

## Supplementary Information

### Contents

1. Supplementary discussion
2. Supplementary Tables 1-4 (Identified cross-links)

### Supplementary discussion

#### *Core peripheral arm subunits*

The fold of all fourteen core subunits is well conserved from the bacterial version, with only slight shifts of subunits as rigid-bodies and minor shifts of helices and  $\beta$ -sheets in most subunits. These relative domain movements are larger in the peripheral arm as compared to the membrane domain. One significant difference in fold is found in the 75 kDa subunit in its less conserved region after residue 630 – whilst in *T. thermophilus* the C-terminus is folded into a  $\beta$ -barrel, the ovine enzyme has two short  $\alpha$ -helices instead.

The hydrophilic arm can be subdivided into two main modules<sup>27</sup>. The dehydrogenase (N-module) comprises the 51 kDa (containing FMN, the NADH binding site and the 4Fe-4S cluster N3 at the start of the redox chain), the 24 kDa (with the 2Fe-2S cluster N1a) and 75 kDa (with the 2Fe-2S cluster N1b and the 4Fe-4S clusters N4 and N5) subunits. The connecting (Q) module connects the N-module with the quinone-binding site and comprises TYKY (coordinating the 4Fe-4S clusters N6a and N6b), PSST (with the 4Fe-4S cluster N2) and two subunits with no cofactors, 30 and 49 kDa. Apart from four 4Fe-4S and two 2Fe-2S clusters coordinated in standard geometry by four cysteines, there are two unusual clusters in complex I, which may not be simple “stepping stones” in the chain. The coordination of the 4Fe-4S cluster N5 by three cysteines and a histidine is preserved in the ovine enzyme. It is separated from the next cluster (N6a) by 13.6 Å, the longest distance in the chain, and has a very low potential<sup>56</sup>. Thus, it represents a bottleneck in the pathway, likely to control the overall rate of electron transfer. The cluster is surrounded by charged and polar residues, some of which (Asp104) directly interact with the Zn<sup>2+</sup>-containing supernumerary 13 kDa subunit. Conserved Tyr86<sup>13-kDa</sup> from the zinc-binding domain interacts with the backbone of residues coordinating N5, so it is likely that the N5 potential depends on proper folding of 13 kDa subunit, which in its turn interacts tightly with the NADPH-containing 39 kDa subunit. It is

possible that the unusual coordination and environment of cluster N5 helps it to “sense” the redox state of downstream clusters and control electron transfer accordingly. Along with cluster N6a it may also respond to the redox status of the mitochondrial matrix via the 13 kDa subunit, as discussed in the main text.

Unique coordination of the terminal 4Fe-4S cluster N2, which donates electrons to quinone, by two consecutive “tandem” cysteines 54 and 55 is also preserved and may be responsible for the backbone flexibility around the cluster and pH dependence of the N2 redox potential. Helices connected to the cluster shift upon its reduction in *T. thermophilus*<sup>31</sup>, suggesting a possible link to conformationally-driven catalytic cycle. Conserved Arg85<sup>49-kDa</sup> is symmetrically dimethylated<sup>57</sup> and its guanidino group is found in direct contact with cluster N2. Therefore this post-translation modification, rendering the residue more hydrophobic, will clearly influence the N2 redox potential.

All edge-to-edge distances in the redox chain are within 14 Å, as required for electron transfer on physiological time scales<sup>58</sup>. The 2Fe-2S cluster N1a is off the main pathway (Fig. 2a) and is fully conserved, although its functional significance is not yet clear. It may prevent excessive reactive oxygen species (ROS) production at the flavin site<sup>1</sup>, stabilize the fold<sup>59</sup> or help to prime the proton pumping mechanism<sup>60</sup>.

### ***Core membrane domain subunits***

The largest core subunits in the membrane domain of complex I are three homologous 14TM antiporter-like subunits, ND2, ND4 and ND5. Uniquely in metazoans, the ND2 subunit lost three N-terminal TM helices and they are not replaced by any protein in our structure, similarly to the bovine enzyme<sup>6</sup>. Thus ND2 has 11 TM helices but we generally refer to the numbering of 14 conserved helices. Distal subunit ND5 contains a C-terminal extension with TM15 followed by amphipathic helix HL traversing most of the membrane domain and ending with TM16, stabilizing matrix side of the domain. The antiporter-like subunits contain two conserved symmetry-related 5TM domains, TM4–TM8 and TM9–TM13, respectively forming the N-terminal matrix-linked and C-terminal IMS-linked half-channels for proton translocation<sup>11</sup>. These half-channels are linked in the middle of the membrane by conserved charged residues, forming a single channel within each of the three antiporter-like subunits. The fourth likely channel (called the E-channel<sup>9</sup>) is formed in a similar fold but combined from smaller subunits, with ND3, ND6 and ND4L contributing the IMS-linked half-channel and

TM2-TM6 of ND1 contributing matrix-linked half-channel (Fig. 4a). These four channels probably account, one each, for four protons translocated per catalytic cycle<sup>1</sup>.

All of the membrane subunits preserve features key for proton translocation<sup>1,9,11</sup>. Extensive mid-membrane breaks/loops in TM helices 7, 8 and 12 of each antiporter like subunit lend flexibility to key protonatable residues: Lys<sup>TM7</sup> from the matrix half-channel, Lys/His<sup>TM8</sup> from the connection and Lys/Glu<sup>TM12</sup> from the IMS half-channel. Conserved Glu<sup>TM5</sup>'s are found in positions suitable for interaction both with Lys<sup>TM7</sup> from the same subunit and Lys/Glu<sup>TM12</sup> from the neighbour, as in bacteria<sup>9,11</sup>. Similar features in the fourth channel include conserved ND1 residues, Glu143 and Glu192 (near the end of short TM5) as possible Glu<sup>TM5</sup> and Lys<sup>TM7</sup> analogues, respectively. Conserved Tyr60 sits on the  $\pi$ -bulge in TM3<sup>ND6</sup> and interacts with essential Glu34<sup>ND4L</sup>. Highly conserved TM3<sup>ND6</sup> probably plays the antiporter's TM12 role in the fourth channel and is a hot-spot for pathological mutations<sup>1</sup>. These key residues are surrounded by conserved charged and polar residues, with cavities sufficient to hold many water molecules<sup>11</sup>, so that a continuous central hydrophilic flexible axis extends from the Q site all the way to the tip of the membrane domain<sup>9</sup> (Fig. 4a). Recent detailed analysis suggests that additional inputs into the antiporter channels are possible along the central TM8 and inter-subunit along Glu<sup>TM5</sup>, maximizing the protonation of central axis residues from the cytoplasm/matrix<sup>1</sup>. Consistent with multiple inputs from the matrix, core subunits on this side of the membrane domain are more conserved than on IMS side (data not shown).

### *Different conformations of the complex and the quinone-binding site*

Apart from the  $\beta 1$ - $\beta 2$ <sup>49-kDa</sup> loop, the Q cavity is lined by charged residues from the TMH5-6<sup>ND1</sup> loop and is also flanked by the TMH1-2<sup>ND3</sup> loop, which embraces PA/MD interface. PSST contains hydroxylated Arg77<sup>57</sup>, a very rare post-translational modification. Intriguingly, this arginine forms a partial constriction about halfway into Q site (Fig. 2d). TMH1-2<sup>ND3</sup> loop contains conserved Cys39<sup>ND3</sup>, which becomes accessible to chemical modification only in the D state of the enzyme<sup>61</sup>. From our structure it is not clear if Cys39<sup>ND3</sup> is accessible for modifications as it is partially occluded by the poly-alanine stretch of ND6 subunit. In the recent bovine enzyme study it was suggested that the class 2 structure represents the A state since the loops around the Q site are better ordered than in class 1 and Cys39<sup>ND3</sup> is inaccessible<sup>15</sup>. The conformation of loops around Q site in the ovine "closed" class is not clear due to lower overall resolution. It is difficult to relate bovine and ovine conformations as

instead of PA rotation (seen in bovine) we observe a hinge-like motion of the PA about the Q site in the “closed” class, accompanied by smaller shifts around the ND5 area (Extended Data Fig. 8). The complex thus is remarkably flexible but at the same time exists in defined states, suggesting that these states may be related to the conformational coupling cycle.

