Structure and Biogenesis of Membrane Proteins

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Abstract

Membrane proteins make up about one-third of the cellular proteome. The diverse roles that membrane proteins have in cells include major life-sustaining processes, making them major drug targets. The respiratory chain comprises a series of complexes of membrane proteins residing in the inner mitochondrial membrane, which serve as major drivers of ATP synthesis. Assembly of the respiratory chain complexes (RCC) requires coordinated synthesis of nuclear and mitochondrial subunits. Cbp3-Cbp6 complex binds to the mitoribosome as translational activator for cytochrome b synthesis and binds the nascent polypeptide to facilitate its hemylation. Cbp3 consists of an N-terminal domain specific to mitochondrial homologues and a conserved C-terminal ubiquinol-cytochrome c chaperone domain. In this thesis I present the first crystal structure of the C-terminal domain from a bacterial homologue that has enabled us to identify the interaction sites of yeast Cbp3 with Cbp6 and cytochrome b using site-specific photo-crosslinking. Our finding suggests that Cbp3 contacts the mitoribosome via the N-terminal domain in a manner that positions the substrate binding site close to the tunnel exit. In the second project, we have analyzed the effects of disease causing cytochrome b mutations, on bc₁ complex assembly. We found that complex III assembly is blocked at either intermediate 0 or I due to impaired insertion of b₁ or b₇ heme respectively, which indicates that assembly processes are involved in disease development. We then focused on NADH, a product of alpha-ketoglutarate dehydrogenase complex (KGDH) catalyzed citric acid cycle reaction and one of the substrates that supply electron to the respiratory chain. Kgd4 is a novel subunit of this enzyme complex and two functional variants (Kgd4S and Kgd4L) of unknown origins exist in yeast. We report in our work that Kgd4L originates from a UUG alternative start site, 90 nucleotides upstream and in frame of the annotated start codon. The sequence context upstream of UUG determines the efficiency of recognition of this alternative start codon. Finally, Na⁺/H⁺ antiporters are present in all species and are involved in regulation of intracellular pH, cell volume and sodium concentration. ATP formed during oxidative phosphorylation serves as energy source for Na⁺/K⁺ ATPase to generate Na⁺ gradient across the inner mitochondrial membrane, which drives local Na⁺/H⁺ antiporters. We show that K305 is involved in proton transport and responsible for the electrogenicity of NapA, while human NHA2 shows electroneutral antiporter activity.

Keywords: Cbp3, cytochrome b, respiratory complex III, alternative translation initiation and sodium/proton exchange.
STRUCTURE AND BIOGENESIS OF MEMBRANE PROTEINS

Mama Ndi
To my family, especially Mussah Ndi and Nvondenga Innocent Ndi, who recently passed away. May your souls rest in peace.
List of publications in thesis.

I Structural basis for Cbp3 interaction with newly synthesized cytochrome b during mitochondrial respiratory chain assembly. 
Mama Ndi*, Geoffrey Masuyer*, Hannah Dawitz*, Andreas Carlström, Mirco Michel, Arne Elofsson, Mikaela Rapp, Pål Stenmark § and Martin Ott§,#. 
(Under revision in JBC).

II Functional characterization of disease-causing Cytochrome b mutations. 
Marin-Buera L, Mama Ndi, Meunier B and Ott M. (Manuscript).

III Alternative Translation Initiation at a UUG Codon Gives Rise to Two Functional Variants of the Mitochondrial Protein Kgd4. 
Manfred Heublein¹, Mama Ndi², Carmela Vazquez-Calvo, F.-Nora Vögtle and Martin Ott. 

IV Dissecting the proton transport pathway in electrogenic Na⁺/H⁺ antiporters. 
Povilas Uzdavinys¹, Mathieu Cöïnçon¹, Emmanuel Nji, Mama Ndi, Iven Winkelmann, Christoph von Ballmoos and David Drew. 

Additional publications

1. Biogenesis of the bc1 complex of the mitochondrial respiratory chain. Mama Ndi¹, Lorena Marin-Buera¹, Roger Salvatori, Abeer Prakash Singh and Martin Ott. 

(Submitted in Scientific Reports).

(Accepted for publication in Journal of Synchrotron Radiation).
Abbreviations

Cytb                  Cytochrome b
Cytc                  Cytochrome c
Cytc1                 Cytochrome c1
UQH2                  Ubiquinol
UQ                    Ubiquinone
1/2UQH2               Semi-ubiquinone (UQH)
Heme bL               Low potential heme
Heme bH               High potential heme
COB mRNA              Cytochrome b messenger RNA
IMM                   Inner mitochondrial membrane
IMS                   Intermembrane space
MTS                   Mitochondrial targeting sequence
TOM                   Translocase of the outer membrane
TIM                   Translocase of the inner membrane
UAAs                  Unnatural amino acids
pBpa                  p-Benzoyl-L-phenylalanine
eIF                   Eukaryotic initiation factor
KGDH                  α-Ketoglutarate dehydrogenase
RCC                   Respiratory chain complexes
NHE                   Sodium proton exchanger
TC                    Ternary complex
PIC                   Preinitiation complex
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Abstract

Membrane proteins make up about one-third of the cellular proteome. The diverse roles that membrane proteins have in cells include major life-sustaining processes, making them major drug targets. The respiratory chain comprises a series of complexes of membrane proteins residing in the inner mitochondrial membrane, which serve as major drivers of ATP synthesis. Assembly of the respiratory chain complexes (RCC) requires coordinated synthesis of nuclear and mitochondrial subunits. Cbp3-Cbp6 complex binds to the mitoribosome as translational activator for cytochrome b synthesis and binds the nascent polypeptide to facilitate its hemylation. Cbp3 consists of an N-terminal domain specific to mitochondrial homologues and a conserved C-terminal ubiquinol-cytochrome c chaperone domain. In this thesis I present the first crystal structure of the C-terminal domain from a bacterial homologue that has enabled us to identify the interaction sites of yeast Cbp3 with Cbp6 and cytochrome b using site-specific photo-crosslinking. Our finding suggests that Cbp3 contacts the mitoribosome via the N-terminal domain in a manner that positions the substrate binding site close to the tunnel exit. In the second project, we have analyzed the effects of disease causing cytochrome b mutations, on bc1 complex assembly. We found that complex III assembly is blocked at either intermediate 0 or I due to impaired insertion of bL or bH heme respectively, which indicates that assembly processes are involved in disease development. We then focused on NADH; a product of alpha-ketoglutarate dehydrogenase complex (KGDH) catalyzed citric acid cycle reaction and one of the substrates that supply electron to the respiratory chain. Kgd4 is a novel subunit of this enzyme complex and two functional variants (Kgd4S and Kgd4L) of unknown origins exist in yeast. We report in our work that Kgd4L originates from a UUG alternative start site, 90 nucleotides upstream and in frame of the annotated start codon. The sequence context upstream of UUG determines the efficiency of recognition of this alternative start codon. Finally, Na+/H+ antiporters are present in all species and are involved in regulation of intracellular pH, cell volume and sodium concentration. ATP formed during oxidative phosphorylation serves as energy source for Na+/K+ ATPase to generate Na+ gradient across the inner mitochondrial membrane, which drives local Na+/H+ antiporters. We show that Lys305 is involved in proton transport and responsible for the electrogenicity of NapA, while human NHA2 shows electroneutral antiporter activity.
Introduction

Mitochondria

Mitochondria are essential double membrane-bound organelles in eukaryotic cells, in which critical, life-sustaining biochemical reactions occur. Mitochondria contain an outer mitochondrial membrane (OMM), which serves as the outermost border and a contact through which solutes, metabolites, signals etc., are exchanged with the cytoplasm (fig. 1). Beneath the OMM is the inner mitochondrial membrane (IMM), which defines the border of an enzyme rich viscous matrix. IMM harbors integral membrane protein complexes of the oxidative phosphorylation system (OXPHOS), responsible for ATP synthesis. Its presence in the last eukaryotic common ancestor (LECA) qualifies it as one of the oldest features of present eukaryotic cells [1]. Its origin has been traced to an aerobic alpha-proteobacterial cell that was engulfed about 2 billion years ago by an anaerobic archaeal cell for mutual survival as the primitive atmosphere became increasingly oxygenated during early evolution of life [2-4]. The notion that mitochondria were once a free living entity is evident by the presence of mitochondrial DNA (mtDNA) and translational machinery operating independently of the rest of the cell. Although most of its genes have progressively been transferred to the nuclear genome, it retains a few but vital genes encoding core subunits of oxidative phosphorylation system and translation [5]. Mitochondria are often described as the “power house” of the cell due to its central role in ATP synthesis. Essential metabolic processes such as the tricarboxylic acid and urea cycles, metabolism of amino acids and cholesterol, beta oxidation of fatty acids, biosynthesis of coenzymes, signaling, thermogenesis and apoptosis regulation are mediated by mitochondria and therefore these organelles remain a crucial component of the cell even in circumstances, where its central role is abrogated [6].

