Supplementary Information for
Sideroflexin 4 is a Complex I assembly factor that interacts with the MCIA complex and is required for the assembly of the ND2 module

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- Supplementary Tables 1-5
Supplementary Methods

Quantitative mass spectrometry and data analysis

For immunoprecipitation experiments, acetone precipitated pellets were resuspended in urea/ABC buffer (8M urea, 50 mM ammonium bicarbonate (ABC)) and solubilised through sonication for 15 minutes in a water bath sonicator. To achieve protein reduction and alkylation, TCEP and CAA were added to the solubilised proteins to final concentrations of 10 mM and 50 mM, respectively. Samples were incubated at 37 °C for 30 minutes with 500 RPM shaking, before being diluted to 2 M urea through the addition of 50 mM ABC buffer. 1 µg of trypsin was added to each sample, which were digested overnight at 37 °C with 500 RPM shaking. Stage-tips (containing 2 x 14G plugs of 3M Empore SDB-XC Extraction Disks (Sigma-Aldrich)) were prepared and activated with 50 µL of 100% acetonitrile, which was centrifuged through the stage tip at 2500 x g. The activated stage tips were equilibrated with 50 µL of 0.1 % (vol/vol) TFA, 2% (vol/vol) acetonitrile. Trypsin digested samples were acidified to 1% TFA, centrifuged at 20000 x g to remove insoluble debris, and transferred to the stage tips. After centrifugation at 2500 x g to allow the supernatant to flow through to the collection tube, the stage tips were washed with 100 µL of 0.1 % (vol/vol) TFA, 2% (vol/vol) acetonitrile, after which bound peptides were eluted using 100 µL of 0.1% (vol/vol) TFA, 80% (vol/vol) acetonitrile. The eluted peptides were dried in a CentriVap Benchtop Vacuum Concentrator (Labconco) and reconstituted in 0.1% (vol/vol) TFA, 2% (vol/vol) acetonitrile for analysis by liquid chromatography (LC)-MS/MS.

LC-MS/MS was performed with a QExactive plus Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nanoESI interface in combination with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000). The LC system was equipped with an Acclaim Prepmap nano-trap column (Dionex C18; 100 Å, 75 µM x 50 cm). The reconstituted peptides were injected into the enrichment column at an isocratic flow of 5 µL/min of 2% (vol/vol) acetonitrile containing 0.1% (vol/vol) formic acid for 5 min before the enrichment column was switched in-line with the analytical
column. The eluents were 5% dimethyl sulfoxide (DMSO) in 0.1% (vol/vol) formic acid (solvent A) and 5% (vol/vol) DMSO in acetonitrile (solvent B). For analysis of mitochondrial samples, the flow gradient was 1) 0-6 min at 3% B, 2) 6-95 min at 3-22% B, 3) 95-105 min at 22-40% B, 4) 105-110 min at 40-80% B, 5) 110-115 min at 80% B, 6) 115-117 min at 80-3% B. Equilibration was performed with 3% B for 10 min before the next sample injection. For analysis of immunoprecipitation eluates, the flow gradient was 1) 0-3 min at 3% B, 2) 2-50 min at 3-26% B, 3) 50-53 min at 26-45 % B, 4) 53-54 min at 45-80% B, 5) 54-55 min at 80% B, 6) 55-60 at 80-3%. Equilibration was performed with 3% B for 10 min before the next sample injection. The mass spectrometer was operated in data-dependent mode. Full MS1 spectra were acquired in positive mode, 70000 resolution, AGC target of 3e6, and maximum IT time of 50 ms. A loop count of 15 on the most intense targeted peptide was isolated for MS/MS. The isolation window was set to 1.2 m/z and precursors fragmented using stepped normalised collision energy of 27, 30 and 32. MS2 resolution was at 17500, AGC target at 2e6, and maximum IT time of 50 ms. Dynamic exclusion was set to be 30 seconds.