Disorder of the 4-TM helix subunit B14.7 in our structure leads to disorder of the nearby C-terminal half of traverse helix HL and TM16 from ND5, as well as TM4 from ND6. However, this area is well ordered in our supercomplex maps<sup>16</sup>, where B14.7 interacts extensively with complex III. This region, including B14.7, is also resolved in bovine CI maps, except for class 3 maps, which are disordered in this area, similarly to ovine classes<sup>15</sup>. Therefore B14.7 “clamps” on the C-terminus of ND5, stabilising the whole contact area. Apart from the better-ordered inter-complex contact areas, there appears to be no major difference in the conformation of ovine CI in the supercomplexes and in the isolated state, except that in the supercomplex it is more similar to the “closed” state. As the supercomplex is a more physiological environment for CI, it is likely that the “closed” state is more similar to the active conformation of the enzyme.

### ***Supernumerary subunits fold***

STMD subunits are defined by a single hydrophobic  $\alpha$ -helical region separating hydrophilic N- and C-terminal domains. Previously, due to the lack of clear tertiary structure motifs, they were particularly challenging to assign. Our cross-linking data indicate that the N-termini of all STMD subunits are located in the mitochondrial matrix. Along with the ACP/LYR pair (SDAP- $\beta$ /B22), the matrix “bulge” on the tip of the MD is formed from the highly intertwined matrix N-terminal domains of STMD subunits B15, ASHI, B12, AGGG, B17 and SGDH, which surround the tip with their TM helices, stabilizing this distal area. Unlike the other STMD subunits, which are all roughly perpendicular to the plane of the membrane, the TM helix of B17 is highly tilted,  $\sim 60^\circ$  relative to the plane of the membrane. The C-terminus of B17 in the IMS forms a short  $\beta$ -strand with the  $\beta$ -hairpin of ND5 generating a three-stranded anti-parallel  $\beta$ -sheet. The C-terminus of AGGG extends as a coil along the length of the long B18 helix. This coil contacts complex IV in the supercomplex structures<sup>16</sup>. B12 forms the very tip of MD, contributing the most distal TM helix and interacting with SDAP- $\beta$ /B22 pair in the matrix. B12 interacts extensively with complex IV in the supercomplex<sup>16</sup>.

The TM helix of SGDH binds on top of a lipid filled cleft between ND4 and ND5. At the C-terminus of the TM helix the polypeptide crosses under PDSW and then swings around it, completely wrapping PDSW before continuing along the IMS side of the complex as a long helix (Extended Data Fig. 7b). These two subunits are thus “locked” via their backbone and together they contain three long  $\alpha$ -helices traversing nearly entire intermembrane surface, strongly stabilizing this side of the membrane domain (Fig. 1c). PDSW contains four globally conserved cysteines, which can form two disulphide bonds further stabilizing the fold in the oxidizing environment of the IMS. Additionally Cys154 from the C-terminal  $\alpha$ -helix likely forms a disulphide with Cys112 of subunit ESSS (Extended Data Fig. 7f); both residues are conserved in mammals.

SGDH may be involved in assembly due to its backbone “interlock” with PDSW. This wrapped structure would require a very specific assembly sequence in which the TM portion of SGDH binds first, with the C-terminal IMS helix remaining extended and unbound to the complex. Next PDSW would have to bind on top of SGDH wrapping around it near the IMS side of its TM helix. Only then could the IMS helix of SGDH swing around PDSW and bind to the IMS surface of complex I. The first part of this assembly likely occurs in an assembly intermediate containing only the ND4 and ND5 core subunits<sup>62</sup> and the C-terminus of SGDH would not swing around and wrap PDSW until this intermediate is connected with the remainder of the membrane arm, allowing interaction between the C-terminus of SGDH and ND2, 15 kDa, ND3 and ND6 subunits.

Three subunits facing the IMS (PGIV, 15 kDa and B18) contain double C<sub>x</sub>C domains (CHCH domain), which stabilise helix-turn-helix structures with two disulphides<sup>63</sup>. Our structure is consistent with the presence of disulphides, processed by the Mia40/Erv1 disulphide relay<sup>64</sup>. The 15 kDa and PGIV subunits together add four shorter helices traversing the IMS side of the membrane domain in addition to the PDSW/SGDH helices. The main part of PGIV is a rigid structure formed by two CHCH domains that clamp the “heel” of the complex, locking subunit B16.6 in place. The C-terminal coil of PGIV reaches all the way to the ND2/ND4 interface, where it buries a C-terminal aromatic FFW motif (Extended Data Fig. 7e), connecting the “heel” to the middle of membrane domain. The rigid disulphide-rich interlocked helices of these subunits appear to replace the extended  $\beta$ -hairpin/helix motif ( $\beta$ H), which stabilizes this side of the domain in bacterial CI<sup>9</sup>. The  $\beta$ H element is almost lacking in

the mammalian enzyme, as the  $\beta$ -hairpin between TM2/3 is deleted from ND2 and is short in ND4.

In subunit B18 one of the helices from the CHCH motif is very long and protrudes conspicuously far out of the complex. This helix and the C-terminal PDSW helix project towards complex III (and IV in the case of B18) in the supercomplex<sup>16</sup>. Since both of these helices contain disulphides, this feature may allow for redox regulation of interactions in the supercomplexes, if the positions of helices change upon reduction of disulphides.

The 42 kDa subunit is a large globular protein, which preserves a nucleoside kinase family fold and is attached to the matrix side of ND2. Subunit B13 (bound to the core 30 kDa subunit) and the SDAP- $\alpha$ /B14 pair form PA extensions which approach the 42 kDa subunit, providing possible additional stabilizing contact between the peripheral and membrane arms. In the “closed” class conformation B13 moves about 3 Å closer to the 42 kDa subunit, allowing for direct contacts (Extended Data Fig. 8).

At the “heel” of the complex subunit MWFE forms extensive interactions in the membrane with ND1 as a helix anti-parallel to TM1<sup>ND1</sup> and in the IMS with PGIV, B16.6 and ND6. B16.6 spans the MD/PA interface and is identical to the cell death regulator GRIM19<sup>65</sup>. It contains a long N-terminal coil interacting with nearly the full extent of the Q-module, followed by an exceptionally long (65 residues)  $\alpha$ -helix traversing the membrane and ending with a loop and short helix interacting with several subunits on the IMS side, in particular the 15 kDa CHCH subunit. Among the TM subunits B16.6 extends furthest into the hydrophilic arm, stabilising the interaction between two arms of the complex. Other subunits stabilizing this interaction are B17.2 and B14.5a, both containing N-terminal amphipathic  $\alpha$ -helices, bound at the membrane interface. Subunits PSST, TYKY and B9 also contain such amphipathic helices, all located at the “back” of the complex (Fig. 1c), probably helping to ensure proper position of the hydrophilic arm over the lipid bilayer. The 39 kDa subunit may provide additional support for the peripheral arm, as it contains an exposed hydrophobic loop (residues 188-196, conserved in mammals) which can dip into lipid bilayer.