Mitochondrial genome and proteome The number of genes in mtDNAs is fairly stable in all organisms, but differences in genome size exist (ranging between 16 to 80 kb) mainly due to variations in the amount of non-coding sequences between and, or within the genes [5], [7]. For instances, S. cerevisiae mtDNA (75 kb) is about 5 times larger in size than in mammals, and this is as a result of the presence of large number of non-coding sequences between and within the genes in the former [8, 9], whereas genes in the latter are closely packed and less interrupted by noncoding sequences [10]. There are approximately 1187 and 1837 proteins in yeast and human mitochondria respectively [11], from two genetic origins. About 99 percent of the proteins are encoded in nuclear genome, transcribed in the nucleus, and translated in the cytoplasm with mitochondrial targeting sequences (MTS), which recruit cytosolic chaperones
for proper translocation into the mitochondria via the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM) complexes [12-14]. In yeast, the mtDNA encodes eight proteins (Cytb, Cox1, Cox2, Cox3, Atp6, Atp8, Atp9 and Var1) and two ribosomal RNAs (21S and 15 S rRNAs) [12, 15]. In human 13 core subunits of the OXPHOS system (complex I, III, IV and the ATP synthase), 22 tRNAs and 2 rRNA are synthesized from its 16.5 kb mtDNA. Hundreds to thousands copies of mtDNA molecules occur in a cell and the number varies with cell types and reflects the energy demand of the cell. This explains why mitochondria in muscle cells contain more copies of mtDNA molecules compare to leukocytes [16].

**Respiratory chain complexes**

The respiratory chain is composed by redox active protein complexes that are functionally connected by increasing order of reduction potential in the IMM through which electrons from NADH/FADH2 flow down-hill to O2, the ultimate acceptor, to form water. They include: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (Cytochrome c reductase or bc1 complex) and complex IV (cytochrome c oxidase). Apart from complex II, which is exclusively assembled from nuclear encoded subunits, each of the other RCC consists of nuclear and mtDNA encoded subunits [17, 18]. The first enzyme in the sequence is complex I which catalyzes the transfer of electrons from NADH to quinone (Q) to form NAD+ and quinol (QH2) [19]. The canonical complex I is absent in S. cerevisiae, but alternative NADH dehydrogenase known as Nde1/Nde2 exists, though functionally limited only to the regeneration of NAD+ from NADH by converting quinone to quinol [20-22].

Figure1: General schematic of mitochondria and the OXPHOS system in the inner membrane.
Complex II is an enzyme that is simultaneously active in two pathways: the citric acid cycle (TCA) and the RCC. In the TCA cycle it catalyzes the formation of fumarate from succinate by directly converting \( Q \) to \( QH_2 \). The quinols from the actions of the first two complexes are re-oxidized to quinones by complex III, which then reduces cytochrome \( e \) (Cyt\( e \)). The last RCC enzyme is complex IV which re-oxidizes Cyt\( e \) by transferring electrons to \( O_2 \), which is reduced to \( H_2O \). The free energy released from the flow of electrons down-hill through the RCC, is utilized at complex I, III and IV to translocate protons (4 \( H^+ \), 4 \( H^+ \) and 2 \( H^+ \), respectively) from the matrix side to the inter membrane space (IMS) and by so doing, a proton gradient across the IMM is maintained. The energy stored in the gradient is used by ATP synthase for phosphorylation of ADP to ATP as it permits protons to flow back to the matrix, driving ATP synthesis [24]. The RCC together with the ATP synthase constitute the oxidative phosphorylation (OXPHOS) machinery. The RCC are not organized in a static manner in IMM but rather they occur as independent components that are flexible enough to interact with each other to form higher structural organizations known as supercomplexes within the IMM. Supercomplexes between complex I and III and another between complex III and IV have been isolated. However, the independent complexes exist alongside the supercomplexes [25, 26].

**bc\(_1\) complex** 

\( bc_1 \) complex plays a central role in RCC by mediating electron transfer from quinol to Cyt\( e \) [27], a reaction which is a major contributor to the proton gradient that drives ATP synthesis. \( bc_1 \) complex is present in the IMM as well as in the plasma membrane of photosynthetic and respiring bacteria [28]. It is a functional homologue of the \( b_6f \) complex in chloroplast, involved in the light driven cyclic and non-cyclic electron transfer [29]. All eukaryotic cells contain the complex except those that lack mitochondrial respiration like the Monocercomonoides sp. [30]. The redox active subunits are common in both mitochondria and bacteria, but the supernumerary units are only present in mitochondrial \( bc_1 \) complex [31]. This enzyme couples electron transfer to proton translocation across IMM via a mechanism called the Q-cycle [32, 33]. Structures of mitochondrial \( bc_1 \) complex (fig. 2), from yeast, and chicken, among others revealed a dimer with each monomeric unit consisting of 10 subunits. Cytochrome \( b \) (Cob), Rieske Iron-sulfur protein (Rip1) and cytochrome \( c_1 \) (Cyt\( c_1 \)) together make up the catalytic center [34], while two core units (Cor1 and Cor2) and five supernumerary subunits (Qcr6, Qcr7, Qcr8 Qcr9, Qcr10) stabilize the complex. Studies have shown that the supernumerary subunits except Qcr10 are vital for the assembly of functional \( bc_1 \) complex [35]. An extra subunit known as subunit 9 (SU9) is part of the mammalian
complex III originating from the cleaved mitochondrial targeting sequence of Rip1, which is processed and retained [36]. The transfer of electrons and protons by the bc1 complex is mediated by four redox prosthetic groups, namely heme $c_1$ of Cyt$c_1$, Fe-S cluster of Rip1 and the two hemes of Cob (fig. 2). In yeast, the homodimer is 670 kDa in size [35, 37]. The mechanism by which the bc1 complex couples electron transfer to proton translocation across the IMM is called the Q-cycle [32, 33].

![Figure 2. Structure of bc1 complex. Crystal structure of bc1 complex (PDB 3CX5). The catalytic subunits are depicted in red for Cyt$b$, orange for Cyt$c_1$ and yellow for Rip1. Soluble cytochrome $c$ is colored in pink. Adapted from [38].](image)

The Q-cycle The trajectory of the two electrons from ubiquinol (UQH$_2$) transferred via the bc1 to cytochrome $c$ is circular hence the name “Q-cycle” (named after quinol) is used to describe the electron pathway in the bc1 complex (fig. 3). One of the two electrons in ubiquinol (UQH$_2$) at the Q$_o$ site goes through the iron-sulphur center of Rip1 and the heme $c_1$ of cytochrome $c_1$ (Cyt$c_1$) to cytochrome $c$ in the IMS. Through two heme prosthetic groups, heme $b_H$ and heme $b_L$ of cytochrome $b$, the second electron reduces ubiqinone to semi-ubiquinone (UQH) at the Q$_i$ site. For every electron that reaches Cyt$c_1$ via Cyt$b$, two protons are translocated into the IMS from the matrix (fig. 3) and this contributes to the build-up of a membrane potential across the IMM which drives ATP synthesis [33].
Figure 3. Representation of the Q-cycle through bc₁ complex catalytic subunits. Reduced quinol (UQH₂) binds to the Qₒ site, two electrons are transported: One electron goes through the redox centers in Rip₁ (Fe-S) and Cytc₁ (heme c₁) and, finally to the soluble cytochrome c. The other electron goes through hemes bₗ and bₜ to the Qᵢ site reducing ubi-quinone (UQ) to semi-ubiquinone (UQH denoted as 1/2UQH₂ for clarity). Qₒ and Qᵢ sites are shown in green. Figure is adapted from [38].

Cytochrome b  Cytb is a transmembrane protein that serves as the central catalytic subunit of the bc₁ complex [39]. Cytb is made up of 385 amino acids in spans of eight transmembrane helices with a theoretical size of about 40 kDa but runs at a lower size (around 30 kDa) due to its highly hydrophobic nature. Cytochrome b is highly conserved across bacteria and eukaryotes [28]. It has two b-types heme groups: the high potential heme group bₜ and the low potential heme group bₗ (fig. 3). Cytb has one reacting center for ubiquinone on each side of the membrane, designated: center Qᵢ (Qᵢₐ, Qᵢₑ, Qᵢₜ, or Qᵢₙ) for proton input and Qₒ (Qₒₘ, Qₒₑ, or Qₒₖ) for proton output [28, 40, 41]. It also has binding sites for inhibitors and analogues of ubiquinone. In human, mutations lead to several complex III deficiency associated diseases [42, 43] while resistances to anti-fungi and anti-malaria drugs have been linked to mutations in the cytochrome b orthologues in the respective parasites [44, 45].

Cytb is the lone mitochondrial synthesized subunit of the bc₁ complex and in S. cerevisiae it is encoded by the mitochondrial COB gene [46], which is transcribed into a bicistronic COB mRNA with varying number of introns depending on the strain [47]. The reference S288C strain for instance contains five introns: bl₁, bl₂, bl₃, bl₄ and bl₅, each of them belonging to either group I (bl₁) or II (bl₂-bl₅) self-splicing introns [48]. The sequence of the mRNA from bl₂ to bl₅ are in frame with the upstream COB exons such that a cytochrome b protein fused
to polypeptide sequences encoded by the introns is also produced as a result of alternative splicing and both COB and COX1 are spliced by the maturase activities contained within these extra polypeptides sequences [47, 49-51]. Also, multiple nuclear-encoded proteins are involved in the maturation and translation of the COB mRNA. For instance, the COB mRNA is co-transcribed with an upstream glutamic acid tRNA, which is processed to form the right 5’ end COB mRNA with the help of the proteins Cbp1 and Cbt1 [52]. Cbp2 is another player which is involved in the splicing of bl5 [53]. Five nuclear encoded proteins described as translational activators (Cbp1, Cbs1 Cbs2, Cbp3 and Cbp6) have been implicated in the translation of the COB mRNA. These translational activators may perform their function by interacting with the 5’ UTR of the transcript as is in the case of Cbp1 which binds to CCG triplet and stabilizes the COB transcript [54] or to the mitoribosome [55] or with the nascent protein near the exit tunnel as is the case of Cbp3-Cbp6 complex [56, 57]. Cbp3 and Cbp6 form a stable Cbp3-Cbp6 complex that binds close to the exit tunnel on the mitoribosome [56] and to the newly synthesized cytochrome b [57]. The function of translational activators could be exerted by either changing their expression levels which then regulates the efficiency of translation of targeted mitochondrial transcript [58] or by adjusting mitochondrial translation of the client transcript depending on the efficiency of assembly of the OXPHOS [59]. The expression level of COB is regulated by means of a feedback loop involving Cbp3-Cbp6 between the assembly line and the translational machinery [57].