The MaxQuant platform (version 1.6.5.0) (1) was used for analysis of raw files, which were searched against the human UniProt database (March 2021) containing both canonical forms and isoforms of reviewed entries. Default search parameters were used for label-free quantitation (LFQ), with matched between runs enabled. An LFQ minimum ratio count of 2 was used for analysis of isolated mitochondria, while an LFQ minimum ratio count of 1 was used in analysis of immunoprecipitation experiments to enable inclusion of MT-ND6, which is only detectable via a single unique peptide. Subsequent analysis was performed using the Perseus platform (Version 1.6.5.0) (2). The proteinGroups.txt output from the MaxQuant search was loaded into Perseus, and all rows that were listed as being ‘Only identified by site’, ‘Reverse’, or ‘Potential contaminant’ were removed. LFQ intensity values were Log2 transformed, grouped according to the experiment group (i.e control or knock-out; control immunoprecipitation or FLAG-tagged protein immunoprecipitation). Subsequent analysis differed depending on whether
mitochondrial samples or immunoprecipitation eluates were being analysed. For analysis of mitochondrial samples, matrices were filtered to only include rows where there were at least 2 valid values present for each experimental group. An annotation was added to indicate whether proteins were present in the Mitocarta2.0 database (3) (matching by gene name), and the matrix was filtered to only include those which were present in the database. A further annotation was added to indicate ‘Known mitochondrial’ proteins from the IMPI database (2017) (4), which were used for normalisation using ‘Subtract row cluster’. Two-sample t-tests were performed using p-value for truncation (threshold p-value p<0.05). Volcano plots were generated as scatter plots with ‘Student’s T-test difference’ on the x-axis and ‘-Log Student’s T-test p-value’ on the y-axis. For analysis of immunoprecipitation experiments, summary statistics were used to calculate the number of valid values present in the FLAG-tagged protein eluate, and the matrix was filtered to only include rows with >2 valid values in this group. Imputation was then used to fill missing values in the control immunoprecipitation group at the limit of detection based on the normal distribution of LFQ values in the experiment. After a Mitocarta2.0 annotation was added and used to remove proteins not present in the database, a two-sided t-test was performed with significance determined using permutation based FDR statistics, which was modified so that the significance threshold excluded all proteins enriched in the control sample.

**Complexome profiling**
Following completion of electrophoresis, the gel containing separated SILAC-labelled mitochondria was immediately transferred into fixing solution (50% (vol/vol) methanol, 10% (vol/vol) acetic acid, 10 mM ammonium acetate) and incubated for 30 minutes, followed by a 30-minute incubation in Coomassie solution (0.025% (w/v) Coomassie blue G250, 10% (v/v) acetic acid). The gel was destained in 10% (vol/vol) acetic acid for several hours until the background was clear, and then washed in water at least three times. Lanes were excised and cut into 60 even slices. Each slice was diced into smaller pieces and placed into individual wells of an Acroprep™ 30-40 μM PP/PE filtered microtiter plate
containing 50 mM ammonium bicarbonate (ABC) for in-gel tryptic digest as previously described (5).

Briefly, ABC was removed by centrifugation and replaced with destain solution (60% (vol/vol) methanol, 50 mM ABC) and incubated for 60 minutes with gentle shaking. All centrifugation wash steps were performed at 1500 g. Destain was repeated 2-3 times as required. Gel pieces were washed in 50 % (vol/vol) acetonitrile (ACN), 50 mM ABC and incubated in 10 mM dithiothreitol, 50 mM ABC for 60 minutes at 56 °C without shaking. Gel pieces were then incubated in 40 mM chloroacetamide, 50 mM ABC for 45 minutes before being washed a further two times in 50% (vol/vol) ACN, 50 mM ABC. Gel pieces were then allowed to air dry for 40-45 minutes without agitation, before 20 µL of digestion solution (10 ng/µL trypsin (Thermo Fisher Scientific), 10 % (v/v) ACN, 0.01% (w/v) ProteaseMAX surfactant (Promega), 1 mM CaCl₂, 50 mM ABC) was added to each well. The plate was incubated at 4°C to allow gel pieces to re-swell and topped up with 80 µL of 50 mM ABC, for overnight incubation at 37°C in a pre-humidified incubator.