From the membrane surface B14.5a extends along a cleft between the 49 kDa, B17.2 and TYKY subunits away from the membrane. Once it reaches the interface of the Q- and N-modules the B14.5a coil turns nearly 90°, continues along the surface of the 49 kDa and 30 kDa core subunits and then turns again back towards membrane domain, binding in a cleft

between the 30 kDa, 49 kDa and B13 subunits. Thus, B14.5a wraps over nearly the entire Q-module (Extended Data Fig. 7a). B17.2 snakes with its C-terminus all the way towards the tip of the large C-terminal domain of the 75 kDa core subunit, bridging the Q-module and N-module. The assembly factor B17.2L is homologous to B17.2 and is known to interact with an assembly intermediate containing the Q-module and MD<sup>66</sup>. It has been proposed that during the final stage of complex I assembly, B17.2L is replaced by B17.2 and the N-module is connected to the Q-module<sup>67</sup>. It is interesting to note that the long C-terminal coil of B17.2, passes underneath the 13 kDa subunit, which is found in the N-module assembly intermediate<sup>67</sup>. The 13 kDa subunit also bridges the N-module and Q-module, with its C-terminal Zn<sup>2+</sup> containing domain bound to the N-module and its N-terminal domain bound to the Q-module. This arrangement suggests a sequence of events during assembly in which the C-terminus of B17.2 (bound at the Q-module via its N-terminal domain) reaches underneath the 13 kDa subunit (bound to the N-module via its C-terminal domain) to contact the N-module and then the N-terminal domain of the 13 kDa subunit “clamps” across the B17.2 coil to interact with the N-module subunits (e.g. 39 kDa subunit). This arrangement traps the C-terminal coil of B17.2 and would stabilize the interaction between the N-module and Q-module. On the opposite side of PA, the 18 kDa subunit plays a similar stabilizing role running nearly the entire length of the PA with a long C-terminal coil (Extended data Fig. 7c). In patients lacking either the 18 kDa or 13 kDa subunits, accumulation of the B17.2L-containing assembly intermediate lacking the N-module is observed<sup>66</sup>. The resulting CI deficiency highlights the importance of these subunits in CI assembly, stability and function.

The small globular subunit B8 adopts a thioredoxin fold<sup>68</sup> and binds at the interface of two sub-domains of the 75 kDa C-terminal domain. B8 is the only matrix subunit that contains a possible disulphide bond (Cys23-Cys57). The cysteines are likely to be reduced in the mitochondrial matrix and they are exposed in the complex, adjacent to interface with 75 kDa subunit. B8 may play a role as a sensor of oxidative damage as in patients with Parkinson’s disease B8 is lost from complex I and this loss is correlated with the accumulation of oxidative damage to the enzyme<sup>69</sup>.

At the tip of PA, the 10 kDa subunit binds along the surface of 24 kDa and 51 kDa core subunits mostly as an extended coil. At its C-terminus it reaches the 75 kDa core subunit and the supernumerary 18 kDa subunit. Given its position at the interface of the three N-module

core subunits and its extended structure the 10 kDa subunit is likely involved in the stabilization of this region of the complex.

### *Supernumerary subunits containing cofactors*

The 39 kDa subunit is wedged into the side of PA, interacting with many subunits (75 kDa, 30 kDa, 13 kDa, TYKY and PSST) near the interface with membrane. It belongs to the short-chain dehydrogenase/reductase family and contains tightly bound NADPH, which does not play a catalytic role<sup>70</sup>. The density for bound NADPH is clear (Fig. 3b). The nicotinamide ring is ~45 Å away from the nearest Fe-S cluster N2, precluding direct redox function. Conserved Arg50<sup>39-kDa</sup> from the Rossmann fold interacts with the phosphate on the 2'-position of adenosine (Fig. 3b), and when this arginine is mutated, NADPH is lost and complex is destabilized<sup>70</sup>. The same phosphate participates in strong hydrogen bonds with the guanidinium of Arg178<sup>PSST</sup>. This arginine is conserved in metazoans and in yeast and sits on the C-terminal helix directly connected to cluster N2. As this helix undergoes conformational change upon cluster reduction<sup>31</sup>, it is conceivable that such a structure allows the complex to sense the redox poise of the cell via the presence of NADP(H) and communicate it to the heart of its machinery at cluster N2. Additionally, the 39 kDa subunit forms extensive contacts with the key TMH1-2<sup>ND3</sup> loop and can be cross-linked to ND3 only in the D state of the enzyme<sup>71</sup>, hence it may be linked to the A/D transition.

There are two ACP copies in mammalian complex I, SDAP- $\alpha$  and - $\beta$ . ACPs in general play an essential role in fatty acid synthesis by carrying intermediates between enzymes participating in the synthesis of a growing acyl chain, attached to ACP via a phosphopantetheine moiety<sup>22</sup>. The acyl chain is provided to ACP partners by the entire chain “flipping” from the hydrophobic cavity between ACP helices into hydrophobic cavities in cognate enzymes<sup>22</sup>. We observe clear density for phosphopantetheine moieties in both copies of SDAP. In SDAP- $\alpha$  it is covalently attached to conserved Ser44<sup>SDAP</sup> and extends in the “flipped out” conformation far into the hydrophobic crevice between three helices of the SDAP- $\alpha$  partner protein, LYR motif containing subunit B14<sup>72</sup>. The density is clear for a 12-carbon acyl chain extension on the phosphopantetheine moiety (Fig. 3d), consistent with experimentally determined values of about 14 carbons<sup>17</sup>. Therefore SDAP- $\alpha$  is bound to B14 mostly due to this large buried hydrophobic surface. SDAP- $\alpha$  only interacts with complex I via B14, both subunits forming a subdomain of the hydrophilic arm pointing towards the 42 kDa



subunit. When the B14 analogue in *Y. lipolytica* is deleted or its LYR motif mutated, the SDAP- $\alpha$  analogue is also lost<sup>72</sup>.

Subunits B14 and B22 belong to the LYR (leucine/tyrosine/arginine) motif family of proteins, mostly found in mitochondria, acting as assembly factors for OXPHOS complexes and involved in Fe-S cluster biogenesis, often in partnership with ACPs<sup>23,72</sup>. Subunits B22 and SDAP- $\beta$  form part of the matrix “bulge” at the MD tip. SDAP- $\beta$  also interacts with B22 through a “flipped out” phosphopantetheine chain buried within the three-helix bundle of B22, although here there is no clear density for an acyl chain extension past the thiol. Thus, both pairs of ACP-LYR proteins are tightly linked via cofactors. In both cases, residues of the LYR motif are clearly involved in interactions with SDAP: Leu (residue 34 in B14 and 18 in B22) forms part of the hydrophobic cavity interacting with the acyl chain, Tyr (residue 35 and 19 respectively) stabilizes the interface between two LYR protein helices that interact with the SDAP helix harbouring the modified Ser44, and Arg (residue 36 and Lys20 respectively) forms a salt bridge with fully conserved Asp64 from SDAP.

To our knowledge, these are the first atomic structures of LYR/ACP protein complexes, revealing a clear structural role for the LYR motif. Previously it was suggested that the negatively-charged phosphate of phosphopantetheine interacts with arginine from the LYR motif<sup>23</sup>. We see that phosphate is mainly interacting with ACP itself, screening out the positive N-terminal dipole of the helix starting with Ser44. Instead, the interactions between ACP and LYR proteins are dependent on acyl chain “flipping” into the core of the LYR protein.