As translation of COB mRNA proceeds, the nascent polypeptide is inserted into the membrane and, as soon as translation is completed, Cbp3-Cbp6 bound Cytb is released from the mitoribosome into the IMM. Then the next phase is the assembly that starts with the incorporation of the two heme b molecules.

**Biogenesis of the mitochondrial bc1 complex (in yeast)**

Like every other component of the OXPHOS system, nuclear and mitochondrial encoded subunits must come together to assemble a functional bc1 complex. Since excess of mitochondrial encoded subunits, not incorporated into complex have been shown to be toxic to the cell [59], translation in both compartments needs to be properly coordinated for efficient synthesis and assembly.

**Early assembly** Cytochrome b is translated on mitoribosomes with bound translational activators; Cbs1, Cbs2 and with Cbp3-Cbp6 complex positioned ideally on the large subunit of the ribosome near the tunnel exit such that it binds to and stabilizes the nascent Cytb.
polypeptide as it exits the ribosome (fig. 4) [56]. Once the fully synthesized nascent Cytb binds to Cbp3-Cbp6, the Cytb-bound complex leaves the mitoribosome and then the first heme group is incorporated at the $b_L$ site (fig. 4) [60]. This is also the case for Cytb from bacteria [61, 62]. It is likely that this first hemylation induces a conformational change that favors the binding of Cbp4 to Cytb. Cbp4 is an assembly factor that binds and stabilizes the partially hemylated Cytb consenting the hemylation of the $b_H$ site [60]. The Cbp3-Cbp6 complex is then released from the fully hemylated Cytb and it returns to the mitoribosome to initiate new rounds of translation of the COB mRNA [57, 60]. Therefore the dual role of Cbp3-Cbp6 (translation initiation and assembly factor) in the assembly of $bc_1$ complex is part of a feedback loop mechanism that coordinates expression levels of Cytb and the assembly of the $bc_1$ complex (fig. 5). Structural subunits Qcr7 and Qcr8 are recruited to stabilize the completely hemylated Cytb to form intermediate II (fig. 4) [60].

![Figure 4](image_url)

**Figure 4.** Early assembly steps in the biogenesis of $bc_1$ complex. Cytb is synthesized by mitochondrial ribosomes with the help of the translational activators Cbs1, Cbs2, Cbp1 and Cbp3-Cbp6. Upon completion of translation, Cbp3-Cbp6 interacts with the newly synthesized Cytb to form assembly intermediate 0. Insertion of heme $b$ into the $b_L$ site triggers recruitment of Cbp4 to stabilize the acquired heme, thus forming intermediate I. Upon insertion of heme $b$ into the $b_H$ site, Cbp3-Cbp6 is released and the two first nuclear encoded subunits, Qcr7 and Qcr8 are joined with Cytb and Cbp4 to form intermediate II, which contains fully hemylated Cytb ready for further assembly. Figure is adapted from [38].

**Early assembly factors Cbp3, Cbp6 and Cbp4**

Cbp3 is a nuclear encoded protein comprising of 335 amino acids synthesized in the cytoplasm with a cleavable N-terminal mitochondrial targeting sequence (MTS) consisting of the first 20 amino acids [63]. It has an ubiquinol-cytochrome $c$ chaperone domain that is highly conserved and a yeast specific uncharacterized N-terminal domain. Cbp3 and Cbp6 form a tight Cbp3-Cbp6 complex so that Cbp3 is destabilized in the absence of Cbp6 and vice versa. Cbp6 consists of 162 amino acids with a non-cleavable MTS. Impaired respiration with complex III deficiency has been observed in Cbp3 or Cbp6 knock-out yeast strains [63].
and Cbp6 play non-redundant role in complex III biogenesis because overexpression of one of them cannot complement the absence of the other [56].

Cbp3 and Cbp6 have human orthologues known as the ubiquinol-cytochrome c reductase complex assembly factors 1 and 2 (UQCC1 and UQCC2) respectively [64]. Just as the yeast homologues, a complex between UQCC1 and UQCC2 has been shown to be involved in complex III biogenesis at the level of cytochrome b synthesis and stability [64]. A pathogenic complex III deficiency in human is reported to be partly due to a splicing defect originating from a homozygous mutation situated three bases upstream to the third exon of UQCC2 which leads to reduced UQCC1 and UQCC2 protein levels [64].

Cbp4 is another early assembly factor which exposes a large domain in the IMS and a transmembrane segment that goes through the IMM with a small portion reaching the matrix [56]. Recruitment of Cbp4 to the bc1 assembly line is triggered by the incorporation of the first heme group into cytochrome b at the $b_L$ site (fig. 4) [56, 60]. Reduced bc1 complex activity has also been reported to be caused by mutations in Cbp4 [65]. Its human orthologue is UQCC3 and a missense mutation (c.59T>A) has been shown to affect cytochrome b synthesis and caused deficiency of bc1 complex despite normal UQCC1 and UQCC2 proteins levels [66].

**Summarized further assembly steps until bc1 is assembled into supercomplexes**

Assembly intermediate II consists of the fully hemylated cytochrome b with Qcr7 and Qcr8 incorporated [57]. The completely hemylated cytochrome b is stabilized by Qcr7 with the other two subunits in the intermediate (Cbp4 and Qcr8) stabilized by the N-terminal domain of Qcr7 [67, 68]. Four more proteins; Cor1, Cor2, mature cytochrome c1 and Qcr6 are incorporated to intermediate II in an unclear order to form intermediate III [57]. The structural subunit Cor1 (431 residues) makes direct contact with cytochrome b and also serves as a link between the catalytic subunits and Cor2 (352 residues) that is involved in the dimer interface of the bc1 complex [69, 70]. Mature cytochrome c1 (CytC1) is one of the catalytic subunits of bc1 complex. CytC1 has a molecular weight of about 30 kDa with a single heme prosthetic group in a domain that is anchored in the IMS by a transmembrane helix in the IMM. It catalyzes electron transfer from Rip1 to cytochrome c in the IMS. CytC1 and Cor1/Cor2 are far apart and make only minor contacts within the bc1 complex. Nonetheless, a sub-complex of yet unknown biological significance has been reported between them [71]. After intermediate III has been formed, the remaining subunits Qcr9, Rip1 and then Qcr10 are
incorporated sequentially [72] to form completely assembled $bc_1$ complex. In the IMM, the assembled $bc_1$ complex exists as a homo-dimer and Rip1 has been implicated in the dimerization process although the actual stage at which dimerization begins has been shown to be independent of Rip1 [73]. The individual complexes of the respiratory chain are not randomly distributed in the IMM; they are rather assembled into supramolecular structures known as respiratory supercomplexes. The homo-dimer of $bc_1$ for instance, is structurally and functionally associated with one and two copies of complex IV in the IMM of yeast [74, 75] and bovine [75] mitochondria. The role of supercomplexes remains unclear but some researchers have proposed that single respiratory enzymes are better stabilized in supercomplexes [76]. It is also proposed that supercomplexes bring the redox centers of the respiratory chain closer together for efficient substrate channeling that enhances respiration [77, 78]. Also, the possibility of electrons passing through the respiratory chain to exit especially at complex I [79, 80] and the $bc_1$ complex [81, 82] (leading to ROS formation) before reaching O$_2$ [83], is reported to be decreased or limited by the presence of supercomplexes [84]. Apart from respiratory chain complex, the IMM is also heavily crowded with other proteins creating possibility of unspecific interactions. Supercomplex formation is proposed to limit the chances for single respiratory chain complexes to be caught up in such unspecific binding interactions [85, 86]. Some organisms express only membrane bound version of Cytc [87], hence close proximity of the respiratory chain complexes is very necessary for transfer of electrons between the $bc_1$ complex via the less flexible membrane bound Cytc to complex IV.

**Regulation of Cytb synthesis via a translational feedback loop**

The biogenesis of the $bc_1$ complex is auto-regulated via a feedback loop, which ensures that incessant synthesis of Cytb in the mitochondria is driven by consecutive assembly to form mature $bc_1$ complex [56]. The Cbp3-Cbp6 complex coordinates translation of the $COB$ mRNA and early steps of $bc_1$ complex assembly [57]. The heterodimeric complex plays a dual role in the biogenesis of $bc_1$ complex; as a translational activator it is essential for efficient synthesis of Cytb and as an assembly factor it is required for early steps of $bc_1$ complex assembly (fig. 5). When Cytb becomes fully hemylated, the structural subunits Qcr7 and Qcr8 are recruited into the assembly complex while Cbp3-Cbp6 is released. The released Cbp3-Cbp6 returns to the mitoribosomes to stimulate another translation round of the $COB$ mRNA. Deletion of nuclear genes that encode structural subunits or assembly factors stalls the assembly of $bc_1$ due to down-regulation of translation of $COB$ mRNA emanating from
Cbp3-Cbp6 sequestration in assembly intermediate I. Consequently, availability of free form of the complex for stimulation of translation of COB mRNA is limited [57]. However, down-regulation of COB mRNA translation was not observed in mutants that lack genes for late assembly factors or subunits and a possible explanation is that biogenesis of bc1 complex could proceed efficiently through early steps to intermediate III; thereby Cbp3-Cbp6 sequestration is circumvented.