Digested peptides were eluted the next day into a fresh microtiter plate by centrifugation before adding an additional elution buffer (30% [v/v] ACN, 3% (v/v) formic acid (FA)) to gel pieces for gentle shaking for a further 20 minutes. The elution buffer was collected by centrifugation and both elutions were pooled. Peptide solutions were dried using a CentriVap concentrator (Labconco) then reconstituted in 2% (vol/vol) ACN, 0.1% (vol/vol) TFA. Samples were desalted on stagetips containing 2x14G plugs of 3M™ SDB-XC extraction discs (Sigma-Aldrich), which were pre-activated with 100% ACN and equilibrated with 2% (vol/vol) ACN, 0.1% (vol/vol) TFA before loading. Stagetips were washed with 2% (vol/vol) ACN, 0.1% (vol/vol) TFA and samples were eluted with 80% (vol/vol) ACN, 0.1% (vol/vol) TFA. All centrifugation steps were performed at 1800g. Elutions were dried and reconstituted in 2% (vol/vol) ACN, 0.1% TFA for LC-MS/MS analysis.
The LC system was equipped with an Acclaim Pepmap nano-trap column (Dinoex-C18, 100 Å, 75 μM x 50 cm). The tryptic peptides were injected to the enrichment column at an isocratic flow of 5 μL/min of 2% (vol/vol) ACN containing 0.05% (vol/vol) TFA for 6 min applied before the enrichment column was switched in-line with the analytical column. The eluents were 5% (vol/vol) DMSO in 0.1% (vol/vol) formic acid (solvent A) and 5% (vol/vol) DMSO in 100% (vol/vol) ACN and 0.1% (vol/vol) FA (solvent B). The flow gradient was (i) 0-6 min at 2% B; (ii) 6-25 min, 2-23% B; (iii) 25-35 min, 23-40% B; (iv) 35-40 min, 40-80% B; (v) 40-42 min, 80-80% B; (vi) 42-42.1 min, 80-2% B; and equilibrated at 2% B for 10 minutes before the next sample injection.

The QExactive plus mass spectrometer was operated in the data-dependent mode, whereby full MS1 spectra were acquired in positive mode, 70000 resolution AGC target of $3 \times 10^6$ and maximum IT time of 50 ms. Fifteen of the most intense peptide ions with charge states ≥2 and intensity threshold of $4 \times 10^4$ were isolated for MSMS. The isolation window was set at 1.2 m/z and precursors fragmented using normalised collision energy of 30, 17500 resolution, AGC target of $5 \times 10^4$ and maximum IT time of 50 ms. Dynamic exclusion was set to be 30 sec.

Raw files were processed using the MaxQuant platform (version 1.6.17.0) against the UniProt human canonical and isoforms database (42,360 entries, August 2020) using settings for the standard identification of light and heavy (Arg-10 and Lys-8) SILAC-labelled peptides. Oxidation of methionine and N-terminal acetylation were specified as variable modifications, and carbamidomethylation of cysteine was set as a fixed modification. Trypsin/P cleavage specificity was used with a maximum of 2 missed cleavages, and a search tolerance of 4.5 ppm was used for MS1 and 20 ppM for MS2 matching. False discovery rates (FDR) were determined through the target-decoy approach set to 1% for both peptides and proteins. The calculation of intensity-based absolute quantification (iBAQ) intensities was enabled with the Log fit function disabled. Outputted heavy and light iBAQ intensities were filtered and annotated for mitochondrial proteins listed in MitoCarta.
3.0 (6) in Perseus (version 1.6.15.0) then imported for visualisation into NOVA (version 0.8.0.0) for complexome analysis. Mass scale calibration was performed by exponential interpolation in NOVA by inputting apparent masses selected from Table S5 from Maclean et al., 2020 (7). Selected proteins of interest underwent hierarchical clustering using default settings, with optimised leaf ordering, average linkage, and a Pearson Correlation Distance function, without normalisation. Exported heatmaps with iBAQ intensities were normalised in Excel, by row average for heatmap generation, and by maximum intensity for profile plot generation. Figures were generated in GraphPad Prism 9.
**A**

| Gene | Gene | Gene | Gene | Gene |
|------|------|------|------|------|
| SFXN1 | SFXN2 | SFXN3 | SFXN4 | SFXN5 |
| EBF1 | PHykpl | RUFY1 | CBY3 | SYK |
| DOCK2 | LDLRAP1 | GLS | ATP1B3 | DOK3 |
| EFCA89 | FGFR4 | ITSN2 | C1orf127 | GCLC |
| C1orf127 | GCLC | IRF4 | CLTB | NCF1 |
| COX6c | MEF2B | **Indels:** | **Predicted protein impact** | **Frequency/Depth** |
| c.[86_107del] | c.[85_95del] | p.[Val29Alafs*15] | p.[Val29Glyfs*19] | 4/6 |

**B**

- **Flp-In™ T-REx™ SFXN4KO**
- **Gene Rank**

**C**

- **Correlation**
- **Cl assembly factor**
- **Cl subunit**
- **Mito translation**
- **Other OXPHOS**
- **Other mitochondrial**
- **Non-mitochondrial**

**D**

- **SFXN4KO**
- **Control**
- **Untreated**
- **Formate**
- **NAC**
- **Cystine**

- **IB:** SDHA (72 kDa)
- **IB:** NDUFA10 (41 kDa)
- **IB:** NDUFA9 (43 kDa)
- **IB:** β-actin (43 kDa)
Supplementary Figure 1.