The B14 subunit interacts intimately with the TMH1-2<sup>ND3</sup> loop, which wraps around the Q site and changes conformation upon the A to D transition<sup>61</sup>, as noted above. This may explain why, when B14 is deleted, the complex becomes inactive even though still assembled<sup>23</sup>, as the deletion may lock the TMH1-2<sup>ND3</sup> loop in the D conformation. This suggests that complex I will not become active until fully assembled with the SDAP- $\alpha$ /B14 pair containing an extended acyl chain, possibly providing a regulatory link between fatty acid synthesis and OXPHOS activity in mitochondria. Nearby subunit B13 is also involved in A/D transitions<sup>73</sup>. On the other side of complex, SDAP- $\beta$ /B22 pair makes numerous close interactions with antiporter-like subunit ND5 and so could in principle regulate proton-pumping activity. For example, Leu63Pro<sup>B22</sup> mutation results in branchio-oto-renal syndrome in humans<sup>74</sup>. Leu63 is situated in one of the  $\alpha$ -helices forming a hydrophobic pocket accommodating acyl chain from phosphopantetheine. Proline will disrupt the helix and this pocket, weakening interactions

between B22 and SDAP- $\beta$ . B22 interweaves between at least five subunits at the tip of the membrane domain and so must be essential for proper assembly in this area.

The 13 kDa subunit is one of the three accessory subunits already found in  $\alpha$ -proteobacteria<sup>75</sup> and harbours a conserved Zn-binding motif, confirmed to bind Zn<sup>2+</sup> in *Y. lipolytica*<sup>25</sup>. We observe a clear density for a Zn<sup>2+</sup> coordinated by three conserved cysteines and a histidine (Fig. 3c). The subunit has an extended structure interacting mostly with TYKY but also with the 49 kDa, 75 kDa, B17.2 and 39 kDa subunits. In our structure Zn<sup>2+</sup> ion is closer to the Fe-S clusters than in *Y. lipolytica*, being within 13.4 Å to cluster N6a and 15.3 Å to cluster N5. The Zn<sup>2+</sup> ion is not redox active<sup>24</sup>, so even though this is within electron transfer distance, the presence of Zn is probably mostly important for the fold of 13 kDa subunit, although some influence on the potential of clusters N6a and N5 is also possible. The deletion of the 13 kDa analogue in *Y. lipolytica* resulted in loss of EPR signal for cluster N6a and concomitant loss of the B17.2 analogue<sup>25</sup>. The latter result can be explained by the fact that part of B17.2 interweaves “under” the 13 kDa subunit (see above discussion) locking B17.2 in place. Loss of cluster N6a likely occurs because, in contrast to the bacterial structure, in the absence of the 13 kDa subunit N6a is exposed to solvent (and so to oxidative damage).

### ***Assignment of supernumerary subunits***

In order to experimentally assign all supernumerary subunits several rounds of mass spectrometric identification of chemically cross-linked peptides (XL-MS) were carried out (Extended Data Fig. 5, Supplementary Tables 1-4). These data in conjunction with our high-resolution EM maps allowed us to unambiguously assign all supernumerary subunits.

Many of the previous assignments of supernumerary subunits were based on indirect biochemical data, secondary structure prediction and homology (B13, B15, B18, PDSW, 18 kDa, 15 kDa, B14.5b<sup>13</sup> and B17.2<sup>16</sup>), and thus, were tentative. Cross-links involving these subunits now provide experimental data confirming their positions. For example, STMD subunit B15 was tentatively assigned in the bovine structure to be associated with ND4 adjacent to helix HL based on its presence in subcomplex I $\beta$  (containing core subunits ND4 and ND5) and the presence of comparable density in *Y. lipolytica*, which harbours a homologue of B15 (NB5M)<sup>13</sup>. This assignment has now been experimentally verified by a cross-link between B15 and PDSW in the mitochondrial matrix. The assignment of subunits PDSW and B18 to the helical density in the IMS has been confirmed by cross-links between

PDSW, B15, ESSS and ASHI. The STMD subunit MNLL was identified by a cross-link to the B14.5b supernumerary subunit, containing two TMHs. MNLL binds to ND4 in the membrane near the interface with ND2.

STMD subunits SGDH, B17, B12 and ASHI are known components of subcomplex I $\beta$ <sup>17,32,76</sup>, so are likely to surround ND4 and ND5. The assignment of the long helix in the IMS to SGDH is supported by cross-links to B14.5b, PGIV and the 15 kDa subunit. After the helix ends the C-terminus of SGDH extends along the IMS side of the complex as a coil interacting with the 15 kDa subunit and reaching to ND3 and ND6. The short N-terminal region of SGDH interacts with both ND4 and ND5 in the matrix. Therefore SGDH spans nearly the entire length of the membrane arm of complex I from ND3 to ND5 (Fig. 1c, Extended Data Fig. 7b).

STMD subunit B17 has been identified in an assembly intermediate that lacks ND4 and ND5 but contains the ND6, ND3, ND4L and ND2 core subunits instead<sup>66,77</sup>. Our cross-linking data unambiguously place B17 adjacent to core subunit ND5. The N-terminus of B17 contains a  $\alpha$ -helix in the matrix that cross-links to the adjacent  $\alpha$ -helix of B22, which binds on the matrix side of ND5. Two cross-links between the N-terminus of B12 and SDAP- $\beta$  place B12 at the tip of the membrane arm, adjacent to AGGG.

Subunit ASHI has also been found in an assembly intermediate that lacks subunits ND4 and ND5<sup>78</sup>. Cross-links from the C-terminus of ASHI and the C-terminus of PDSW in the IMS place ASHI on the same side of the membrane arm as B15 and B14.7, claiming the last remaining unassigned TM helix density on this side of complex I. ASHI has a large N-terminal globular domain, and cross-links are observed between this N-terminal domain of ASHI and B15 and ESSS in the matrix. The density in this region is sufficient only for a poly-alanine model for the N-terminal domain of ASHI. The TM helix is partially disordered but a partial atomic model was build for the C-terminal half of the helix interacting with ND5. The C-terminus of ASHI in the IMS forms an extended coil, which nearly wraps around the long C-terminal helix of B18.

Previously there was ambiguity between the positions of B9 and MWFE, both STMD subunits known to associate with core subunit ND1. Cross-links between the N-terminus of PGIV and the C-terminus of MWFE and the N-terminus of TYKY and the N-terminus of B9 unambiguously place these subunits into the structure.

The 10 kDa subunit is known from biochemical studies to be part of subcomplex I $\lambda$ , which contains only the hydrophilic arm of the complex<sup>32,79</sup> and to be part of an assembly intermediate made up of the N-module<sup>66</sup>. Cross-links between the 10 kDa subunit and the 24 kDa and 51 kDa core subunits locate it to a cleft between those subunits. Supernumerary subunit B14.5a is also known to be a component of subcomplex I $\lambda$ <sup>32,79</sup>, but has not thus far been identified in any assembly intermediates. Cross-links of B14.5a with B17.2, B16.6, 75 kDa, 49 kDa, 30 kDa and B13 across its entire length (from Lys22 to Lys101) unambiguously place it in extended density along the surface of the Q-module.

In addition to these newly assigned subunits, the positions of large extensions to the previously assigned subunits were also identified by cross-linking. The C-terminus of PGIV extends as a coil along the IMS side of the matrix arm until it reaches the cleft between ND2 and ND4. This C-terminal extension is confirmed by cross-links between the C-terminus of PGIV, B14.5b and SGDHI (Extended Data Fig. 7e). The position of the long N-terminal coil of PDSW is confirmed by an intra-subunit cross-link between PDSW residues Lys6 and Lys126. The N-terminus of B15 contains a long  $\alpha$ -helix and its assignment is supported by a cross-link between the N-termini of B15 and adjacent ASHI. This B15 helix interacts with complex III in the supercomplex<sup>16</sup>.