According to ribosome profiling, regulation of mitochondrial translation is activated when cells are adapted from fermentation to respiratory growth [88]. The synthesis of Cytb, Cox1 and some subunits of ATP synthase (Atp6, Atp8 and Atp9) is subjected to regulation. Cytosolic translation controls this dynamic regulation especially for Cytb and Atp6. For instance, translation of ATP6 is up-regulated when cytosolic translation is inhibited, probably due to down-regulation of expression of its repressor, Smt1 [89]. However, inhibition of cytosolic translation down-regulates COB translation severely [88], which could be explained by either a decrease in the synthesis of translational activators that determine the rate of translation of COB mRNA [88], or a decreased import of nuclear encoded subunits, which reduces the efficiency of assembly of mitochondrial encoded subunits. Upon respiratory adaptation, there are delayed changes in COB mRNA translation compared to most other subunits of complex III [88]. This delay is possibly due to the fact that COB translation is linked to bc1 complex assembly via a feedback mechanism (fig. 5) [57]. At steady state Cytb accumulates at assembly intermediate I [57], representing a reservoir of the protein for subsequent assembly into functional bc1 complex. New rounds of COB mRNA translation are activated only when the quantities of this intermediate I are reduced by ongoing assembly by allowing the liberated Cbp3-Cbp6 complex to stimulate translation of its client mRNA. Thereby, the Cbp3-Cbp6 mediated feedback loop regulates Cytb translation, which responds to the mitochondrial influx of nuclear encoded subunits.
Figure 5. Translational feedback loop regulating Cytb synthesis in mitochondrial. Nuclear gene expression of mitochondrial biogenesis genes determines the rate of Cytb synthesis in two ways: First, it determines the levels of translational activators inside the mitochondria, as they are rate limiting for COB mRNA translation and directly regulate Cytb synthesis. For acute translation regulation, Cbp3-Cbp6 availability modulates synthesis of Cytb depending on the efficiency by which Cytb can assemble. Upon formation of intermediate I, Cbp3-Cbp6 is sequestered and not available to stimulate translation. Once Cytb in intermediate I progresses further in the assembly, Cbp3-Cbp6 is released to drive another round of Cytb synthesis. Blockage in assembly due to lack of subunits caused by a down regulation of their expression by nuclear signaling mechanisms will therefore provoke stalling of assembly and consequently down regulation of Cytb synthesis. Figure adapted from [38].

Therefore, Cbp3-Cbp6 interacts with the mitoribosome as a translational activator, and with Cytb as an assembly factor for bc1. To understand at the molecular level how these dual roles are accomplished by the Cbp3-Cbp6 complex, knowledge of Cbp3/ Cbp6 structures, residues essential for their interactions in Cbp3-Cbp6 complex as well as with other proteins are required. At the start of my work, information on the structure of Cbp3 and the interaction surfaces was not available.

Yeast strains lacking Cbp3 are respiratory deficient due to inefficient translation of Cytb and assembly [56]. In human, knock down of UQCC1 (human Cbp3 homolog) leads to loss of UQCC2 (human Cbp6 homolog) and reduced levels of assembled bc1 complex [64]. Apart from translational and assembly factors, some mutations in synthesized Cytb also affect assembly of bc1 complex resulting in mitochondrial disease [90, 91].

Mitochondrial Disease
Mitochondrial disease is a collective term for a group of genetic disorders that are associated with defective OXPHOS, caused by mutations in mitochondrial or nuclear genes involved in mitochondrial function [92]. Mitochondrial diseases originate when cells contain inefficient or completely inactive mitochondria with the symptoms and severity determined by the number and location of affected cells in the body. Organs with high energy demands such as the heart, liver, brain, kidney and muscles are often the most affected [92]. When a particular organ or system is affected to a significant extend, the disease is given a specific name such as Leigh’s disease, Alper’s disease, mitochondrial encephalopathy lactic acidosis and stroke [MELAS] [93]. Exercise intolerance is one of the most common symptoms among those suffering from mitochondrial diseases. Some of the causes of exercise intolerance include cytochrome b mutations, respiratory disorders and heart conditions. Mitochondrial disease is a thought-provoking area of genetics because two genomes may contribute to the pathogenesis [94]. Approximately 1158 proteins are required for proper mitochondrial function [95]. Therefore, the nuclear genome has a great influence on mitochondrial disease. Its prevalence per one hundred thousand persons stands at 9.6 cases for mtDNA mutations and 2.9 cases for nDNA mutations [96]. Mutations in the COB gene are among those implicated in mitochondrial disease.

Cytochrome b in human and S. cerevisiae share just 55% sequence similarity, but amino acids residues of the extramembrane loops (130-150 and 170-190), which constitutes part of the Q₀ (quinol oxidation site), and amino acids segment 20-40, which is part of the Q₁ (quinone reduction site) reaction centers, are highly conserved [40]. Intramembrane hydrophobic regions harboring the heme-ligating histidines are also conserved, but the extramembrane segment to the histidine residues ligating the bL heme is more conserved [40]. Some mutations in Cytb like Y279C, G291D and an in-frame deletion of residues 252-259, have been isolated from patients suffering from exercise intolerance [90, 91, 97], but the molecular impact of these mutations on mitochondrial function is not known. Interestingly, the protein segments of Cytb harboring these mutations (fig. 6) are conserved and therefore we could use the yeast model to analyze how these mutations contribute to the pathogenesis of the disease.
Figure 6. Topological representation of cytochrome b in the IMM. Helices from N to C terminal labelled A-H. Positions of mutations indicated in red.

**Photo-crosslinking to test protein-protein interactions**

To fully understand the mechanism of RCC assembly, details of protein-protein interaction need to be determined. Methods like yeast 2-hybrid (Y2H), certain fluorescence microscopy techniques and affinity purification have been widely employed in protein complex interaction studies, but there are limitations with each of these approaches [98]. With affinity purification and Y2H approaches, proteins interactions are studied under non-native conditions with increased likelihood to identify false interactions, while genuine interactions that require physiological conditions might be overlooked. Photo-activated crosslinking approach, in contrast, provides a way of recognizing and characterizing proteins interactions in intact living cells [99], with possibility of monitoring sequential resolution of interaction events [100]. Also, affinity purification and Y2H approaches are biased towards strong protein-protein interactions, whereas the photo-crosslinking approach involves formation of new covalent bond called crosslink, which eliminates discrimination between strong and weak affinity interactions. Lately, diazirines, aryl azides and benzophenone (Bpa) have become the most popular classes of photo-crosslinkers. In the absence of UV radiation, they remain stable and therefore can be incorporated as unnatural amino acids and localized within living cells. Upon exposure to UV radiation, they form highly reactive intermediates that react with C-H bonds of a nearby molecule forming a covalent crosslink (fig. 7). Because of the high reactivity of the intermediates, they can react and form bonds with nearby molecules irrespective of their functional group. Besides, high reactivity is related to short half-life of the intermediates, which lessens non-specific interactions. Thirdly, should the interacting
partner be absent, the intermediate reduces non-specific interaction by either falling down to
ground state, which can be activated again as in Bpa (fig. 7) [101], or react with water
molecules or react with itself as in diazirines and aryl azides (fig. 7) [99]. I established and
applied site directed photo-crosslinking approach in our laboratory to determine residues in
Cbp3 involved precisely in interaction with Cbp6, Cytb and other partner proteins.

Figure 7. Comparison of photocrosslinking groups. The structures in black at the top are the stable
photochemicals precursors. Highly reactive photochemical intermediates are shown in red. Figure is
adapted from [99].

**Incorporation of unnatural amino acids into proteins** Site-directed photo-crosslinking
requires incorporating photoactive unnatural amino acids (UAAs) into growing polypeptide
chains during translation in host cell. UAAs can be incorporated into proteins either by
metabolic or genetic approach. Metabolic (residue specific) approach is when the UAAs (e.g
photo-Leu) and its natural analog (e.g Leu) are quite similar so that they share a common
tRNA/aminoacyl-transfer RNA synthetase pair and with the help of the host’s translational
machineries, the UAAs is incorporated into cellular proteins in place of its natural analog
[102]. The genetic (position-specific) approach is achieved via expansion of the genetic code
by engineering an orthogonal aminoacyl-tRNA synthetase, which recognizes and charges only
its corresponding suppressor tRNA with a photo-reactive unnatural amino acid. This is
incorporated into the protein via suppression of TAG amber stop codon at specified positions
This approach depends on modification of an existing synthetase resulting in a mutant that is capable of charging a suppressor tRNA with a specific UUAs, which subsequently can incorporate at an amber stop codon [104-106]. Therefore, application of the genetic approach demands the following requirements; genes of the mutant target protein and tRNA/synthetase pair are introduced and expressed in the cell, introduction of UAAs into cell through growth medium and an efficient light delivery system into the cell [107]. Because the genetic approach involves orthogonal tRNA/synthetase pair, it is slower and less flexible compared to the metabolic approach. However, the strength of this technique is that it has increased the possibility of utilization of UAAs in different model systems such as in mice [108-110], Drosophila [111], zebrafish [109], yeast [112]), Caenorhabditis elegans [113] and mammalian cell lines [108, 114]. There are UAAs with varied chemical properties such as those that are light-sensitive, fluorescent, redox-active, and some with bioconjugatable side chains [104, 115], hence they can be applied to studying protein to protein or ligand interaction, and monitoring of ion channel gating [116]. The use of UAAs for cross-linking studies is accompanied by a number of advantages; they are incorporated during protein synthesis, hence independent of post-translational modifications, they are site tolerant due to their small size, and retain their properties while in cell [117]. Some tRNA-aminocyl tRNA synthetase pairs that can be used to adapt amber stop codon into codon have been developed [103, 118-121]. Cells are therefore transfected with two plasmids, one containing genes encoding engineered tRNA/tRNA synthetase pair and the other containing the gene encoding the amber (TAG) version of the targeted protein and grow in the presence of the UAAs.
During suppression of amber stop codon, its surrounding nucleotide sequence ensures that translation is momentarily paused at the TAG once recognized by the machinery and there is initiation of codon-anticodon complementary base paring between the TAG of the mRNA and its complement, the orthogonal tRNA charged with the UAAs. That results to the incorporation of the UAAs into the protein as shown in figure 8 a.

