*for **PNAS***

Classification – Major: Biological Sciences

Classification – Minor: Biochemistry

Supporting Information

The human mitochondrial holocytochrome *c* synthase's heme binding, maturation determinants, and complex formation with cytochrome *c*

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Key Words

CCHL, cytochrome *c*, HCCS, heme, mitochondria

Supporting Materials and Methods

Construction of strains and plasmids. All oligonucleotide primer sequences, plasmids, and strains are given in Supplemental Table 2. *E. coli* strains TB1 and HB101 were used as host strains for cloning. To generate N-terminal hexahistidine-tagged HCCS, the human HCCS gene was PCR amplified from a cDNA clone (Origene) as described previously (1), digested, and ligated into the NcoI and HindIII sites in pET Blue-2 (EMD Biosciences) to generate pRGK402. To generate N-terminal GST-tagged HCCS, the human HCCS gene was PCR amplified from pRGK402, digested and ligated into the EcoRI and XhoI sites in pGEX 4T-1 (GE Healthcare) to generate pRGK403. To generate C-terminal Intein-tagged HCCS, the human HCCS gene was PCR amplified from pRGK402, digested, and ligated into the NdeI and SapI sites in pTXB21 (NEB) to generate pRGK404. The human cytochrome *c* gene (CYCS) was PCR amplified from cDNA clone MGC12367 (ATCC), digested, and ligated into the EcoRI and PstI sites in pRGK330 (2) to generate pRGK405. The cytochrome c_2 gene from *Rhodobacter capsulatus* (CYCA) was PCR amplified from pRGK389 (1), with an engineered initiation codon to exclude the native periplasmic signal sequence as depicted in Figure 4, digested, and ligated into the NcoI and XbaI sites of pRGK330 (2) to generate pRGK406. All nucleotide substitutions and insertions were engineered using the QuikChange I Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacturer's recommendations, and oligonucleotides were synthesized by Sigma-Aldrich. Each of the final constructs was sequenced to verify the mutation(s).

Proteomics analysis. Identification of GST-HCCS and co-purified cytochrome *c* was achieved by ESI-MS by the Donald Danforth Plant Science Center Proteomics and Mass Spectrometry Facility. Briefly, Coomassie-blue stained bands corresponding to 57 kDa GST-HCCS and the co-purified 12 kDa cytochrome *c* for the WT, C15S, and C18A variants were excised, washed and digested with trypsin overnight. Tryptic peptides were run on a 1hr gradient LC-MS/MS using an LTQ-Orbitrap. The data was searched using Scaffold (for GST-HCCS) or Mascot (for the cytochrome *c* variants) against a custom database including the sequences provided for each of the cytochrome *c* variants and the following variable modifications: deamidation on Asn and Glu, pyroglutamate formation, glutathione and heme on Cys, and Met oxidation. To confirm covalent heme attachment to the C18A variant of cytochrome *c*, the 12 kDa band corresponding to cytochrome *c* was excised, washed, and digested with trypsin overnight as described in (3). Digested samples were subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) using an IonSpec ProMALDI FT-MS 7.4 T mass spectrophotometer by the Washington University in St. Louis NCRR Mass Spectrometry Facility.

Supporting Figure Legends

Figure S1. Full-length GST-HCCS localizes largely to the membrane. Anti-GST immunoblot showing cleared sonicate (CS), soluble (S) and DDM-solubilized membrane (M) fractions from *E. coli* cells expressing N-terminal GST-tagged HCCS. Note the increase in full-length 57 kDa GST-HCCS in the DDM-solubilized membrane fraction (M, lane 3) relative to the soluble fraction (S, lane 2).

Figure S2. Identification of purified human HCCS by mass spectrometry. The results of LC-MS/MS on trypsin-digested, full-length 57 kDa GST-HCCS. 23 unique peptides were identified corresponding to human HCCS covering 57 % of the HCCS protein sequence. The sequence of each unique peptide along with the modification and the mass (in Daltons) is given.

Figure S3. Multiple sequence alignment of HCCS and HCC_1S genes from *Homo sapiens*, *Saccharomyces cerevisiae*, *Mus musculus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Plasmodium falciparum*, *Dictyostelium discoideum*, *Volvox carteri*, *Chlamydomonas reinhardtii*, and *Neurospora crassa*. Two histidine residues (boxed, arrows), His154 and His211 in the human HCCS, are completely conserved.

Figure S4. The X-ray crystal structures of **(A)** *E. caballus* cytochrome *c* (pbd HRC1) and **(B)** *R. capsulatus* cytochrome c_2 (pdb 1c2r) exhibit similar three-dimensional folding (4). Selected oxygen atoms are shown in red, nitrogens in blue, sulfurs in yellow, the heme macrocyle is in pink, and the Fe atom at the heme center is orange. Note the similar three-dimensional localization of the indicated residues (arrows) with respect to the heme group in the two structures. With the exception of *R. capsulatus* cytochrome c_2 Met95, amino acid designations in (A) and (B) refer to the *H. sapiens* cytochrome *c* N-terminal Met, as in Fig 3 and 4A (even though the initiation Met is cleaved off). PyMOL was used for displays.