## Supplementary Table 1.

### Summary of cross-linking experiments.

Cross-linker	Number of experiments	Detergent	Spacer length (Å)	Approx. max length (Å)	Total cross-links observed	Inter-subunit cross-links	Intra-subunit cross-links	False positive cross-links	False positive rate
<b>DSS</b>	4	DDM	11.4	23.4	174	82	67	25	0.14
<b>DSS</b>	2	LMNG	11.4	23.4	72	14	42	16	0.22
<b>DSS</b>	2	LDAO/DDM	11.4	23.4	72	37	20	15	0.21
<b>BS3</b>	1	DDM	11.4	23.4	25	10	10	5	0.20
<b>DSA</b>	1	DDM	8.9	20.9	14	8	4	2	0.14
<b>SDH</b>	1	DDM	13.5	25.4	28	19	5	4	0.14
<b>ADH</b>	1	DDM	11.1	23.0	26	12	6	8	0.31
Total Observed					411	182	153	75	0.18
Total unique					218	87	73	58	0.27

## Supplementary Table 2. Inter-subunit cross-links.

Solvent accessible surface distances (SAS) calculated using Xwalk<sup>55</sup> are shown. If the SAS distance is less than 8 Å over the approximate maximum cross-link length the distance is shown in red. These are considered true cross-links because of the flexibility of the side chains and were each checked manually in the structure. Residues in red are not modelled in the structure. Distances that could not be calculated because of unmodelled residues are shown as dashed lines.

Subunit 1	Residue	Subunit 2	Residue	Cross-linker	Euclidian Distance (Å)	SAS (Å)	Number of times observed	Best Score
51kDa_NDUFV1	Lys8	10kDa_NDUFV3	Lys6	DSS	-	-	1	39.92
51kDa_NDUFV1	Lys8	10kDa_NDUFV3	Lys17	DSS	-	-	1	32.05
51kDa_NDUFV1	Lys34	10kDa_NDUFV3	Lys6	DSS	-	-	2	40.70
51kDa_NDUFV1	Lys34	10kDa_NDUFV3	Lys17	DSS	-	-	2	37.83
51kDa_NDUFV1	Lys34	10kDa_NDUFV3	Lys22	DSS	-	-	2	30.38
51kDa_NDUFV1	Lys34	10kDa_NDUFV3	Lys23	DSS	-	-	3	39.59
51kDa_NDUFV1	Glu158	10kDa_NDUFV3	Glu31	SDH	-	-	1	28.79
51kDa_NDUFV1	Lys397	75kDa_NDUFS1	Lys87	DSS	20.8	29.0	2	31.34
51kDa_NDUFV1	Lys397	75kDa_NDUFS1	Lys88	DSS	10.1	12.3	1	31.68
24kDa_NDUFV2	Lys180	10kDa_NDUFV3	Lys17	DSS	-	-	1	36.92
24kDa_NDUFV2	Lys183	10kDa_NDUFV3	Lys23	DSS	-	-	1	28.87
75kDa_NDUFS1	Lys61	30kDa_NDUFS3	Lys223	DSS	-	-	3	40.12
75kDa_NDUFS1	Lys61	B14.5a_NDUFA7	Lys79	DSS	-	-	2	35.52
75kDa_NDUFS1	Glu297	B14_NDUFA6	Asp116	ADH	11.8	15.4	1	30.51
75kDa_NDUFS1	Glu297	39kDa_NDUFA9	Asp220	SDH	10.6	11.8	1	27.55
75kDa_NDUFS1	Asp486	B8_NDUFA2	Glu37	SDH	13.8	15.3	1	27.03
75kDa_NDUFS1	Asp504	B8_NDUFA2	Asp49	SDH/ADH	20.8	-	2	26.73
75kDa_NDUFS1	Lys516	B8_NDUFA2	Lys12	DSS	-	-	1	30.00
75kDa_NDUFS1	Lys598	B14_NDUFA6	Lys115	DSS	16.5	19.3	1	29.43
75kDa_NDUFS1	Glu624	B8_NDUFA2	Asp59	SDH/ADH	9.2	12.3	2	43.52
75kDa_NDUFS1	Lys650	B8_NDUFA2	Lys45	DSS	13.1	15.1	1	29.43

49kDa_NDUFS2	Lys334	B16.6_NDUFA13	Lys6	DSS	11.0	17.3	1	31.00
49kDa_NDUFS2	Lys334	B14.5a_NDUFA7	Lys39	DSS/BS3/DSA	9.8	9.9	7	33.87
49kDa_NDUFS2	Lys404	B14.5a_NDUFA7	Lys79	DSS	-	-	1	25.63
30kDa_NDUFS3	Lys20	B14.5a_NDUFA7	Lys79	BS3	-	-	1	37.58
30kDa_NDUFS3	Lys20	B14.5a_NDUFA7	Lys92	DSS/BS3	13.7	16.3	5	45.74
30kDa_NDUFS3	Glu158	B14_NDUFA6	Glu108	ADH	18.4	24.0	1	28.42
30kDa_NDUFS3	Glu158	B14_NDUFA6	Glu110	SDH/ADH	12.0	12.1	1	30.63
30kDa_NDUFS3	Asp178	PSST_NDUFS7	Glu109	SDH	12.6	14.4	1	34.49
30kDa_NDUFS3	Glu189	B14_NDUFA6	Glu108	SDH	22.0	35.4	2	35.35
30kDa_NDUFS3	Lys196	18kDa_NDUFS4	Lys56	DSS	9.0	10.4	1	31.17
30kDa_NDUFS3	Lys223	B13_NDUFA5	Lys45	DSS	-	-	1	29.47
30kDa_NDUFS3	Lys223	18kDa_NDUFS4	Lys53	DSS	-	-	1	31.96
30kDa_NDUFS3	Lys223	B14.5a_NDUFA7	Lys79	DSS	-	-	3	33.18
30kDa_NDUFS3	Lys224	18kDa_NDUFS4	Lys53	DSS	-	-	2	31.17
30kDa_NDUFS3	Lys224	18kDa_NDUFS4	Lys112	DSS	-	-	2	29.62
PSST_NDUFS7	Lys17	39kDa_NDUFA9	Lys78	DSS	-	-	2	30.05
PSST_NDUFS7	Lys17	39kDa_NDUFA9	Lys144	DSS	-	-	2	33.47
TYKY_NDUFS8	Lys15	B9_NDUFA3	Lys9	DSS	11.1	15.4	1	30.62
TYKY_NDUFS8	Lys54	B17.2_NDUFA12	Lys43	DSS	19.4	19.9	1	29.83
18kDa_NDUFS4	Lys31	B14_NDUFA6	Lys68	DSS	11.1	13.0	1	27.97
18kDa_NDUFS4	Lys31	B14_NDUFA6	Lys120	DSS	12.1	12.9	1	33.32
15kDa_NDUFS5	Lys53	SGDH_NDUFB5	Lys117	DSS	8.9	10.9	1	30.45
15kDa_NDUFS5	Glu101	B16.6_NDUFA13	Glu91	SDH/ADH	-	-	2	25.99
15kDa_NDUFS5	Asp102	B16.6_NDUFA13	Glu88	SDH	-	-	1	29.29
15kDa_NDUFS5	Asp102	B16.6_NDUFA13	Glu91	SDH	-	-	1	28.28
13kDa_NDUFS6	Asp22	39kDa_NDUFA9	Glu162	ADH	23.6	27.4	1	32.67
MWFE_NDUFA1	Lys64	PGIV_NDUFA8	Lys18	DSS/BS3/DSA	7.9	11.9	6	35.25
B13_NDUFA5	Lys39	B14.5a_NDUFA7	Lys79	DSS	-	-	2	37.96
B13_NDUFA5	Lys45	B14.5a_NDUFA7	Lys79	DSS/BS3/DSA	-	-	7	44.62
B13_NDUFA5	Lys45	B14.5a_NDUFA7	Lys91	DSS	10.5	11.2	2	48.38
B13_NDUFA5	Lys45	B14.5a_NDUFA7	Lys92	DSS/BS3/DSA	14.7	21.2	7	45.83
B13_NDUFA5	Lys65	B14.5a_NDUFA7	Lys101	DSS	16.8	18.0	1	29.78
B14.5a_NDUFA7	Lys22	B17.2_NDUFA12	Lys53	DSS	16.3	19.2	1	43.39
B14.5a_NDUFA7	Lys32	B17.2_NDUFA12	Lys101	DSS	11.9	13.0	1	26.08
B14.5a_NDUFA7	Lys32	B17.2_NDUFA12	Lys43	DSS	19.3	25.5	3	37.85