**Translation Initiation in eukaryotes**

Translation initiation is the assembly of 80S ribosome with an established codon-anticodon base-pairs between the initiation tRNA and the initiation codon at the P-site of the ribosome, capable of elongation. It comprises of two steps involving about nine initiation factors: formation of the 48S complex and then joining of the 60S ribosomal subunits to the 48S.
initiation complex. The cap-dependent mechanism of initiation is most frequent and it begins with recruitment of the ternary complex [eukaryotic initiation factor 2 (eIF2), GTP and a methionyl-initiator transfer RNA (Met-tRNA\textsubscript{Met})], eIF1, eIF1A, eIF3 and eIF5 to the small subunit of the ribosome (40S) to form the 43S preinitiation complex (PIC) [123]. Binding of eIF1 and eIF1A to the 40S subunit triggers a conformational change in the ribosome, involving opening of the mRNA entry site [124]. Next is the loading of the 43S PIC on the 5’ cap of the mRNA by eIF4, followed by hydrolysis of the GTP but its products (GDP and P\textsubscript{i}) remained bound to eIF2. Then the 43S PIC scans the mRNA downstream of the cap from the 5’ untranslated region (UTR) toward the 3’ direction until it comes across the initiation codon where the eIF1, P\textsubscript{i}, and eIF2-GDP-eIF5 are released (from the 43S PIC) and replaced by eIF5B-GTP that recruits the 60S ribosomal subunits to the complex. This is followed by hydrolysis of the GTP and the release of eIF5B and eIF1A from an assembled elongation competent 80S ribosome. There is also Internal Ribosome Entry Site-mediated initiation which is an alternative initiation mechanism that involves direct recruitment of ribosomes to a site within the 5’ UTR [123].

Scanning of the mRNA entails two coupled processes; unwinding of secondary structural element, within the 5’ UTR of the mRNA and movement of the ribosome along the mRNA. The ribosome requires just the right conformation (induced by eIF1A and eIF1) [124], to move itself along the mRNA [125]. However, in the presence of the least secondary structure within the 5’ UTR, ATP and eIF4 A/B/G would be required to unwind this region in order to facilitate movement of the 43S and the extent of the secondary structure determines the amount of ATP and eIF4A required [126, 127]. Apart from recruiting and attaching the ternary complex (TC) with the 43S, eIF3 is involved in scanning of the mRNA [128].

**Recognition of the initiation codon** During scanning, the 43S PIC must have a mechanism to prevent partial base pairing between triplets at the 5’ UTR and the initiator tRNA in favor of that with the authentic initiation codon, which is often AUG within an optimum context [GCC(A/G)CCAUGG, purines at -3 and +4 positions] [129]. The fidelity of translation initiation is sustained to a significant extend by eIF1 via assisting 43S scanning complex to discriminate against non-AUG triplets and AUG triplets located within poor sequence context or within first 8 nucleotides from the 5’end of mRNA. It is also capable of dissociating ribosomes that are assembled at such triplets in its absence [125, 130, 131]. Hence a mutant eIF1 that dissociates more rapidly from 48S subunit promotes initiation of translation from a non-AUG codon [132]. eIF1 has been identified as a determinant of initiation codon
recognition in yeast [133]. Once 43S PIC locates an optimal initiation codon, the interaction between eIF1A and 40S is strengthened [134], followed by displacement of eIF1 (which is in close proximity to the P site) [135, 136]. The displacement, leads to a switch of 43S from an “open” to a “closed” conformation which is required to keep the 43S locked on the spot at the mRNA so that a stable codon-anticodon base pair is established. The eIF1 has to be released because it antagonizes the close conformation of PIC. The C-terminal tail (CTT) of eIF1A promotes the “open” conformation of 43S scanning complex and increases the stringency of start codon selection, whereas its N-terminal tail (NTT) enhances the “closed” conformation and decreases the accuracy of start codon selection [137].

**Alternative Translation Initiation** The use of non-AUG translation start sites is a phenomenon that contributes immensely to the generation of multiple protein isoforms from a single gene and this is common event in bacteria, viruses, and has also been reported in eukaryotes [138-140]. According to a recent study in yeast, 4457 out of 6664 genes analyzed have either AUG or near cognate non-AUG upstream to the annotated start sites, that are potential alternative initiation start sites, capable of generating 12958 additional protein isoforms with N-terminal extension [11]. This means that alternative translation initiation is very common in yeast and contributes significantly to the complexity of its proteome. It enables different variants of a protein to simultaneously reach or target different cellular organelles. Codons that differ from AUG by one nucleotide are potential alternative translation initiation sites (aTIS) [141] and the weak base pairing between the non-AUG initiator codon and the anticodon of the initiator tRNA seems to be compensated by interaction of neighboring nucleotides especially purines (A/G) at position -3 and G at +4 [129, 131]. In *S. cerevisiae* for instance, ACG in *ALA1* (encoding alanyl-tRNA synthetase) and TTG in *GRS1* (encoding glycyl-tRNA synthetase) are alternative translation initiation codons [142]. It has been shown that TTG, CTG, ACG and ATT can also initiate translation of *ALA1* at ~50% rate relative to ATG [142]. Apart from sequence context, presence of a stable hairpin structure 12~15 nucleotides downstream of non-AUG initiator codon may also enhance its recognition by the scanning complex [143]. Non-AUG triplets are used to initiate translation at low efficiency in yeast [144]. Detection of the alternative forms of proteins is often challenging due to the low frequency of translation initiation from the non-canonical codons [142]. Initiations from non-AUG codon in certain scenarios allow for dual protein targeting like the case of the extended non-AUG codon initiated forms of histidine tRNA.
Composition and function of alpha-ketoglutarate dehydrogenase (α-KGDH)

The Krebs cycle plays a central role in cellular metabolisms, providing intermediates for biosynthetic pathways and channeling electrons from reduced metabolites to the OXPHOS system for efficient ATP synthesis. One of the Krebs cycle’s electron channeling reactions is catalyzed by alpha-ketoglutarate dehydrogenase (α-KGDH), a multienzyme complex that uses thiamine pyrophosphate as a cofactor to catalyze an oxidative decarboxylation reaction where succinyl-CoA, NADH and CO₂ are formed from α-ketoglutarate, coenzyme A and NAD⁺ (fig. 9). α-KGDH is a major site of regulation of the Krebs cycle and in humans, it is reported to be inhibited by products of its catalytic actions, succinyl-CoA and NADH [147, 148]. Like pyruvate dehydrogenase (PDH) and isocitrate dehydrogenase, α-KGDH is also activated by calcium [149, 150] leading to an increased supply of NADH to the respiratory chain [151]. Apart from its products, the ratio of NADH/NAD⁺, ATP/ADP, calcium and availability of its substrates are also involved in its regulation. The multienzyme complex consists of three catalytic subunits; a thiamine pyrophosphate dependent dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2), a dihydrolipoamide dehydrogenase [152, 153] and a structural subunit, Kgd4 which was recently identified by our group [154].

Figure 9. Alpha-ketoglutarate dehydrogenase catalyzed synthesis of succinyl-CoA and NADH from alpha-ketoglutarate. Figure adapted from reference [214].

One of the products, succinyl-CoA, could either be converted to succinate by succinyl-CoA synthetase or used as a substrate in heme synthesis. The genes for E1, E2 and E3 of the enzyme complex were first identified in yeast and were termed Kgd1, Kgd2 and Lpd1 respectively [155-157].

Kgd4 In the course of identifying the composition and organization of α-KGDH in yeast, recently, a novel subunit was discovered and named Kgd4 with evidences supporting its involvement in the efficient recruitment of E3 subunit into the E1-E2 catalytic core of α-
KGDH in yeast (fig. 10 a) [154]. It has two functional domains; a C-terminal domain that interacts with subunit E1 (Kgd1) and an N-terminal domain that interacts with subunit E3 (Lpd1)[154]. In yeast there is a single open reading frame for Kgd4 with no introns in the mRNA [158, 159] and therefore alternative splicing could not justify the presence of two forms; long (Kgd4L) and short (Kgd4S) with relative molecular weights of 20 and 15 kDa, respectively [154, 160]. Deletion of Kgd4 leads to decrease activity of α-KGDH without destabilizing the E1-E2 catalytic core (fig. 10 b). The role of Kgd4 is evolutionarily conserved from fungi to mammals [154].

Figure 10. Model for the organization of KGDH and the role of Kgd4. (a) Model. The novel subunit Kgd4 recruits the E3 subunit to the core of KGDH, formed by E1 and E2 subunits. The C-terminus of Kgd4 contacts Kgd41-Kgd2 core, while the N-terminus interacts with Lpd1. (b) In the absence of Kgd4, the contact of E3 to the core is dramatically reduced. Figure adapted from reference [154].