(A) Top 20 most strongly correlated genes with SFXN1, SFXN2, SFXN3, and SFXN5. Genes are colour coded by functional category. (B) Plot indicating number of OXPHOS genes within the top 100 most strongly correlated genes for SFXN1, SFXN2, SFXN3, SFXN4 and SFXN5. (C) Schematic representation of the CRISPR/Cas9 editing system used to generate SFXN4<sup>KO</sup> Flp-In T-Rex 293 cell line used in the study. The resulting indels are indicated. (D) Whole cell extracts from control cells, SFXN4<sup>KO</sup> cells, and SFXN4<sup>KO</sup> cells treated for 24 hours with 1 mM formate, 2.5 mM NAC, or 1.25 mM cystine were analysed by SDS-PAGE and immunoblotting.
A

| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|----|----|----|----|----|----|----|----|----|
| TX-100 Swelling | +  | +  | +  | -  | -  | -  | -  | -  |
| PK | -  | +  | +  | -  | -  | -  | -  | -  |
| SFXN4 KO | -  | -  | -  | -  | -  | +  | +  | +  |
| SFXN4 HA | -  | +  | +  | -  | -  | -  | -  | -  |
| SFXN4 MYC | -  | +  | +  | -  | -  | -  | -  | -  |
| SFXN4 KO + SFXN4 HA | -  | -  | -  | -  | +  | +  | +  | +  |
| SFXN4 KO + SFXN4 MYC | -  | -  | -  | -  | +  | +  | +  | +  |

IB: SFXN4 (36 kDa)
IB: FLAG (36 kDa)
IB: Mfn2 (86 kDa)
IB: OPA1 (80-100 kDa)
IB: NDUFAF2 (20 kDa)

B

| 1  | 2  | 3  | 4  |
|----|----|----|----|
| Control | + SFXN4 KO HA | + SFXN4 KO MYC |
| IB: SDHA (72 kDa) | IB: SFXN4 (low) (38 kDa) | IB: SFXN4 (high) (38-40 kDa) |
| IB: HA (40 kDa) | IB: MYC (40 kDa) | IB: NDUFA10 (40 kDa) |
| IB: NDUFV2 (23 kDa) | IB: NDUFA10 (40 kDa) | IB: NDUFV2 (23 kDa) |

Jackson et al., 2021 Supplementary Figure 2
Supplementary Figure 2.

(A) SFXN4 and FLAG-SFXN4 subfractionation and carbonate extraction. Mitochondria were isolated from SFXN4\textsuperscript{KO} + SFXN4 or SFXN4\textsuperscript{KO} + FLAG-SFXN4 HEK293 cells. Intact mitochondria, mitoplasts, or solubilised mitochondria were treated with or without proteinase K (PK). For carbonate extractions, mitochondria were solubilised in Na\textsubscript{2}CO\textsubscript{3} and separated into pellet (P) and supernatant (S) fractions through centrifugation. Samples were analysed by SDS-PAGE and immunoblotting. (B) Whole cell extracts from control, SFXN4\textsuperscript{KO}, SFXN4\textsuperscript{KO} + SFXN4\textsuperscript{HA}, and SFXN4\textsuperscript{KO} + SFXN4\textsuperscript{MYC} cells were analysed by SDS-PAGE and immunoblotting.
Jackson et al., 2021 Supplementary Figure 3

A

**SFXN4 coessentiality**

- **SFXN4**
- **NDUFAF1**
- **ACAD9**
- **ECSIT**

B

- **SFXN4 isoform 1** (Q6P4A7-1)
- **SFXN4 isoform 3** (Q6P4A7-3)