B14.5a_NDUFA7	Lys32	B17.2_NDUFA12	Lys53	DSS	7.0	8.2	1	36.39
B14.5a_NDUFA7	Lys39	B16.6_NDUFA13	Lys6	DSS	10.9	13.3	1	42.73
PGIV_NDUFA8	Glu5	B16.6_NDUFA13	Glu91	ADH	15.8	18.3	1	30.86
PGIV_NDUFA8	Glu10	B16.6_NDUFA13	Glu91	ADH	10.8	12.6	1	34.43
PGIV_NDUFA8	Lys18	B16.6_NDUFA13	Lys78	DSS	19.4	24.9	1	35.3
PGIV_NDUFA8	Glu148	B14.5b_NDUFC2	Asp16	SDH/ADH	18.5	24.5	2	39.9
PGIV_NDUFA8	Glu148	B14.5b_NDUFC2	Glu99	ADH	14.4	16.1	1	32.81
PGIV_NDUFA8	Glu152	B14.5b_NDUFC2	Glu17	SDH/ADH	11.1	12.6	2	36.13
PGIV_NDUFA8	Glu152	B14.5b_NDUFC2	Asp100	SDH/ADH	21.7	29.4	2	36.24
PGIV_NDUFA8	Asp156	B14.5b_NDUFC2	Glu17	SDH/ADH	7.0	7.2	2	34.99
PGIV_NDUFA8	Lys158	B14.5b_NDUFC2	Lys95	DSS	12.7	13.5	3	44.15
PGIV_NDUFA8	Lys158	B14.5b_NDUFC2	Lys104	DSS	27.5	29.5	1	38.98
PGIV_NDUFA8	Lys158	SGDH_NDUFB5	Lys106	DSS	13.6	13.9	5	46.33
42kDa_NDUFA10	Lys87	ESSS_NDUFB11	Lys17	DSS	-	-	3	33.42
MNLL_NDUFB1	Lys29	B14.5b_NDUFC2	Lys25	DSS	11.9	12.2	1	23.68
B12_NDUFB3	Lys12	SDAP_NDUFAB1	Lys20	DSS	-	-	2	32.79
B12_NDUFB3	Lys19	SDAP_NDUFAB1	Lys20	DSS	6.4	6.9	1	36.87
B15_NDUFB4	Lys30	ASHI_NDUFB8	Lys25	DSS/BS3/DSA	22.7	26.5	6	39.21
B15_NDUFB4	Lys119	PDSW_NDUFB10	Lys170	DSS/BS3/DSA	26.7	30.7	7	38.09
SGDH_NDUFB5	Lys106	B14.5b_NDUFC2	Lys95	DSS	12.6	14.6	3	37.89
SGDH_NDUFB5	Lys106	B14.5b_NDUFC2	Lys104	DSS	14.3	15.8	8	44.04
SGDH_NDUFB5	Lys106	B14.5b_NDUFC2	Lys106	DSS	11.4	12.1	2	39.56
SGDH_NDUFB5	Lys106	B14.5b_NDUFC2	Lys107	DSS	19	21.1	2	33.65
B17_NDUFB6	Lys23	B22_NDUFB9	Lys120	DSS	10.6	13.3	1	31.11
ASHI_NDUFB8	Lys25	ESSS_NDUFB11	Lys17	DSS	-	-	6	34.91
ASHI_NDUFB8	Lys49	ESSS_NDUFB11	Lys17	DSS/BS3/DSA	-	-	1	27.74
ASHI_NDUFB8	Lys148	PDSW_NDUFB10	Lys126	DSS	-	-	1	31.02
ASHI_NDUFB8	Lys148	PDSW_NDUFB10	Lys167	DSS	-	-	1	29.02
ASHI_NDUFB8	Lys148	PDSW_NDUFB10	Lys170	DSS	-	-	2	32.55
B22_NDUFB9	Lys137	ESSS_NDUFB11	Lys17	DSS/BS3/DSA	-	-	6	36.24
PDSW_NDUFB10	Glu165	ESSS_NDUFB11	Glu109	SDH	17.1	19.1	1	32.16



### Supplementary Table 3. Intra-subunit cross-links.

Solvent accessible surface distances (SAS) calculated using Xwalk<sup>55</sup> are shown. If the SAS distance is less than 8 Å over the approximate maximum cross-link length the distance is shown in red. These are considered true cross-links because of the flexibility of the side chains and were each checked manually in the structure. Residues in red are not modelled in the structure. Distances that could not be calculated because of unmodelled residues are shown as dashed lines.

Subunit 1	Residue	Subunit 2	Residue	Cross-linker	Euclidian Distance (Å)	SAS (Å)	Number of times observed	Best Score
51kDa_NDUFV1	Lys16	51kDa_NDUFV1	Lys8	DSS	8.7	10.5	2	38.06
51kDa_NDUFV1	Lys61	51kDa_NDUFV1	Lys78	DSS	13.6	24.7	1	30.07
51kDa_NDUFV1	Lys61	51kDa_NDUFV1	Lys84	DSS	15.0	17.9	4	46.78
24kDa_NDUFV2	Lys29	24kDa_NDUFV2	Lys36	DSS	6.7	7.7	2	35.59
24kDa_NDUFV2	Glu40	24kDa_NDUFV2	Glu32	SDH	11.8	11.8	1	38.17
24kDa_NDUFV2	Lys122	24kDa_NDUFV2	Lys167	DSS	17.2	21.9	1	28.76
75kDa_NDUFS1	Lys61	75kDa_NDUFS1	Lys75	DSS	17.5	19.7	3	32.37
75kDa_NDUFS1	Lys61	75kDa_NDUFS1	Lys85	DSS	11.4	11.6	1	35.52
75kDa_NDUFS1	Lys64	75kDa_NDUFS1	Lys85	DSS	12.0	18.9	1	32.99
75kDa_NDUFS1	Lys275	75kDa_NDUFS1	Lys686	DSS	12.9	13.1	1	27.89
75kDa_NDUFS1	Lys508	75kDa_NDUFS1	Lys516	DSS	8.1	8.1	7	38.03
49kDa_NDUFS2	Glu302	49kDa_NDUFS2	Glu227	ADH	16.6	24.4	1	32.51
30kDa_NDUFS3	Asp178	30kDa_NDUFS3	Glu189	SDH/ADH	22.8	-	1	30.87
30kDa_NDUFS3	Glu179	30kDa_NDUFS3	Glu189	SDH	17.9	32.3	1	29.17
30kDa_NDUFS3	Lys217	30kDa_NDUFS3	Lys223	DSS	-	-	2	42.78
PSST_NDUFS7	Lys17	PSST_NDUFS7	Lys32	DSS	12.1	18.2	2	36.22
PSST_NDUFS7	Lys17	PSST_NDUFS7	Lys102	DSS	-	-	1	25.57
10kDa_NDUFV3	Lys6	10kDa_NDUFV3	Lys17	DSS	-	-	2	34.46
10kDa_NDUFV3	Lys6	10kDa_NDUFV3	Lys23	DSS	-	-	1	25.17
10kDa_NDUFV3	Lys16	10kDa_NDUFV3	Lys23	DSS	-	-	1	26.12
18kDa_NDUFS4	Glu15	18kDa_NDUFS4	Asp91	ADH	23.5	26.1	1	35.81
18kDa_NDUFS4	Lys53	18kDa_NDUFS4	Lys109	DSS	18.7	27.4	1	38.56
18kDa_NDUFS4	Lys112	18kDa_NDUFS4	Lys126	DSS	17.0	18.4	1	33.81