**Transporters**

Whole genome sequence analysis suggests that genes coding for membrane proteins constitute about 30 % of the open reading frames (ORFs) and this proportion increases with genome size [161]. About 50% of all pharmaceutical drugs target membrane proteins [162] because they are involved in vital processes in cells: transports, enzymatic activity, signal transduction, cell-cell recognition, intercellular joining and attachment to the cytoskeleton and extracellular matrix etc. Membrane proteins that are permanently attached via hydrophobic, electrostatic or non-covalent interactions to the phospholipid bilayers of cells are referred to as integral membrane proteins. If the protein completely penetrates across the lipid bilayers, it
is described as a transmembrane protein. Peripheral membrane proteins do not penetrate the hydrophobic core of the lipid bilayer and are temporarily attached to the cell membrane. Hydrophobic organic molecules diffuse through the lipid bilayers of membranes [163] which are rather selectively permeable to charged and polar molecule (ions and organic solutes) and their movement across is facilitated by the presence of pores (Channels) and carriers (Transporters) within the membranes [164, 165]. Proteins that form pores for passive movements of ions/solutes across membranes down their concentration gradients are called channel proteins and they are classified based on the solutes [166], e.g; sodium, potassium, chloride and water (aquaporins) channels, or based on their mode of activation e.g; ligand gated and voltage gated channels [167]. On the other hand, transporters carry solutes/ions against their concentration gradients using energy and they are classified as either primary or secondary transporters based on their energy sources. Transporters that use energy from the hydrolysis of ATP are referred to as primary active transporters (e.g ABC transporters like maltose transporter, F-type, P- type, and V- type ATPases) whereas secondary active transporters (e.g Na⁺/H⁺ antiporters) use potential energy of an existing chemical gradient for transport [168, 169].

Secondary active transporters use potential energy stored in existing electrochemical gradients to transport solutes against concentration gradients [170] and are classified into symporters and antiporters. Those that transport solutes and ions in same direction across the membrane are known as symporters and a good example is the lactose permease Lac Y from *E. coli* [171], while antiporters like the transporter NapA from *T. thermophilus* transport Na⁺/2H⁺ ions in opposite directions across the membrane [172].

**Sodium-proton antiporter** Na⁺/H⁺ antiporters are present in all species from prokaryotes to eukaryotes and are involved in the maintenance of intracellular pH, sodium concentration and cell volume [173-175]. They belong to the monovalent cation:proton antiporter (CPA) superfamily that includes CPA1 and CPA2, among others [174]. Members of the CPA1 (NHE1-9 in mammals) clade are generally considered to catalyze electroneutral Na⁺/H⁺ exchange using Na⁺ gradient, while CPA2 (NHA1-2 in mammals) members are thought to catalyze electrogenic exchanges, driven by proton gradient [174] with 2H⁺:Na⁺ and 3H⁺:2Na⁺ transport stoichiometry as reported [176, 177]. While electrogenic antiporters are sensitive to membrane electrical potential, electroneutral antiporters are not [178].
In mammals, dysfunction in Na\textsuperscript{+}/H\textsuperscript{+} antiporters is associated with number of diseases such as diabetes [179], hypertension [180], epileptic-like seizures, retarded growth and ataxia [181]. Human NHE is also involved in cell growth and differentiation [182]. NHE activity has been reported in both plasma membrane and IMM of mammalian cells [183, 184]. By removing intracellular protons from epithelial cells in exchange for extracellular sodium ions (fig. 11b), NHE helps in neutralizing damaging effects of excess acids that accumulate in them [183, 184] and also helps in the reabsorption of NaCl across renal and intestinal tracks [185-188]. Hence, better understanding of their molecular mechanisms of actions could reveal some details of their roles in various diseases and how they can be exploited as potential therapeutic targets [174]. In bacteria, Na\textsuperscript{+}/H\textsuperscript{+} antiporters use proton-motive force to extrude Na\textsuperscript{+} ions (fig. 11a) to maintain homeostasis of the cell and contribute to pathogenesis [163]. Bacterial homologues are often used in studies of mechanisms of their actions because of the associated challenges involved in production and purification of their mammalian counterparts.
Figure 11. An illustration of the cation pumps; (a) the F-type H\(^+\) ATPase that provides the driving force for bacterial Na\(^+\)/H\(^+\) antiporters (Nha) and (b) the Na\(^+\)/K\(^+\) ATPase that provides the driving force for mammalian Na\(^+\)/H\(^+\) exchangers (NHE).

The most studied homologue is NhaA from *E. coli* and it contains the NhaA fold [189, 190], which consists of a dimer domain and a core domain, with a pH sensitive transport activity for Na\(^+\) and Li\(^+\) that begins at pH 6.5 and goes up to the maximum rate of 1500 ions per seconds at pH8.5 [191]. The core domain binds and translocates ions, while the dimer domain holds the monomers together in the dimeric form. Its crystal structure revealed an inward-facing negatively charged funnel consisting of highly conserved Asp163, Asp164, Asp133, Thr132 and Lys300 residues, proposed to form the ion binding site [192]. Mutation of the strictly conserved Asp163 or Asp164 led to abolishment of activity, whereas mutation of any of the other three led to impaired activity [193]. It was previously assumed that for electrogenic...
antiporters, two (one for electroneutral antiporters) strictly conserved aspartate residues (e.g D163 and D164 in NhaA) in the binding site carry out exchange of the ions across the membrane via direct protonation of their carboxyl groups on one side of the membrane and the release of the protons in exchange for sodium ions on the other side of the membrane [194, 195], although there was no evidence of direct measurement of transport activities of these residues to back the claim. The crystal structure of NhaA dimer revealed a salt bridge between one of the conserved aspartate residues Asp163 and a membrane embedded Lys300 [196]. Also the crystal structure of another electrogenic antiporter NapA from *Thermus thermophilus* revealed the highly conserved D156 and D157 positioned for ion binding, with also a salt bridge present between D156 and a membrane embedded K305 [172]. The presence of the salt bridge between one of the purported proton carrier aspartate residues and a nearby lysine residue suggested a transport mechanism likely to involve the membrane embedded lysine as a proton carrier [196]. Residues around the purported substrate binding site were mutated in the past and were shown to retain some antiporter activities for Na⁺ and Li⁺ [195]. However, antiporter activities of these mutants were not properly characterized.

**The Elevator alternating access mechanism** It is established that the sodium ion and proton compete for a common binding site in NapA [197]. Generally, to transport solutes across membranes the primary or secondary transporter has to undergo conformational changes that lead to opening and closing of the binding site on either side of the membrane [198]. To exchange Na⁺ and proton, NapA undergoes conformational changes involving large movement of the core domain harboring the binding site relative to the dimerization domain that is fixed by oligomerization, such that transport is carried out only by the core domain and is referred to as the elevator alternating access model (fig. 12 and 13).
Figure 12. Alternating access model for Na’+/H’ antiporter. Surface depiction of a section through the outward facing structure of NapA (left) and an inward-facing model of NapA (right). The position of the conserved Asp157 denoted with a pink in asterisk and only one molecule is shown for the sake of clarity. Figure is adapted from [172].

Figure 13. Schematic presentation of the proposed transport mechanism, illustrating the conformational changes with the Core domain moving against the Dimerization domain. Protons (shown in blue) bind to the Core domain in the outward-facing conformation (left) causing it to switch to the inward-facing conformation (right). On the inside, protons are exchanged for sodium (green) and the Core domain moves back to the outside. During this process, Asp157 (shown in red) which is critical for binding of one of the protons and the sodium ion, moves approximately 10 Å. Other residues involved in ion binding are not shown. Figure is adapted from [172].

Beside the “elevator” alternating access mechanism, other transport models have been identified in transporters like the “rocker-switch” mechanisms (fig. 14 a) for transporters with two structurally similar domains. A good example of such proteins operating by this model is the proton-coupled lactose symporter LacY from E. coli [171]. Transporters with structurally divergent domains operate by what is known as the “rocking-bundle” mechanism (fig. 14 b)
and the bacterial leucine transporter LeuT is the best characterised transporter operating by this mechanism [199].

**Figure 14.** Illustration of alternating access mechanisms observed in protein transporters (a) Rocker-switch, (b) rocking-bundle and (c) elevator mechanism. The movement of domains represents the biggest differences between mechanism. Figure adapted from [200].
During photophosphorylation, the electron transport chain in the thylakoid membrane of the chloroplast uses redox electron transfer reactions to extract energy from sunlight by converting NADP\(^+\) to NADPH and water to oxygen. This reaction is accompanied by translocation of H\(^+\) across the thylakoid membrane to generate a proton motive force that is used to synthesize ATP from ADP and P\(_i\). In the next phase, sugars are formed from CO\(_2\) in the presence of ATP and NADPH initially produced. In the IMM where oxidative phosphorylation (OXPHOS) occurs, the electron transport chain here known as respiratory chain (RC) also uses redox reactions to extract energy by transferring electrons from sugar (e.g. glucose) to O\(_2\) to form water, which is accompanied by translocation of H\(^+\) across the IMM to the IMS to generate proton motive force that drives ATP synthesis from ADP and P\(_i\). Therefore, proton motive force is vital for both synthesis and oxidation of sugar. Electrons from sugars are supplied to the respiratory chain in the form of NADH and succinate, which are some of the products of the citric acid cycle. Beside succinyl-CoA, NADH is also one of the products of the KGDH catalyzed reaction. Electron from NADH goes through complex I to ubiquinone while that from succinate goes through complex II to ubiquinone (fig. 15). ATP generated during OXPHOS is used by Na\(^+\)/K\(^+\) ATPase to transfer 3Na\(^+\) ions across the membrane where they are released in exchange for 2K\(^+\) [178]. Its action creates an inward directed Na\(^+\) gradient (fig. 15) which is used by the Na\(^+\)/H\(^+\) exchanger to transport H\(^+\) out in exchange for Na\(^+\) [178]. Although Na\(^+\)/H\(^+\) exchangers do not bind or use ATP directly, they depend indirectly on the availability of ATP for optimal action. This is justified by reports of reduced and complete termination of function of some Na\(^+\)/H\(^+\) exchangers due to acute ATP depletion, despite presence of high H\(^+\) gradient across the membrane [201-203]. Sodium proton antiporters make use of proton motive force to exchange H\(^+\) for Na\(^+\) across membranes. In mammals about nine isoforms of sodium proton exchanger (NHE) have been identified and their activities have been reported in plasma and inner mitochondrial membranes [183, 184].
Figure 15. Illustration of interconnection between the function of KGDH, OXPHOSE and the Na⁺/H⁺ exchanger. How electrons leave NADH and succinate to the OXPHOS, generating the proton motive force that drives ATP synthesis. Na⁺/K⁺ ATPase uses energy from ATP hydrolysis to generate an inward directed Na⁺ gradient that drives the exchange of proton for sodium ion by Na⁺/H⁺ exchanger.
Aims