- Missing in isoform 3

- **TM domain**

C

**SFXN4 gene**

- **Exon**
  - 1 2 3 4 5 6 7 8 9 10 11 12 13 14

- **Isoform 1**
- **Isoform 3**

- **Original CRISPR**
- **New CRISPR**

- **Indels:**
  - c.[433dupA]
  - c.[435_443del]

- **Predicted protein impact**
  - p.[Met145Asnfs*16]
  - p.[Met145Phe148delinsIle]

- **Frequency/Depth**
  - 2/6
  - 4/6

- **Indels:**
  - c.[428_434del]
  - c.[429_433del]

- **Predicted protein impact**
  - p.[Ala143Glyfs*18]
  - p.[Tyr144Glyfs*15]

- **Frequency/Depth**
  - 3/6
  - 2/6
  - 1/6

D

- **Log2(ratio) SFXN4KO/control**
- **#1**
- **#2**
- **CI**
- **CII**
- **CIII**
- **CIV**
- **CV**

- ****** **** ns ns ns ns ns ns ** *"}

- **Flp-In™ T-REx™ all isoform SFXN4KO #1**
- **Flp-In™ T-REx™ all isoform SFXN4KO #2**

- **Log2(ratio) SFXN4/KO/control**

- **CI**
- **CII**
- **CIII**
- **CIV**
- **CV**

- **#1**
- **#2**

- ****** **** ns ns ns ns ns ns ** *"}
Supplementary Figure 3.

(A) Graph plotting interacting partners identified in the FLAG-SFXN4 immunoprecipitation experiment against genes that are coessential with SFXN4. The MCIA complex subunits NDUFAF1 and ECSIT (indicated in red) rank in the top 5 as both interacting proteins and coessential genes. (B) Schematic depiction of SFXN4 isoform 1 and SFXN4 isoform 3. SFXN4 isoform 3 arises by use of an alternate initiating methionine and lacks the first 116 amino acids of the canonical isoform. (C) Schematic depiction of the gene editing strategies used to target the SFXN4 gene. The new gRNA targeted exon 8, a region of the SFXN4 gene present in all theoretical isoforms. The new SFXN4KO cell lines were generated in the background of WT 293 Flp-In T-Rex cells. Indels from the two sequenced clones are indicated. (D) Mitochondria from control cells and the two new SFXN4KO clones were subjected to label-free quantitative mass spectrometric analysis (n=3 for #1, n=2 for #2). The relative abundance of respiratory chain complexes (Complexes I-V) in the SFXN4KO cells as compared to control were plotted. Mean ± 95% CI is depicted. * p<0.05, ** p<0.01, **** p<0.0001.
Supplementary Figure 4.

(A) Mitochondrial lysates from control cells, SFXN4KO cells, and SFXN4KO cells expressing untagged or N-terminally FLAG-tagged SFXN4 were analysed by SDS-PAGE and immunoblotting. (B) Mitochondria isolated from control cells, SFXN4KO cells, and SFXN4KO cells expressing untagged or N-terminally FLAG-tagged SFXN4 were solubilised in 1% digitonin containing buffer and analysed by BN-PAGE and immunoblotting. (C) Mitochondrial lysates from control, SFXN4KO, ACAD9KO, and NDUFAF1KO HEK293 cells were analysed by SDS-PAGE and immunoblotting. (D, E) Mitochondria isolated from control and SFXN4KO + FLAGSFXN4 HEK293 cells were solubilised in 1% digitonin and subjected to anti-FLAG immunoprecipitation. Eluates were analysed by BN-PAGE (D) or SDS-PAGE (E) and immunoblotting. (F) Mitochondria isolated from control and ACAD9KO + ACAD9FLAG HEK293 cells were solubilised in 1% digitonin and subjected to anti-FLAG immunoprecipitation. Eluates were analysed by SDS-PAGE and immunoblotting. I, input; E, elution.
Supplementary Figure 5.

Profile plots depict relative protein abundances normalised to the maximum value within each presented set, whereas heatmaps present protein iBAQ intensities normalised to the average intensities for each protein. (A). Complexome heatmap and corresponding profile plot of MCIA assembly factors and SFXN4, obtained from mitochondria isolated from SILAC-labelled wild-type and SFXN4\textsuperscript{KO} HEK Flp-In cells. Data presented represents a single complexome experiment. (B) Complexome heatmap and corresponding profile plot of MCIA factors and SFXN4, obtained from mitochondria isolated from wild-type 143B osteosarcoma cells and rho0 cells lacking mtDNA. Data acquired from Guerrero-Castillo et al., (2021). Data presented represents four independent complexome profiles.
Supplementary Figure 6.