Figure S5. Human cytochrome *c* variants co-purify with GST-HCCS. Heme stains showing the co-purification of each cytochrome *c* variant (WT, F11A, C15S, C18A, and H19A) with GST-HCCS. CS, cleared sonicate; S, soluble fraction; L, load (detergent-solubilized membranes); W1-W3, wash 1-wash 3; E, elution; M, molecular weight standards. In all cases, 12 kDa cytochrome *c* co-purified with HCCS (E, lanes 7). 12 kDa holocytochrome *c* is indicated by arrows. Note the abundance of holocytochrome *c* in the detergent-solubilized membrane fractions (L, lanes 3) and in the subsequent purified elution fractions (E, lanes 7) for the C15S and C18A variants relative to that in the soluble fractions (S, lanes 2). Relative levels of holocytochrome *c* bound to HCCS are shown in Fig 5A and quantified in Table 1.

Figure S6. Analysis of GST-HCCS and co-purified human cytochrome *c*. (A) Coomassie stain showing concentrated elution fractions for each of the complexes of HCCS with cytochrome *c*. Full-length 57kDa GST-HCCS and the co-purified 12 kDa cytochrome *c* variants are indicated by arrows. Note the abundance of the C15S and C18A variants relative to the WT, F11A, and H19A variants, quantified in Table 1. (B) Coomassie stain, (C) anti-GST immunoblot, and (D) heme stain showing a representative co-purification of a cytochrome *c* variant (C18A) with GST-HCCS. Note the absence of the 12 kDa cytochrome *c* in the wash fractions of the heme stain (panel D, lanes 5-7), indicative of a stable interaction between the co-purified cytochrome *c* and HCCS.

Figure S7. Identification of co-purified cytochrome *c* by mass spectrometry. The results of LC-MS/MS identifying WT, C15S, and C18A variants of cytochrome *c* co-purified with GST-HCCS. These data confirmed that the 12 kDa species co-purified with HCCS were variants of human cytochrome *c*. The sequence of each unique peptide along with the modification and the mass (in Daltons) is given.

Figure S8. Heme is stably attached (covalent) to the co-purified cytochrome *c*. Anti-GST immunoblot, anti-cytochrome *c* immunoblot, and heme stain of purified elution fractions for each cytochrome *c* variant co-purified with HCCS(WT) comparing unboiled samples (lanes 2-7) to those boiled and treated with 8 M urea (lanes 9-14). The full-length 57 kDa GST-HCCS and 12 kDa cytochrome *c* (apo- and holo- forms) are indicated by arrows. Boiling and treatment with 8 M urea did not remove the heme from the co-purified cytochrome *c* variants, suggesting that heme binding to cytochrome *c* in the complex with HCCS is covalent.

Figure S9. Spectroscopic analyses of the indicated HCCS:heme:cytochrome *c* complexes. UV-Vis absorption spectra (360 nm-700 nm) for the complexes of HCCS with (A) WT, (B) F11A, (C) C15S, and (D) C18A cytochrome *c* variants. Maxima are indicated by arrows, and the region from 500 nm to 700 nm has been multiplied by a factor of three.

Figure S10. Spectroscopic analyses of the indicated HCCS:heme:cytochrome *c* complexes in the presence of imidazole. UV-Vis absorption spectra (360 nm-700 nm) for the complexes of HCCS with (A) WT, (B) F11A, (C) C15S, and (D) C18A cytochrome *c* variants in the presence of imidazole (100 mM). Imidazole addition did not alter the spectra of any complex of HCCS with cytochrome *c*, with the exception of the H19A variant (Figure 5B). Maxima are indicated by arrows, and the region from 500 nm to 700 nm has been multiplied by a factor of three.

Figure S11. Heme is covalently attached to the HCCS-co-purified cytochrome *c*(C18A) variant. The results of MALDI-TOF mass spectrometry on trypsin-digested 12 kDa C18A cytochrome *c* variant co-purified with HCCS. The peak corresponding to the species with mass of 1617.381 Daltons was within 0.3 Daltons of the calculated neutral monoisotopic mass predicted for a covalent heme-containing peptide of the mutated heme binding motif in the C18A variant of cytochrome *c* (CSQAHTVEK). The presence of this species indicates that heme is bound covalently to the C18A variant of cytochrome *c* (through Cys15) in the complex with HCCS.

Figure S12. Predicted structural outcomes of the *R. capsulatus* cytochrome c_2 variants engineered in this study. Comparison of the PEP-FOLD-generated three-dimensional structures of the N-termini (19-20 residues) of (A) WT *R. capsulatus* cytochrome c_2 (B) cyt c_2(Ala-ins) and (C) cyt c_2(E8K/E10I/Ala-ins). Selected oxygen atoms are shown in red, nitrogens in blue, and sulfurs in yellow. Each structure has been oriented such that residue Cys15 is in the same position. Amino acids important for maturation are indicated by arrows. For clarity, amino acid designations refer to the *H. sapiens* cytochrome c N-terminal Met. Neither WT cyt c_2 nor cyt c_2(Ala-ins) were detectably matured by human HCCS, while cyt c_2(E8K/E10I/Ala-ins) was a robust substrate for the human HCCS. Note the shift in orientation of E8, E10, and F11 in (B) as a result of the insertion of an Ala residue. Also note that in each case, residue His19 was not part of the predicted helix, suggesting that it may have some flexibility, possibly related to its function as a ligand to the heme in HCCS.