18kDa_NDUFS4	Lys114	18kDa_NDUFS4	Lys126	DSS	16.9	19.5	2	31.85
15kDa_NDUFS5	Lys79	15kDa_NDUFS5	Lys84	DSS	15.6	25.3	1	32.98
15kDa_NDUFS5	Lys79	15kDa_NDUFS5	Lys87	DSS	19.3	23.1	3	40.95
15kDa_NDUFS5	Lys79	15kDa_NDUFS5	Lys90	DSS	22.9	30.0	1	37.70
13kDa_NDUFS6	Glu9	13kDa_NDUFS6	Asp19	ADH	10.5	26.9	1	24.92
13kDa_NDUFS6	Lys10	13kDa_NDUFS6	Lys33	DSS	6.5	7.4	1	28.36
B8_NDUFA2	Lys12	B8_NDUFA2	Lys74	DSS/BS3	-	-	3	38.11
B8_NDUFA2	Lys12	B8_NDUFA2	Lys97	DSS/BS3/DSA	-	-	5	32.69
B8_NDUFA2	Lys38	B8_NDUFA2	Lys45	DSS/BS3	15.2	15.7	5	42.23
B8_NDUFA2	Glu42	B8_NDUFA2	Glu91	SDH/ADH	10.1	11.4	2	36.53
B8_NDUFA2	Lys74	B8_NDUFA2	Lys97	DSS/BS3	-	-	8	42.40
B13_NDUFA5	Lys6	B13_NDUFA5	Lys65	DSS	11.6	13.2	2	30.26
B13_NDUFA5	Lys24	B13_NDUFA5	Lys59	DSS/BS3/DSA	5.4	5.4	9	44.79
B13_NDUFA5	Lys39	B13_NDUFA5	Lys45	DSS	6.7	7.1	3	34.82
B14_NDUFA6	Lys56	B14_NDUFA6	Lys68	DSS	20.7	25.2	1	35.17
B14_NDUFA6	Lys67	B14_NDUFA6	Lys84	DSS	21.0	26.5	1	29.51
B14_NDUFA6	Lys68	B14_NDUFA6	Lys84	DSS	10.6	11.6	1	30.39
B14_NDUFA6	Lys84	B14_NDUFA6	Lys120	DSS	26.7	31.2	1	28.85
B14.5a_NDUFA7	Lys22	B14.5a_NDUFA7	Lys32	DSS/BS3/DSA	20.2	23.0	6	34.86
B14.5a_NDUFA7	Lys32	B14.5a_NDUFA7	Lys39	DSS	23.6	26.6	3	34.08
B14.5a_NDUFA7	Lys79	B14.5a_NDUFA7	Lys92	DSS	-	-	1	39.81
PGIV_NDUFA8	Lys40	PGIV_NDUFA8	Lys50	DSS	17.7	-	1	29.26
PGIV_NDUFA8	Lys40	PGIV_NDUFA8	Lys131	DSS	12.8	-	1	32.48
PGIV_NDUFA8	Glu138	PGIV_NDUFA8	Glu152	ADH	26.2	28.1	1	42.97
39kDa_NDUFA9	Lys78	39kDa_NDUFA9	Lys122	DSS	11.3	24.9	2	38.48
39kDa_NDUFA9	Asp79	39kDa_NDUFA9	Glu86	SDH	12.6	15.5	1	34.29
39kDa_NDUFA9	Lys140	39kDa_NDUFA9	Lys149	DSS	16.5	-	1	31.25
39kDa_NDUFA9	Lys140	39kDa_NDUFA9	Lys154	DSS/BS3	11.0	13.5	8	38.22
39kDa_NDUFA9	Lys144	39kDa_NDUFA9	Lys149	DSS	13.8	-	3	38.39
39kDa_NDUFA9	Lys149	39kDa_NDUFA9	Lys283	BS3	14.2	20.7	1	41.33
42kDa_NDUFA10	Lys35	42kDa_NDUFA10	Lys185	DSS	20.2	25.5	1	34.62
42kDa_NDUFA10	Lys35	42kDa_NDUFA10	Lys186	DSS	14.3	16.1	1	41.18
42kDa_NDUFA10	Lys37	42kDa_NDUFA10	Lys45	DSS	16.2	19.8	2	36.39
42kDa_NDUFA10	Lys37	42kDa_NDUFA10	Lys185	DSS	14.4	15.8	1	28.54
42kDa_NDUFA10	Lys37	42kDa_NDUFA10	Lys186	DSS	10.4	11.4	3	37.80

42kDa_NDUFA10	Lys40	42kDa_NDUFA10	Lys49	DSS	12.9	16.9	2	37.25
42kDa_NDUFA10	Lys40	42kDa_NDUFA10	Lys186	DSS/BS3	16.8	18.9	7	43.10
42kDa_NDUFA10	Lys87	42kDa_NDUFA10	Lys193	DSS	18.1	21.0	1	40.29
42kDa_NDUFA10	Lys245	42kDa_NDUFA10	Lys255	DSS	20.4	-	1	25.53
B15_NDUFB4	Lys113	B15_NDUFB4	Lys119	DSS	18.6	22.9	2	31.92
B18_NDUFB7	Lys111	B18_NDUFB7	Lys123	DSS	-	-	1	25.82
B18_NDUFB7	Lys112	B18_NDUFB7	Lys123	DSS	-	-	1	27.64
ASHI_NDUFB8	Lys6	ASHI_NDUFB8	Lys25	DSS	19.9	35.5	5	34.09
ASHI_NDUFB8	Lys6	ASHI_NDUFB8	Lys26	DSS	16.0	20.5	1	31.32
B22_NDUFB9	Lys51	B22_NDUFB9	Lys58	DSS	19.1	26.3	1	38.30
PDSW_NDUFB10	Lys6	PDSW_NDUFB10	Lys126	DSS	15.8	18.5	1	32.69
B17.2_NDUFA12	Lys43	B17.2_NDUFA12	Lys101	DSS	13.5	22.6	1	32.59
B17.2_NDUFA12	Lys43	B17.2_NDUFA12	Lys107	DSS	22.5	25.9	1	30.15
SDAP_NDUFAB1	Lys12	SDAP_NDUFAB1	Lys20	DSS	11.7	11.7	1	37.22
B14.5b_NDUFC2	Lys104	B14.5b_NDUFC2	Lys107	DSS	6.2	7.6	1	26.21

### Supplementary Table 4. False positive cross-links.

False positive cross-links are too far apart in the structure and likely result from inter-complex cross-linking.