1. Cbp3-Cbp6 complex plays a dual role in the biogenesis of \(bc_1\) complex. Lack of Cbp3 or Cbp6 leads to inefficient translation of Cytb and assembly of \(bc_1\) complex [56]. However, there is very limited understanding of how these mitochondrial respiratory chain assembly factors work. A major aspect of the difficulties in unravelling the molecular mechanisms of their functions is to determine their structures. Here, the aim is to determine the structure of Cbp3 and identify its interaction sites with Cbp6, cytochrome \(b\) and other proteins involved in the biogenesis of \(bc_1\) complex. Cbp3-Cbp6 is involved in the feedback loop that links Cytb translation to \(bc_1\) complex assembly. New round of Cytb synthesis requires that Cbp3-Cbp6 complex is released from intermediate I [57]. Therefore it is important to determine if shuttling of free Cbp3-Cbp6 during \(bc_1\) biogenesis is altered in respiratory deficient Cytb mutants.

2. Cytb as a core catalytic subunit of the \(bc_1\) complex plays a vital role in the generation of ATP in the mitochondrial. Cytb mutations associated with deficient \(bc_1\) complex affect the overall function of the mitochondria. However, the exact step at which \(bc_1\) assembly is targeted in the mutants is unclear. Our aim is to determine where and how \(bc_1\) assembly is impaired in respiratory deficient Cytb mutants. Electrons passing through the mitochondrial respiratory chain come from NADH and succinate. KGDH catalyses the formation of NADH and succinyl-CoA in the citric acid cycle. The activity of KGDH is inhibited by NADH and succinyl-CoA [148, 204]. Therefore the activity of KGDH is related to the efficiency of mitochondrial respiratory chain.

3. Kgd4 is a novel subunit of KGDH, existing in two functional variants of unknown origins in yeast; a long (Kgd4L) and a short (Kgd4S) forms. Also, it is confirmed that in yeast there is a single intronless mRNA of Kgd4 [158, 159], thus, alternative splicing can not be responsible for the two isoforms. The main goal of this work is to determine the origin of the two isoforms of Kgd4. Electron from NADH via the respiratory chain builds the driving force for ATP synthesis. The energy from ATP hydrolysis is used by \(Na^+\)/K\(^+\) ATPase to build \(Na^+\) gradient that drives \(Na^+\)/H\(^+\) exchange across the IMM.

4. Sodium-proton antiporters of the CPA1 and CPA2 clades have two strictly conserved aspartate residues, assumed to be responsible for the transfer of protons across membranes in exchange for sodium ion [194, 195]. However, structures of NhaA and NapA revealed a salt bridge between one of the conserved aspartate residues and a
membrane embedded Lys305. This suggests a different transports mechanism that may involve Lys305. Our mission was to determine the effect of pH to Na\(^+\) and K\(^+\) driven transport activity of NapA wildtype, mutants of Lys305 and other residues around the proposed ion binding site of NapA and also to determine the electrogenicicity of human NHA2.
Summary of papers

Paper I

Structural basis of Cbp3 interactions with newly synthesized cytochrome b during mitochondrial respiratory chain assembly.

After cloning and screening several bacterial and eukaryotic (including human) homologues for expression, purification and crystalization for structure determination, Cbp3 from Brucella abortus (BaCbp3) turned out to be the best candidate and its atomic structure was determined at 1.4 Å, representing the first structure of the ubiquinol-cytochrome c chaperone domain family. The BaCbp3 structure was also used as a template to investigate further the properties of the Cbp3 family and most importantly to shed light on the interactions of Cbp3 with other components of the bc1 complex. Using SWISS-MODEL server [205], a homology model for Cbp3 from Saccharomyces cerevisiae (ScCbp3) was calculated and the resulting model of the yeast’s ubiquinol-cytochrome c chaperone domain corresponded to residues 115-294, with most of the yeast specific N-terminal domain lacking. The core of the ScCbp3 model is similar to that of BaCbp3 with strongly conserved central hydrophobic residues. ScCbp3 and BaCbp3 present similar profile of surface potential, although the negatively charged cavity holding the Mg²⁺ binding sites in BaCbp3 is less prominent and appears more shallow and hydrophobic in ScCbp3. The interactions of the ubiquinol-cytochrome c chaperone domain of ScCbp3 was investigated using photocrosslinking, which is a decent method that has been used for identification and characterization of transient interactions during chaperone-assisted protein folding [206-208], such as the case of Cbp3-Cbp6 in Cytb synthesis and assembly [56]. The integrity of cellular proteins is maintained by chaperones via transient interactions with misfolded or nascent proteins to promote their folding [209, 210]. Cbp3-Cbp6 like other protein complexes, are not static and, in the presence of partner proteins or ligands, conformational changes may alter the topology and or protein-protein interface. Such local and transient changes in protein-protein interactions may be vital for their functions and therefore in vivo photo-crosslinking approach is useful to unravel the dynamics of momentary interactions in living cells [211]. Here we have mapped the interaction sites of Cbp3 with Cbp6 and cytochrome b which shows that two adjacent, nonoverlapping segments of Cbp3 interact with Cbp6 and cytochrome b concurrently. Based on our results a potential mode of interaction of Cbp3 during cytochrome b synthesis could involve the N-terminal domain binding to the mitoribosome, positioning the ubiquinol-cytochrome c chaperone domain in
proximity to the peptide tunnel exit (TE) to allow efficient recognition of nascent cytochrome 
b polypeptide chain emerging at the TE.

**Paper II**

Functional characterization of disease-causing cytochrome b mutations.

Cytochrome b plays a central role in energy conversion in mitochondria. Hence mutations in 
Cytb that impair its functions or assembly into the bc1 complex would have negative impacts 
on ATP production and hence on overall mitochondrial performances. Cytb mutations like 
Y279C in extramembrane segment [90], in frame deletion of residues 252-259 [91] and 
G291D [97] have been isolated in patients suffering from exercise intolerance. However, the 
molecular consequences of these mutations are not known. Cytochrome b in human and S. 
cerevisiae share just 55% sequence similarities, but consensus amino acids residues of the 
extramembrane loops (130-150 and 170-190) constitute part of the Qo (quinol oxidation site) 
and amino acids segment 20-40, which is part of the Qi (quinone reduction site) reaction 
center are highly conserved [40]. The intramembrane hydrophobic regions harboring the 
heme-ligating histidines, and the extramembrane segment to the histidine residues ligating the 
bL heme are also conserved [40]. Because these human mutations are located within protein 
segments that share high percent identity with yeast’s and relevant for catalyzes (Qo and Qi 
reaction centers and heme-ligating histidine), S. cerevisiae was used to analyze the molecular 
effects of the mutations on bc1 assembly and function. Our results show that the following 
Cytb mutants; T46K+G252D, E272V, E272P, Y297W, Y279C, G291D and 252-259 deletion 
are respiratory deficient although to varying extends. We showed that in mutants 
T46K+G252D, E272V and E272P the observed respiratory defect is related to blockage of bc1 
assembly at intermediate 0 due to impaired hemylation at the bL site. Assembly of bc1 is 
blocked at intermediate I in mutants Y279C and G291D due to impaired insertion of the high 
potential heme group at bh site.

**Paper III**

Alternative translation initiation at a UUG codon gives rise to two functional 
variants of Kgd4.

Our recent findings showed that the difference between both Kgd4 isoforms is in their N-
termini [160]. Translation initiation from a non-methionine start codon had been reported in
yeast [142]. Sequencing around the five prime untranslated region (5’ UTR) of Kgd4 mRNA revealed extra codons upstream and in frame with the annotated start site, with no interrupting stop codon [160]. We revealed a UUG located 90 nucleotides upstream of the authentic start site as an alternative translation initiation start site. The translation machinery scans through Kdg4 mRNA from the 5’ end reading first the UUG codon and then the AUG, but only when it fails to initiate translation at the alternative start site [160]. Kgd4L is formed when translation initiation occurs at UUG, while Kgd4S is formed when initiation occurs at the authentic start site [160]. We showed that the context of the nucleotides sequence upstream of UUG determines the efficiency of translation initiation from this site. Strains expressing only the Kgd4S variant showed more activity than the wildtype whereas Kgd4L strain showed wildtype activity. Both isoforms are imported into the mitochondria and support the stability and function of KGDH complex.

**Paper IV**

Dissecting the proton transport pathway in electrogenic Na⁺/H⁺ antiporters.