Complexome migration profiles of complex I subunits and assembly factors, obtained from mitochondrial isolated from SILAC-labelled wild-type and SFXN4\textsuperscript{KO} HEK Flp-In cells. Proteins are arranged according to complex I modules as presented in Guerrero-Castillo et al., (2017). Protein iBAQ intensities are normalised by the average intensities for each protein. Data presented represents a single complexome experiment.
Jackson et al., 2021 Supplementary Figure 7

A

IB: SFXN4

50 µg/mL CAP (days) 0 1 2 3 4

IB: NDUFAF1

NDUFA9

SDHA

kDa

669 - 440 - 132 - 66 -

0 1 2 3 4

0 1 2 3 4

0 1 2 3 4

B

CAP release (h) - 0 1 2 4 8 24

IB: SFXN4

ACAD9

NDUFAF1

NDUFA9

SDHA

kDa

669 - 440 - 132 - 66 -

1 2 3 4 5

1 2 3 4 5

1 2 3 4 5

8 9 10 11 12 13 14

8 9 10 11 12 13 14

15 16 17 18 19 20

15 16 17 18 19 20

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

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Supplementary Figure 7.

(A) Mitochondria isolated from HEK Flp-In cells treated with 50 μg/mL chloramphenicol (CAP) for 0, 1, 2, 3 or 4 days were solubilised in 1% digitonin containing solubilisation buffer and analysed by BN-PAGE and immunoblotting.

(B) HEK cells were treated with 50 μg/mL CAP for 2 days and were harvested at the indicated timepoints following the removal of CAP. Mitochondria isolated from these cells as well as untreated cells were solubilised in 1% digitonin containing buffer and analysed by BN-PAGE and immunoblotting.
**A**

- **SFXN4KO** + **FLAG SFXN4**
- 2 day CAP treatment
- 0, 4, 8, 24 hour chase
- IP SFXN4
- SDS-PAGE and BN-PAGE

**B**

|          | Total (5%) | Elution |
|----------|------------|---------|
| Control  | 1-2-3-4-5  | 1-2-3-4 |
| SFXN4    | 6-7-8-9-10 | 6-7-8-9 |
| SFXN4KO  | 1-2-3-4-5  | 1-2-3-4 |
| FLAG     | 6-7-8-9-10 | 6-7-8-9 |

- IB: MT-ND6 (low) (15 kDa)
- IB: MT-ND6 (high) (15 kDa)
- IB: ACAD9 (64 kDa)
- IB: NDUFAF1 (32 kDa)
- IB: TIMMDC1 (29 kDa)
- IB: Mfn2 (86 kDa)

**C**

- IB: MT-ND6
- Total (10%)
- Elution

|          | Control | FLAG SFXN4 |
|----------|---------|------------|
| 0, 4, 8, 24 | 1-2-3-4-5 | 1-2-3-4 |

Jackson et al., 2021 Supplementary Figure 8
Supplementary Figure 8.

(A) Schematic depicting the workflow used in the chloramphenicol treatment + chase immunoprecipitation experiments. (B, C) Mitochondria isolated from control and SFXN4^KO + ^FLAG^ SFXN4 cells treated with 50 µg/mL CAP for 2 days and chased for various timepoints were solubilised in 1% digitonin and subjected to anti-FLAG immunoprecipitation. Eluates were analysed by SDS-PAGE (D) or BN-PAGE (E) and immunoblotting.
anti-FLAG immunoprecipitation + mass spectrometry analysis of eluate from both cell lines

### B

| Protein | Control #1 | Control #2 | SFXN4KO #1 | SFXN4KO #2 |
|---------|------------|------------|-------------|-------------|
| NDUFA9  |            |            | *           |             |
| NDUFAF1 |            |            |             |             |
| ACAD9 (l.e.) | | | | |
| ACAD9 (h.e.) | | | | |
| SDHA    |            |            |             |             |

