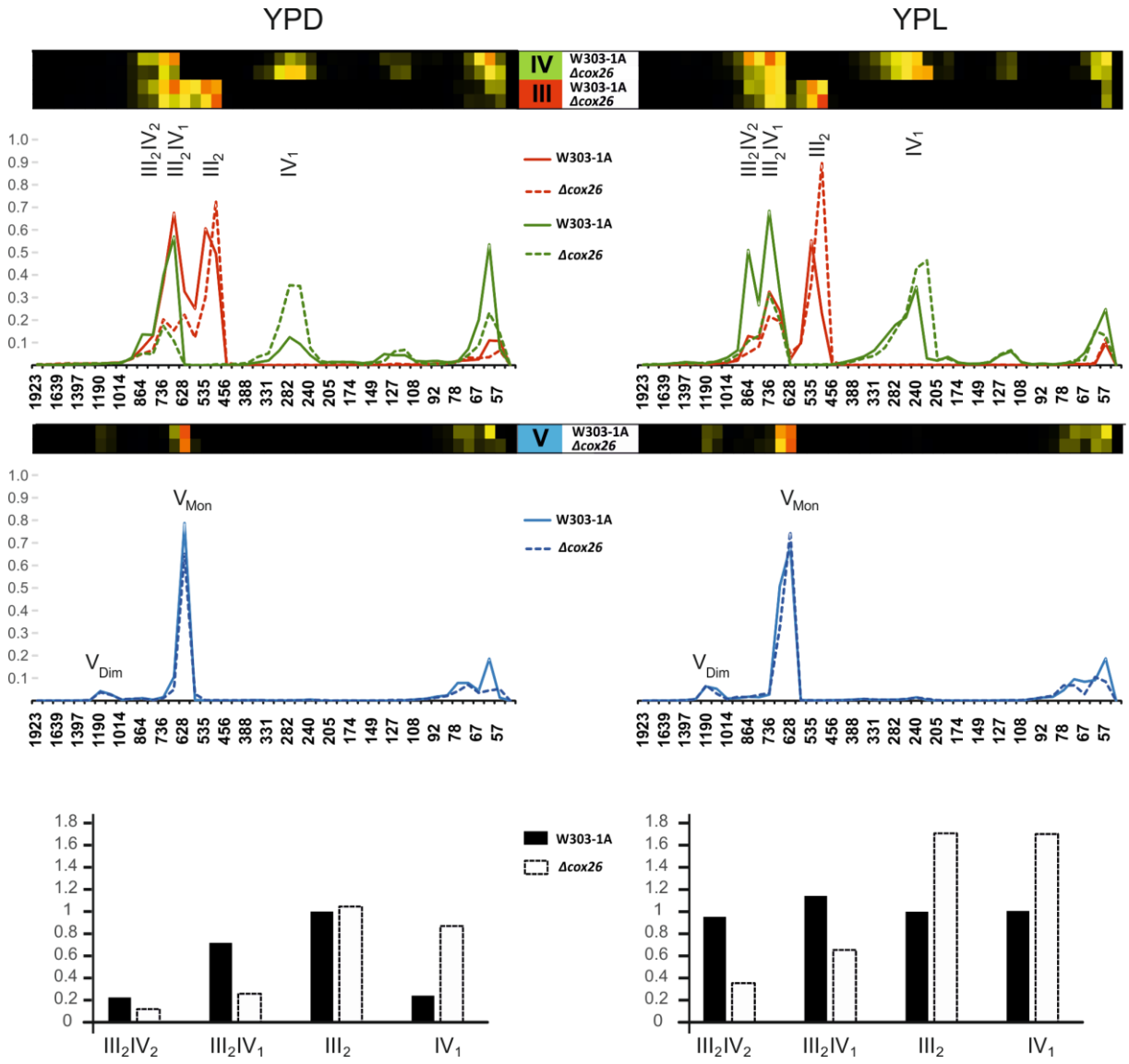
Supplementary material

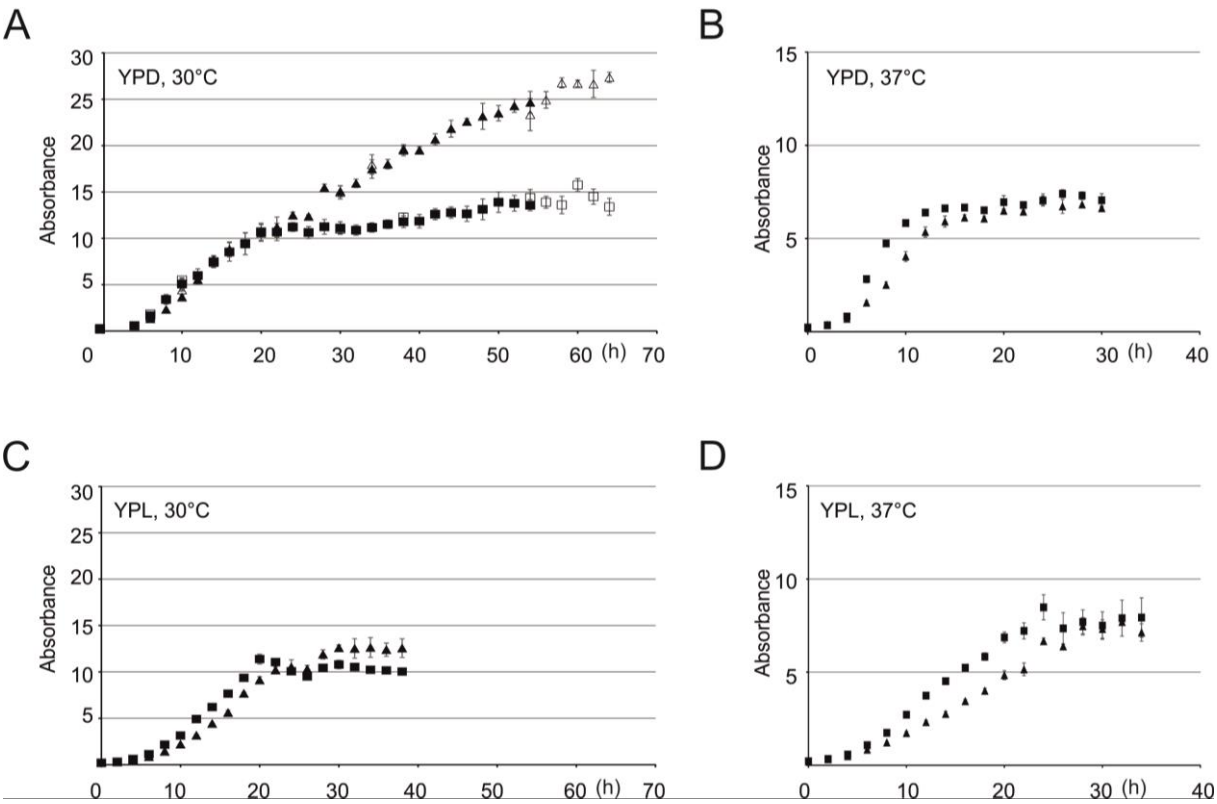
Supplementary Table 1. Estimation of the catalytic activities of complex III in the mitochondrial membrane, measured as NADH:cytochrome *c* oxidoreductase activity, and of respiratory complex IV, determined as cytochrome *c* oxidase activity. Cells were cultured in YPL and catalytic activities were analyzed according to [1].

Mitochondria	NADH:cytochrome c oxidoreductase (µmol/min x mg)		Cytochrome c oxidase (µmol/min x mg)	
	no detergent	+ deoxycholate	no detergent	+ deoxycholate
W303-1A	0.83	0.16	1.05	1.26
Δcox26	0.64	0.13	1.04	1.47



