NDUFA11
knockout mice. Proteins were digested using trypsin and fractionated by SDSPAGE
on 10–20% acrylamide gradient gels in Tris-glycine buffer (Life Technolo-
gies), and proteins in gel slices were digested with trypsin. Peptides were dissolved in 5% (vol/vol) aqueous acetonitrile containing 0.1% (vol/vol) formic
acid). The effluent was passed into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher) operating in data-dependent MS/MS mode, with a mass scan range of 400–2000 Da for precursor ions and MS/MS of the top 10 highest abundance ions selected from the full scan. Each tryptic peptide produced a peptide ion pair differing by either 10.01 or 8.01 Da for peptides with C-
terminal arginine or lysine, respectively. Peptide pairs were located by MaxQuant and identified with Andromeda by comparison of fragment ion masses in tandem mass spectra and the human UniProt database (45). A heavy/light ratio was calculated with MaxQuant. The median peptide ratio was taken to be the protein ratio, using at least two ratios for each protein. The ratios from each experiment were plotted on horizontal and vertical axes respectively, of a “scatter plot”, the log base 2 value. Thus, a fourfold change in the abundance of a protein in both experiments becomes 2 on each axis, and the protein is represented by a point in the top right quadrant of the scatter plot. The horizontal and vertical axes represent 2 raised to the power of zero, a ratio of 1, and no changes in abundance. Proteins un-
affected by experimental conditions cluster around the origin, and those with consistent increases or decreases in abundance occur in the top right or bottom left quadrants, respectively. A diagonal line from the top right to bottom left represents a perfect correlation between the two experi-
ments. Statistically significant proteins in both orientations of labeling were identified with Perseus (46) (P < 0.05). Points in the two other quadrants represent proteins where the differences are irreproducible in the replicate experiments. Those in the top left quadrant contain exoge-
 nous contaminants.

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Supporting Information

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Fig. S1. Comparison of morphology of mitochondria in control cells and in cells in which expression of subunit NDUFA11 has been suppressed transiently. (A) Human 143B cells transfected with (A) control siRNA and (B) siRNA targeted against subunit NDUFA11. The cells were stained with MitoTracker 72 h after transfection, fixed, and visualized by confocal microscopy. (Scale bars, 10 μm.)

Fig. S2. Reestimation of molecular masses of subcomplexes of human complex I by blue native (BN)-PAGE. The gels were calibrated with the following proteins and protein complexes with their calculated molecular masses given in kilodaltons in parentheses: 1, bovine complex I (971); 2, apoferritin 1 (720); 3, bovine ATP synthase (597); 4, bovine complex III (482); 5, apoferritin 2 (480); 6, bovine complex IV (205); 7, B-phycoerythrin (242); 8, lactate dehydrogenase (146); and 9, BSA (66). ▲ standard proteins and complexes; ● subcomplexes of complex I; their molecular masses as measured here are as follows, with previous estimates for a–d (1) in parentheses: a, 815 (830) kDa; b, 550 (650) kDa; c, 370 (460) kDa; d, 315 (400) kDa; and e, 200 kDa.

Fig. S3. Colocalization of C3orf1 and TMEM126B with mitochondria, and members of the C3orf1 protein family. The C-terminal FLAG and StrepII-tagged C3orf1 (A, i–iii) and C-terminal FLAG-tagged TMEM126B (B, i–iii) were visualized in HeLa cells. Plasmid pcDNA5/FRT/TO containing the C-terminal-tagged sequence of interest was transfected into HeLa cells, which were treated with MitoTracker 24 h later, fixed, permeabilized with Triton X-100, and immunostained. (A, i and B, i) Alexa Fluor 488 anti-mouse secondary antibody conjugated to mouse anti-FLAG antibody; (A, ii and B, ii) MitoTracker Orange staining; (A, iii) A, i and A, ii merged; (B, iii) B, i and B, ii merged. (Scale bar in each merged image, 10 μm.) (C) Alignment of sequence sections of C3orf1 and other protein family members NDUFA11, TIM17, TIM22, and TIM23 (PFAM: PF02466). The positions of transmembrane α-helices predicted with HMMTOP (1) are indicated by red bars beneath the sequences. A Clustal X color scheme is used for aligned residues: hydrophobic (A, C, I, L, M, F, W, and V), blue; basic (R and K), red; acidic (D and E), magenta; amide (N and Q) and nucleophilic (S and T), green; H and Y, cyan; G, orange; and P, yellow.

Fig. S4. Presence of NDUFAF3, ACAD9, and NDUFAF2 in subassemblies of complex I. Human 143B cells were treated for 96 h with siRNAs against TMEM126B (100 nM) and NDUFA11 (30 nM). Mitoplast proteins from those cells were separated by BN-PAGE and Western blotted with antibodies against: (A) NDUFAF3, ACAD9, and complex II and (B) NDUFAF2 and complex III.

Fig. S5. Suppression of expression of ATP5SL and DNAJC11 in human 143B cells. Western blot analyses of mitoplasts from human 143B cells prepared 96 h after addition of siRNAs (50 nM) targeted against ATP5SL and DNAJC11, both singly and together. The proteins were fractionated by SDS/PAGE (A) and BN-PAGE (B). Detection with antibodies against, in A, DNAJC11 and ATP5SL, with the membrane stained with Coomassie blue as a loading control, and in B, against the NDUFS2 subunit, a component of subcomplex Iα and subunit NDUFB8, a component of subcomplex Iβ. The levels of complexes II and III are shown on the Right.
Fig. S6. Fragment ion mass spectra of three peptides from TMEM126B that are diagnostic of isoforms 1 and 5. The N-terminal region of isoform 5 is truncated by 30 residues relative to isoform 1. (A) N-terminal peptide (m/z 671.32$^2$) of TMEM126B isoform 1, lacking the initiator methionine residue; (B) peptide (residues 14–27; m/z 692.85$^2$) from isoform 1-specific region of TMEM126B; (C) N-terminal peptide (m/z 846.39$^2$) lacking initiator methionine from TMEM126B isoform 5. The N-terminal alanine is acetylated.
Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)