Na⁺/H⁺ antiporters are members of a large superfamily of monovalent cation:proton antiporter (CPA) which includes CPA1, CPA2 clades and others. It is generally assumed that members of CPA1 clade catalyze electroneutral exchange activity with 1H⁺:1Na⁺ or 2H⁺:2Na⁺ stoichiometry reported while members of CPA2 clades catalyze electrogenic exchange activity with 2H⁺:1Na⁺ or 3H⁺:2Na⁺ stoichiometry reported in some cases. The most studied antiporter so far is NhaA, a member of the CPA2 clade from *Escherichia coli*. It has two conserved aspartate residues assumed to be crucial for transport and responsible for the transport of two protons across membrane while just one aspartate residue is conserved in electroneutral antiporters [194, 195]. Crystal structures of NhaA and NapA revealed a salt bridge between one of the conserved aspartate (Asp156) residues and a lysine residue (Lys305), which suggests a possible role of K305 in proton transport. To verify this, we mutated Lys305 into positive, negative and neutral residues. Except for the histidine variant that showed wild type activity, all Lys305 variants tested showed inability; (i) to be stimulated by dissipation of membrane potential (ii) to be driven by an electric gradient and (iii) to generate an electric potential when driven by ions gradients. Our findings show that Lys305 is essential for electrogenic transport activity. Also we showed that an inactive Asp156Asn (D156N) mutant could be rescued by an additional mutation of the Lys305Gln (K305Q) that restored the salt bridge.
Like in NhaA and NapA, human NHA2 (SLC9B2) has two equivalent aspartate residues in its ion binding site. Unlike NhaA and NapA, the equivalent residue to Lys305 has been replaced by arginine. The K305R NapA mutant show poor electroneutral antiporter activity which is consistent for a residue that can bind and release protons at neutral pH. If Lys305 is essential for proton transport, then it would mean that the activity of NHA2 should be electroneutral due to the presence of lysine to arginine mutation. Our data suggests that NHA2 is electroneutral. We also showed that K305 is essential in proton transport and for conferring electrogenicity of the antiporters.
**Conclusion and outlook**

Efficient synthesis of cytochrome \( b \) and early assembly of the \( bc_1 \) complex depend on the complex formed between Cbp3 and Cbp6 [57]. To achieve this, Cbp3-Cbp6 complex has to position itself in proximity to the polypeptide exit tunnel on mitoribosomes translating \( COB \) mRNA [56]. To understand mechanistically how this dual function is accomplished, knowledge on the structure and interactions of Cbp3 with Cbp6, cytochrome \( b \) and the mitoribosome is instrumental. In an effort to address this, we have solved the first atomic structure of the ubiquinol-cytochrome \( c \) chaperone domain of Cbp3 from *Brucella abortus* (*BaCbp3*). We used the *BaCbp3* structure as template to model and map the interaction surfaces of Cbp3 from *S. cerevisiae* (*ScCbp3*) with Cbp6 and cytochrome \( b \). One previous work showed that Cbp6 is only stable in the presence of Cbp3 [56], and we show here that only full length Cbp3 can rescues respiratory impaired \( \text{cbp3} \Delta \) yeast strains. Based on our data, our current model suggests that the mitochondrial specific N-terminal domain is involved in interaction with mitoribosome such that the ubiquinol-cytochrome \( c \) chaperone domain is positioned along the tunnel exit, where it binds the nascent cytochrome \( b \) (*Paper I*). In the future, it would be interesting to map potential interaction sites in the N-terminal domain of *ScCbp3* and determine if it indeed makes contact with any of the ribosomal proteins that have been reported to be located around the tunnel exit of the mitoribosome such as Mrpl4, Mrpl22, Mrpl20 and Mrpl40 [56, 212]. Efforts should also be put to determine the structure of Cbp3-Cbp6 bound nascent cytochrome \( b \) on mitoribosome by cryo-EM to have an insight of the process.

In the second project, we were able to analyze and determine the molecular impact of some disease causing mutations in cytochrome \( b \), using yeast as a model. Our findings showed that each of the mutations affected \( bc_1 \) assembly at either intermediate 0 due to impaired insertion of \( b_L \) heme or at intermediate I due to impaired insertion of \( b_H \) heme. Also, all mutants showed reduced cytochrome \( b \) content compared to the wildtype (*Paper II*). Because cytochrome \( b \) from human and yeast share just 55 % similarity, our results with the yeast model may not directly depict what happens in human. These mutations should be analyzed further in higher eukaryotes such as mouse.

In the third project, we investigated the biogenesis of Kgd4, a recently identified subunit of alpha-ketoglutarate dehydrogenase complex of the citric acid cycle [154]. Yeast contains an intron-less mRNA for Kgd4, but two isoforms of Kgd4 exist that cannot be justified by
alternative splicing. The use of non-AUG as alternative translation initiation codon has been reported in bacteria, viruses and yeasts and has accounted for numerous truncated isoforms of proteins in cells [138-140]. Our objective was to determine the origin of the two functional variants of Kgd4. We have shown that the large form originates from a UUG alternative start codon which is 90 nucleotides upstream and in frame of the annotated start codon. We also showed that the sequence context directly upstream of UUG determines its recognition, that both forms are imported into the mitochondria, and that they stabilize and contribute to the activity of KGDH (Paper III). Those findings would imply that the PIC scans through the mRNA of Kgd4 in the 5’ to 3’ direction and Kgd4L is produced only when translation is initiated at UUG, while Kgd4S is produced only when initiation at UUG failed and AUG is used instead. Under stress conditions such as hypoxia or nutrient deprivation, alternative translation initiation mechanisms mediated via upstream open reading frame (uORF) or internal ribosomal entry site (IRES) is favored [213]. We observed that Kgd4L showed wildtype activity while Kgd4S showed activity higher than wildtype. This may suggest that the extra N-terminal segment in Kgd4L could have a regulatory role on the overall activity of the complex such that it maintains basal activity when physiological needs of any of the products of KGDH catalyzed reactions (NADH, CO2 and succinyl-CoA) are low. The frequency of translation initiation at UUG depends on the sequence context upstream of UUG. Therefore, at any given time the ratio of Kgd4L/Kgd4S in the cell is determined by the success rate of translation initiation at UUG. In future, it would be very interesting to determine the role of the N-terminal extension. Efforts to determine high resolution structures of the complex with the short and long isoforms may throw more light on the physiological role of the N-terminal extension.

Finally, the crystal structure of NhaA from E. coli and NapA from T. thermophiles revealed a salt bridge between one of the two conserved aspartate residues said to be responsible for proton transport (D156) with a membrane embedded lysine K305. We generated mutants of K305 whose transport activities were screened and characterized and our result showed that, contrary to past assumptions, K305 is the carrier of one of the protons and that it was responsible for the electrogenicity of NapA. To confirm this, we produced human NHA2, which contains the two conserved aspartate residues as in NapA but lacks K305 showed electroneutral antiporter activity (Paper IV). In future it would be interesting to determine the structure of human NHA2 to determine if any of the conserved aspartate residues is involved in the salt bridge.
Sammanfattning på svenska

Mitokondrier är som kraftverk, där det mesta av energin som eukaryota celler använder, känt som Adenosin trifosfat (ATP) produceras. Mitokondrier är omgivna av två skyddande membran; ett inre mitokondriellt membran (IMM) och ett yttre mitokondriellt membran (OMM) som fungerar som säkerhetscheckpunkter för vad som går in och ut. Bortsett från cellens övergripande informationscenter (genomiskt DNA eller gDNA) innehåller mitokondrier sitt eget informationscenter (mtDNA). Olika proteiner från gDNA och mtDNA samlas för att bilda en maskineri som kallas OXPHOS i IMM, som använder syret vi andas för att tillverka ATP. Som nämnt innan består OXPHOS maskineriet av ett antal proteiner. Cytokrom b (Cytb), är ett sådant protein och primära subenheten för bc1-komplexet som spelar en central roll i OXPHOS. Två proteiner; Cbp3 och Cbp6, sammankommer för att bilda Cbp3-Cbp6-komplexet som är väsentligt för syntesen av Cytb och sammanställning av bc1-komplexet. I det första projekten min avhandling presenterar jag den första kristallstrukturen för Cbp3 från Brucella abortus (BaCbp3) som vi har använt som modell för att visa hur Cbp3 från Sacharomyces cerevisiae (ScCbp3) associerar med Cbp6 och Cytb under syntes och montering av bc1-komplexet. I det andra projektet analyserade vi mutationer i Cytb som orsakar sjukdomar och här har vi visat att sjukdomsutvecklingen i mutanterna är relaterad till försämrad införing av hem molekyler (en organisk molekyl med en järnatom i mitten) i Cytb. Det tredje projektet rör Kgd4, en ny subenhet av ett enzym involverad i citronsyrcyclen; α-ketoglutaratdehydrogenas (KGDH). KGDH katalyserar syntesen av NADH, som är en av många substrat i OXPHOS. Här identifierade vi att översättningen av ett alternativt startkodon, UUG, lokaliseras uppströms den anoterade startkodonet är Kgd4L-isoformens ursprung (en längre form av Kdg4) medan Kgd4S (en kortare form av Kgd4) produceras från det anoterade startkodonet först när initiering vid UUG misslyckas. Vi observerade även att båda formerna importereras till mitokondrier och ansluter sig med KGDH-komplexet. Det fjärde projektet rör ett protein som byter ut protoner (H⁺) mot natrium joner (Na⁺) i cellerna. Dessa transportörer är närvarande i alla organismer och är involverade i reglering av intracellulärt pH, cellvolym och Na⁺-jon koncentration. Vi rapporterar här att aminosyran K305 i NapA (en transportör från bakterien Thermus termophilus) deltar i protontransport och är ansvarig för utbytet av 2H⁺ för 1Na⁺ medan human NHA2 byter ut 1H⁺ för 1Na⁺.
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