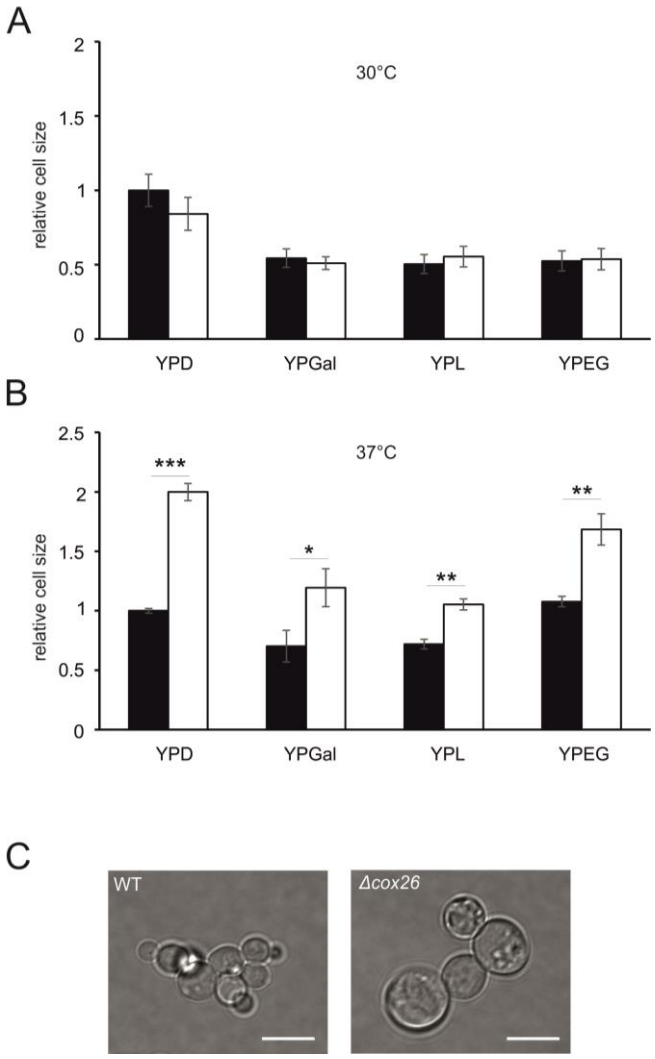
Supplementary Figure 1.

Reference profiles were calculated as average from all subunits of complex III, IV (excluding Cox26) and V and showed as 2-D profiles for cells grown in YPD (left panel) and YPL (right panel). Each complex was quantified by adding values according to their appearance and normalized to complex V monomer. The reference profile of complex IV was used for quantification of supercomplexes. Bar graphs were depicted relative to complex III monomer.

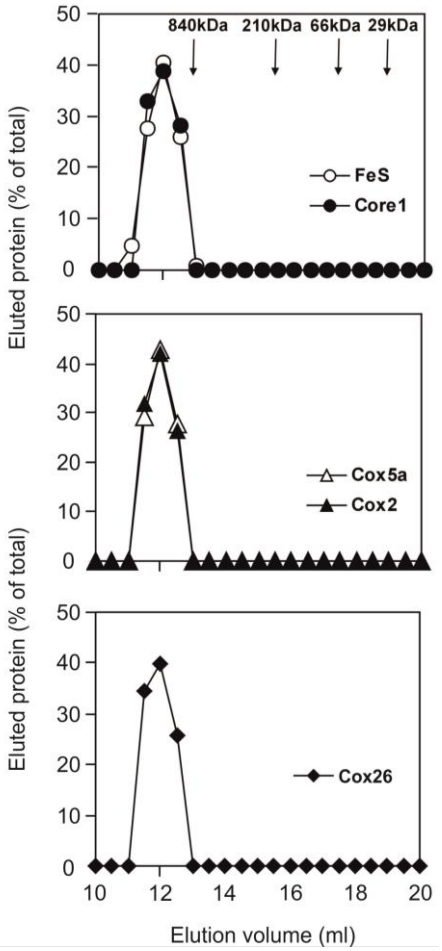


Supplementary Figure 2.