### C

| Protein | Control #1 | Control #2 | SFXN4KO #1 | SFXN4KO #2 |
|---------|------------|------------|-------------|-------------|
| IB: ACAD9 (64 kDa) | | | | |
| IB: SFXN4 (36 kDa) | | | | |
| IB: NDUFA9 (37 kDa) | | | | |
| IB: NDUFA10 (38 kDa) | | | | |
| IB: COXIV (17 kDa) | | | | |
| IB: SDHA (72 kDa) | | | | |

### D

- **Depleted in SFXN4KO** assembly intermediates:
  - MTHFD1
  - NDUFS1
  - NDUFS6
  - MCCC2
  - PDK2
  - MT-ND6
  - NDUFA7
  - MTG1
  - ATP5D
  - GRIPEL1

- **Enriched in SFXN4KO** assembly intermediates:
  - NDUF53
  - NDUF52
  - NDUF51
  - TIMM17A
  - NDUF55
  - SLC25A24
  - NDUF58
  - NDUFV2
  - CHCHD2
  - SCO1

Jackson et al., 2021 Supplementary Figure 9
Supplementary Figure 9.

(A) Workflow for analysis of stalled MCIA containing complex I intermediates in SFXN4\(^{\text{KO}}\) cells. A SFXN4\(^{\text{KO}}\) cell line was generated in the background of an ACAD9\(^{\text{KO}}\) cell line complemented with ACAD9\(^{\text{FLAG}}\). Anti-FLAG immunoprecipitation was performed with mitochondria isolated from ACAD9\(^{\text{KO}}\) cells complemented with ACAD9\(^{\text{FLAG}}\) and SFXN4\(^{\text{KO}}\) ACAD9\(^{\text{KO}}\) cells complemented with ACAD9\(^{\text{FLAG}}\). Eluates were processed for and analysed by mass spectrometry.

(B) Mitochondria isolated from control cells (ACAD9\(^{\text{KO}}\) + ACAD9\(^{\text{FLAG}}\)) and two ACAD9\(^{\text{KO}}\) + ACAD9\(^{\text{FLAG}}\) SFXN4\(^{\text{KO}}\) clones were solubilised in 1% digitonin containing buffer and analysed by BN-PAGE and immunoblotting. 

(C) Mitochondrial lysates from control cells (ACAD9\(^{\text{KO}}\) + ACAD9\(^{\text{FLAG}}\)) and two ACAD9\(^{\text{KO}}\) + ACAD9\(^{\text{FLAG}}\) SFXN4\(^{\text{KO}}\) clones were analysed by SDS-PAGE and immunoblotting. Clone #2 (indicated in red) was used in the subsequent anti-FLAG immunoprecipitation mass spectrometry experiment.

(D) Anti-FLAG immunoprecipitation was performed using mitochondria isolated from control (ACAD9\(^{\text{KO}}\) + ACAD9\(^{\text{FLAG}}\)) and ACAD9 + ACAD9\(^{\text{FLAG}}\) SFXN4\(^{\text{KO}}\) cells. Eluates were analysed by label-free quantitative mass spectrometry. The volcano plot depicts the levels of ACAD9 interacting partners in SFXN4\(^{\text{KO}}\) cells relative to control cells (n=3). Proteins on the right of the volcano were more abundant in the ACAD9\(^{\text{FLAG}}\) immunoprecipitation from the SFXN4\(^{\text{KO}}\) cell line, and proteins on the left of the volcano were more abundant in the ACAD9\(^{\text{FLAG}}\) precipitation from the control cell line. Horizontal cut-off represents a p-value of 0.05, vertical cut-offs represent 1.5x
fold change. Proteins are colour-coded according to which complex I assembly module they belong.
A

Sequence Identity

|       | SFXN1 | SFXN2 | SFXN3 | SFXN4 | SFXN5 |
|-------|-------|-------|-------|-------|-------|
| SFXN1 | 100   | 54.83 | 76.32 | 22.51 | 37.66 |
| SFXN2 | 100   | 55.14 | 100   | 21.22 | 39.56 |
| SFXN3 | 100   | 55.14 | 100   | 21.29 | 37.78 |
| SFXN4 | 100   | 55.14 | 100   | 21.29 | 37.78 |
| SFXN5 | 100   | 55.14 | 100   | 21.29 | 37.78 |

Sequence Identity:

- < 30%
- > 30%
- > 50%
- > 70%
Supplementary Figure 10.

(A) Percent identity matrix generated by Clustal2.1. Sequence identity determined from number of identical residues between each query following multiple sequence alignment (MSA).
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