Subunit 1	Residue	Subunit 2	Residue	Cross-linker	Euclidian Distance (Å)	SAS (Å)	Number of times observed	Best Score
51kDa_NDUFV1	Lys34	PSST_NDUFS7	Lys102	DSS	128	-	1	26.57
51kDa_NDUFV1	Lys202	75kDa_NDUFS1	Lys508	DSS	63.4	-	1	26.55
51kDa_NDUFV1	Lys202	15kDa_NDUFS5	Lys70	DSS	158	-	1	22.78
24kDa_NDUFV2	Glu40	B17_NDUFB6	Glu16	ADH	180	-	1	35.50
75kDa_NDUFS1	Glu138	B13_NDUFA5	Glu53	ADH	88.3	-	1	28.33
75kDa_NDUFS1	Asp333	39kDa_NDUFA9	Asp79	ADH	62.1	-	1	25.34
75kDa_NDUFS1	Asp504	75kDa_NDUFS1	Asp536	SDH	27.9	-	1	24.94
49kDa_NDUFS2	Lys334	PGIV_NDUFA8	Lys131	DSS	128.2	-	1	28.86
49kDa_NDUFS2	Lys339	30kDa_NDUFS3	Lys224	DSS	-	-	1	27.75
30kDa_NDUFS3	Glu179	PSST_NDUFS7	Glu109	SDH	20.9	-	1	24.80
PSST_NDUFS7	Lys102	MWFE_NDUFA1	Lys64	DSS	83.1	-	1	28.29
PSST_NDUFS7	Lys102	B17.2_NDUFA12	Lys43	DSS	58.2	-	1	41.53
PSST_NDUFS7	Glu170	B17.2_NDUFA12	Asp32	ADH	28.6	59.7	1	24.94
TYKY_NDUFS8	Lys3	B14.5a_NDUFA7	Lys101	DSS	25.1	42.5	2	33.76
TYKY_NDUFS8	Lys15	B13_NDUFA5	Lys65	DSS	33.9	37.1	1	24.47
TYKY_NDUFS8	Lys15	15kDa_NDUFS5	Lys87	DSS	103.6	-	1	29.19
10kDa_NDUFV3	Lys9	SGDH_NDUFB5	Lys23	BS3	-	-	1	26.92
10kDa_NDUFV3	Lys9	39kDa_NDUFA9	Lys144	DSA	-	-	1	22.12
10kDa_NDUFV3	Lys17	B8_NDUFA2	Lys12	BS3	-	-	1	22.08
10kDa_NDUFV3	Lys17	42kDa_NDUFA10	Lys35	DSS	-	-	1	24.84
10kDa_NDUFV3	Lys22	42kDa_NDUFA10	Lys35	DSS	-	-	2	25.03
10kDa_NDUFV3	Lys23	B17_NDUFB6	Lys48	DSS	-	-	1	25.44
10kDa_NDUFV3	Lys23	B14_NDUFA6	Lys68	BS3	-	-	1	25.75
18kDa_NDUFS4	Asp14	18kDa_NDUFS4	Glu97	SDH/ADH	24.5	36.4	2	39.93
18kDa_NDUFS4	Lys34	39kDa_NDUFA9	Lys154	DSS	61.2	-	1	29.40
18kDa_NDUFS4	Lys34	18kDa_NDUFS4	Lys112	DSS	30.9	37.6	1	36.71
18kDa_NDUFS4	Lys56	39kDa_NDUFA9	Lys154	DSS	72.6	-	3	43.30
15kDa_NDUFS5	Lys53	SGDH_NDUFB5	Lys138	DSS	30.9	52.5	1	31.50

15kDa_NDUFS5	Lys53	PGIV_NDUFA8	Lys158	DSS	34.6	42.1	2	28.19
15kDa_NDUFS5	Lys70	ND2	Lys314	DSS	-	-	1	27.29
13kDa_NDUFS6	Lys10	B14.5a_NDUFA7	Lys32	DSS	46.7	57.7	1	26.55
B13_NDUFA5	Lys24	PDSW_NDUFB10	Lys157	BS3	137.2	-	1	22.26
B14.5a_NDUFA7	Lys22	B14.5a_NDUFA7	Lys39	DSS	39.7	45.2	1	27.66
B14.5a_NDUFA7	Lys22	PGIV_NDUFA8	Lys101	DSS	56.1	-	1	33.43
B14.5a_NDUFA7	Lys22	B17.2_NDUFA12	Lys43	DSS/BS3	33.0	41.5	3	43.91
B14.5a_NDUFA7	Lys32	B16.6_NDUFA13	Lys6	DSS	27.7	35.5	3	38.30
B14.5a_NDUFA7	Lys91	B14.5a_NDUFA7	Lys101	DSS	29.1	33.5	2	35.18
PGIV_NDUFA8	Lys18	B22_NDUFB9	Lys51	DSS	172.6	-	2	25.33
PGIV_NDUFA8	Lys18	B22_NDUFB9	Lys54	DSS	169.9	-	1	28.95
PGIV_NDUFA8	Lys74	42kDa_NDUFA10	Lys233	DSS	126.4	-	1	26.33
PGIV_NDUFA8	Lys101	B17.2_NDUFA12	Lys43	DSS	75.0	-	2	30.23
PGIV_NDUFA8	Glu148	B14.5b_NDUFC2	Glu17	ADH	26	32.4	1	36.65
PGIV_NDUFA8	Asp156	B14.5b_NDUFC2	Asp100	SDH	26.1	35.5	1	30.71
39kDa_NDUFA9	Lys154	B14.5b_NDUFC2	Lys95	DSS	134.8	-	1	44.16
39kDa_NDUFA9	Lys154	B15_NDUFB4	Lys119	DSS	145.7	-	1	27.11
39kDa_NDUFA9	Lys154	B22_NDUFB9	Lys58	DSS	129.0	-	1	25.64
42kDa_NDUFA10	Lys37	ASHI_NDUFB8	Lys25	DSS/DSA	79.8	-	2	27.33
42kDa_NDUFA10	Lys87	ASHI_NDUFB8	Lys25	DSS	49.2	-	1	30.69
42kDa_NDUFA10	Lys146	SDAP_NDUFAB1	Lys38	DSS	38.6	42.7	1	33.43
42kDa_NDUFA10	Glu216	PDSW_NDUFB10	Glu165	SDH	116	-	1	25.50
B15_NDUFB4	Lys30	ASHI_NDUFB8	Lys6	DSS	39.7	50.8	2	31.92
B15_NDUFB4	Lys30	ASHI_NDUFB8	Lys15	DSS	34.1	37.6	2	33.07
B15_NDUFB4	Lys30	B22_NDUFB9	Lys137	DSS	36.6	42.3	1	32.09
B18_NDUFB7	Asp120	PDSW_NDUFB10	Glu165	ADH	60.8	-	1	24.78
ASHI_NDUFB8	Lys15	ASHI_NDUFB8	Lys25	DSS	26.2	38.1	2	34.08
ASHI_NDUFB8	Lys25	B22_NDUFB9	Lys54	DSS	40.2	44.3	1	28.98
ASHI_NDUFB8	Lys26	B14.5b_NDUFC2	Lys106	DSS	82.0	-	1	23.82
B14.5b_NDUFC2	Lys107	B22_NDUFB9	Lys111	DSS	94.7	-	1	22.32