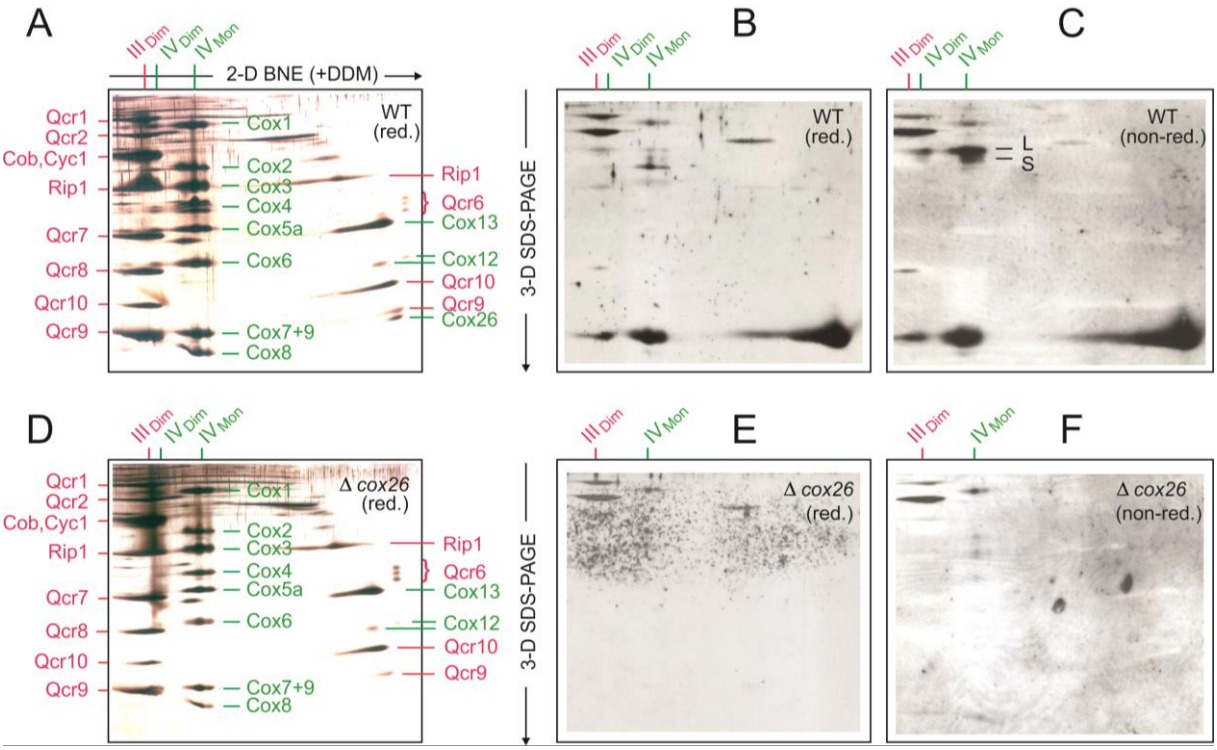
Growth curves of W303-1A and Δ*cox26* strains on fermentable and non-fermentable media. (A and B) Growth of W303-1A (■) and Δ*cox26* (▲) strains on glucose containing medium (YPD) at a temperature of (A) 30ºC and (B) 37ºC with a starting optical density of 0.2. An additional experiment was started for the analysis of *Δcox26* in YPD media at 30 °C to record late time points, depicted with empty symbols (□) - W303-1A and (Δ) - *Δcox26*. (C and D) Growth of W303-1A (■) and Δ*cox26* (▲) strains on lactate containing medium (YPL) at a temperature of (C) 30ºC and (D) 37ºC.



Supplementary Figure 3. Relative cell size of W303-1A (black bars) and Δ*cox26* strains (white bars) under various growth conditions. (A) Growth for 24 h at a temperature of 30 ºC in yeast peptone (YP) media containing 3% glucose (YPD), 2% galactose (YPGal), 2% lactate (YPL) and 2% ethanol, 3% glycerol (YPEG). (B) Growth for 24 h at a temperature of 37ºC. (C) Comparison of the relative size of W303-1A and Δ*cox26* strains after growth for 24 hours at 37 ºC, in YPD medium. Scale bars, 10 µm. We quantified cell size using ImageJ 1.44 from at least 80 cells of each experiment. The mean of each experiment was used to evaluate differences between conditions and strains. Error bars indicate SD; n=3; *, significant $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

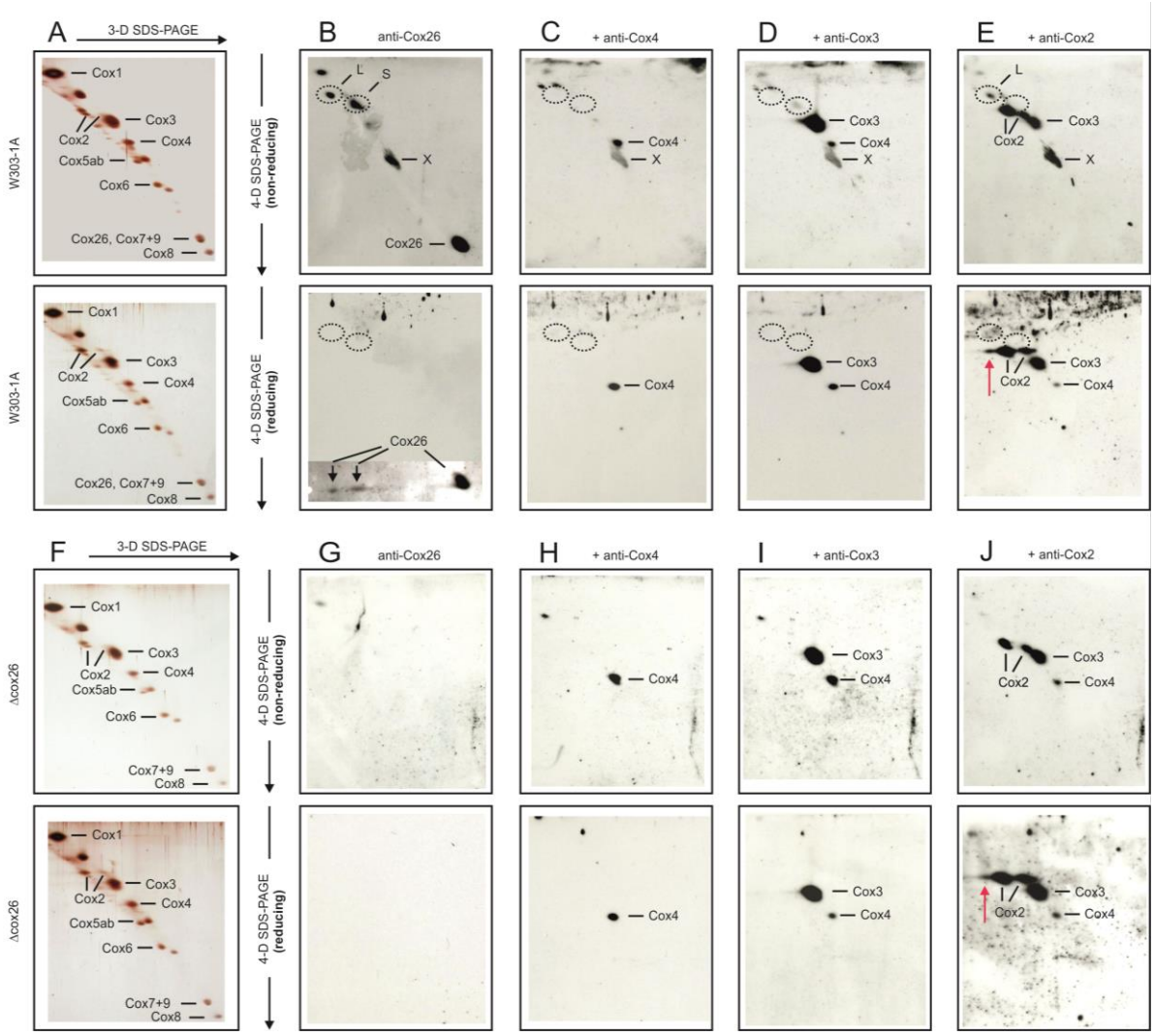


Supplementary Figure 4. Cox26 protein is associated with respiratory supercomplexes and is not present as individual free protein in yeast mitochondria. Mitochondria from W303-1A (1 mg protein) were solubilized in digitonin buffer and subjected to gel filtration analysis as described previously [2]. Cox26 protein eluted together with respiratory complexes III and IV. Antibodies detecting Core protein 1 (●, Core 1), the "Rieske" iron-sulfur protein of respiratory complex III (○, FeS), subunits Cox5a and Cox2 of complex IV (△), and Cox26 protein (◆) were used to identify the relevant proteins and complexes. Commercially available marker proteins were used for mass calibration: bovine erythrocyte carbonic anhydrase (29 kDa); bovine serum albumin monomer (66 kDa); cytochrome b2 (210 kDa); and Hsp60 (840 kDa).



Supplementary Figure 5. Evidence for hydrophobic and covalent interactions of Cox26 protein and complex IV. Assignment of complexes and subunits as in Fig. 1. Cox26 is the smallest of all detergent-labile proteins removed from respirasomes (assigned on the right side of figure part A). Respiratory supercomplexes from wild type (WT) and Δ*cox26* strain were isolated by 1-D BNE (not shown). The individual complexes and detergent-labile subunits were then released by 2-D modified BNE (+DDM) and resolved by 3-D Tricine-SDS-PAGE using 16% acrylamide gels containing 6 M urea. Silver-stained gels from (A) wild type and (D) Δ*cox26* strain and Western blots for wild type (B, C) and Δ*cox26* strain (E, F) are shown. Reducing conditions were applied for 3-D SDS-PAGE except for C and F that were processed under non-reducing conditions. Anti-Cox26 antibody identified Cox26 at three positions at the bottom of figure part C, which can be explained by dissociation of hydrophobic interactions under non-reducing conditions. Specifically, Cox26 protein was released from respirasomes by 2-D modified BNE (largest spot on the right side of figure part C), and from

monomeric and dimeric complex IV (smaller spots on the left side of figure part C). Detection of two bands with masses around 25-30 kDa (L and S in figure part C) and loss of these signals in figure part B using reducing conditions suggested cleavage of a disulfide bond between minor amounts of Cox26 protein and complex IV.



Supplementary Figure 6. Evidence for covalent interaction of Cox26 and Cox2 proteins in isolated complex IV. (A-E) Analysis of W303-1A wild type strain. (F-J) Analysis of Δ*cox26* strain. Gel pieces from 2-D gels (BNE/modified BNE, not shown) containing complex IV were resolved under non-reducing conditions by 3-D Tricine-SDS-PAGE using 9% acrylamide gels. 3-D gel strips were then incubated under non-reducing (upper panels) or

reducing conditions (lower panels) followed by 4-D Tricine-SDS-PAGE using 16% acrylamide gels containing 6 M urea. The 4-D gels were silver-stained (A and F) or blotted onto PVDF membranes (B-E and G-J). Polyclonal anti-Cox26 antibody identified individual Cox26 protein and two bands, L and S, under non-reducing conditions (B, upper panel), and two Cox26 protein spots that were dissociated from bands L and S under reducing conditions (B, lower panel). A local defect of the PVDF membrane (marked X) was also recognized by the antibody. Circles mark the actual or expected positions of bands L and S. (C) Anti-Cox4 and (D) anti Cox3 antibodies that were added consecutively, without using stripping protocols, to the same blot membranes identified individual Cox4 and Cox3 proteins but no bands L and S. Reusing the same blots, an anti-Cox2 antibody finally recognized two spots corresponding to individual Cox2 protein and also band L under non-reducing conditions (E, upper panel) in addition to the protein spots recognized before by the anti-Cox4 and anti-Cox3 antibodies. Performing 4-D SDS-PAGE under reducing conditions (E, lower panel), band L was no longer detected but a third Cox2 spot (red arrow) appeared, as expected upon dissociation of Cox2 from band L. However, since a faint spot was also observed in the corresponding Δ*cox26* control gel (red arrow in J, lower panel) part of the third Cox2 spot in Fig. E, lower panel, may be artificial. (F-J) Gallery of control gels using the Δ*cox26* strain.

[1] C.M. Cruciat, S. Brunner, F. Baumann, W. Neupert, R.A. Stuart, The cytochrome *bc*1 and cytochrome c oxidase complexes associate to form a single supracomplex in yeast mitochondria, J. Biol. Chem. 275 (2000) 18093-18098.

[2] Cruciat, C.-M., Hell, K., Fölsch, H., Neupert, W., Stuart, R.A. Bcs1p, an AAA-family member, is a chaperone for the assembly of the cytochrome *bc*(1) complex. *EMBO J.* 1999, *18*, 5226-33.