Comment on the evolutionary implications of cytochrome c_2 maturation by human HCCS.

The evolution of the HCCS enzyme occurred after the endosymbiotic event that led to the eukaryotic mitochondrion. This is supported by the fact that the mitochondria of some eukaryotes (e.g., all plants and some protozoa) have retained CCM for the synthesis of *c*-type cytochromes. CCM is utilized by alpha proteobacteria, which are the ancestors of the eukaryotic mitochondrion. CCM is "promiscuous" with regard to substrate specificity, requiring little else than the conserved CXXH heme binding motif for recognition and heme attachment. Most alpha proteobacterial cytochromes *c* (~90 %, or 173 out of 200 analyzed) have a two-residue spacing between conserved Phe and the CXXCH motif. However, for all major groups of alpha proteobacteria, there are some cytochromes *c* with a three-residue spacing, and some that lack the Phe altoghether. Thus, there is not a strictly conserved N-terminal architecture in the alpha proteobacterial cytochromes *c*, which is not unexpected given the flexible substrate requirements of CCM (e.g., Phe is not a requirement for CCM recognition and heme attachment). Indeed, the fact that 90 % of the alpha proteobacteria have retained any conservation of N-terminal architecture (other than CXXCH) is surprising, and may reflect the importance of the conserved Phe, for example, in cytochrome *c* function(s) post-maturation by CCM.

By contrast, all eukaryotic mitochondrial cytochromes *c*, regardless of whether they are matured by CCM or HCCS, contain a conserved N-terminal architecture that consists of a three-residue spacing between conserved Phe (F11) and the CXXCH motif. Although the conservation of this architecture in the CCM-containing mitochondria is surprising, the fact that all HCCS-containing mitochondria possess cytochromes *c* with a three-residue spacing supports the idea that HCCS evolved with this specific architecture, and it is now "locked" into using it for attaching heme to its substrate. Furthermore, the conservation of N-terminal architecture in cytochromes *c* throughout eukarya suggests that the three-residue spacing may have originated in the cytochrome *c* of the mitochondrial progenitor. Consequently, the three-residue spacing is now embedded as part of the specificity determinants for recognition and maturation by HCCS. Animal HCCSs, like the human HCCS studied here, are less discriminatory, recognizing motifs within cytochrome *c* and cytochrome c_1 (e.g., such that Tyr11 can replace Phe11).

It is of note that the organisms from the Euglenozoa phylum, for which no cytochrome *c* assembly system has been discovered, contain cytochromes *c* with this same conserved N-terminal architecture (although lacking the first Cys of the heme binding motif). Allen and colleagues (5) have shown that a Euglenozoa (*T. brucei*) cytochrome *c* is matured by yeast HCCS in *E. coli* at very low levels (less than 1 % WT), even when the cytochrome has been engineered to contain the first Cys of the heme binding motif. *T. brucei* cytochrome *c* has the equivalent of Glu8 (as in *R. capsulatus* cytochrome c_2-see Figure 4), which might explain the low levels of maturation, but this has not been explored.

Figure S1

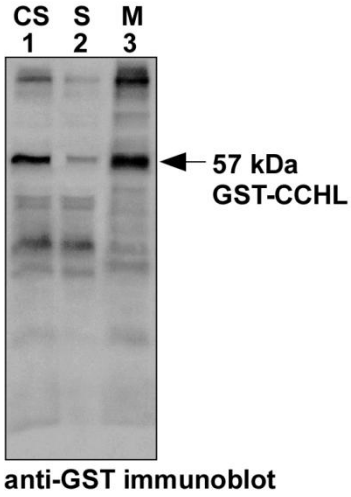
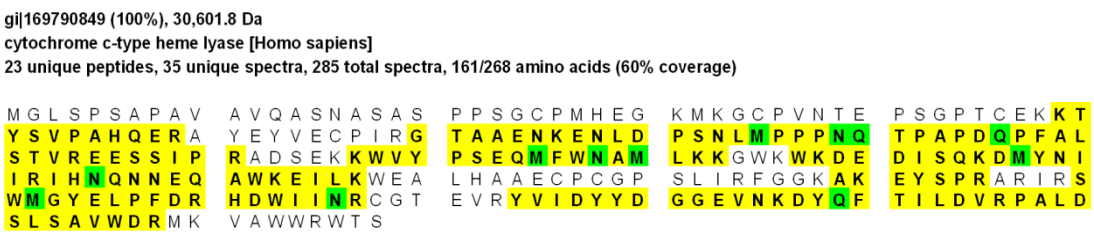
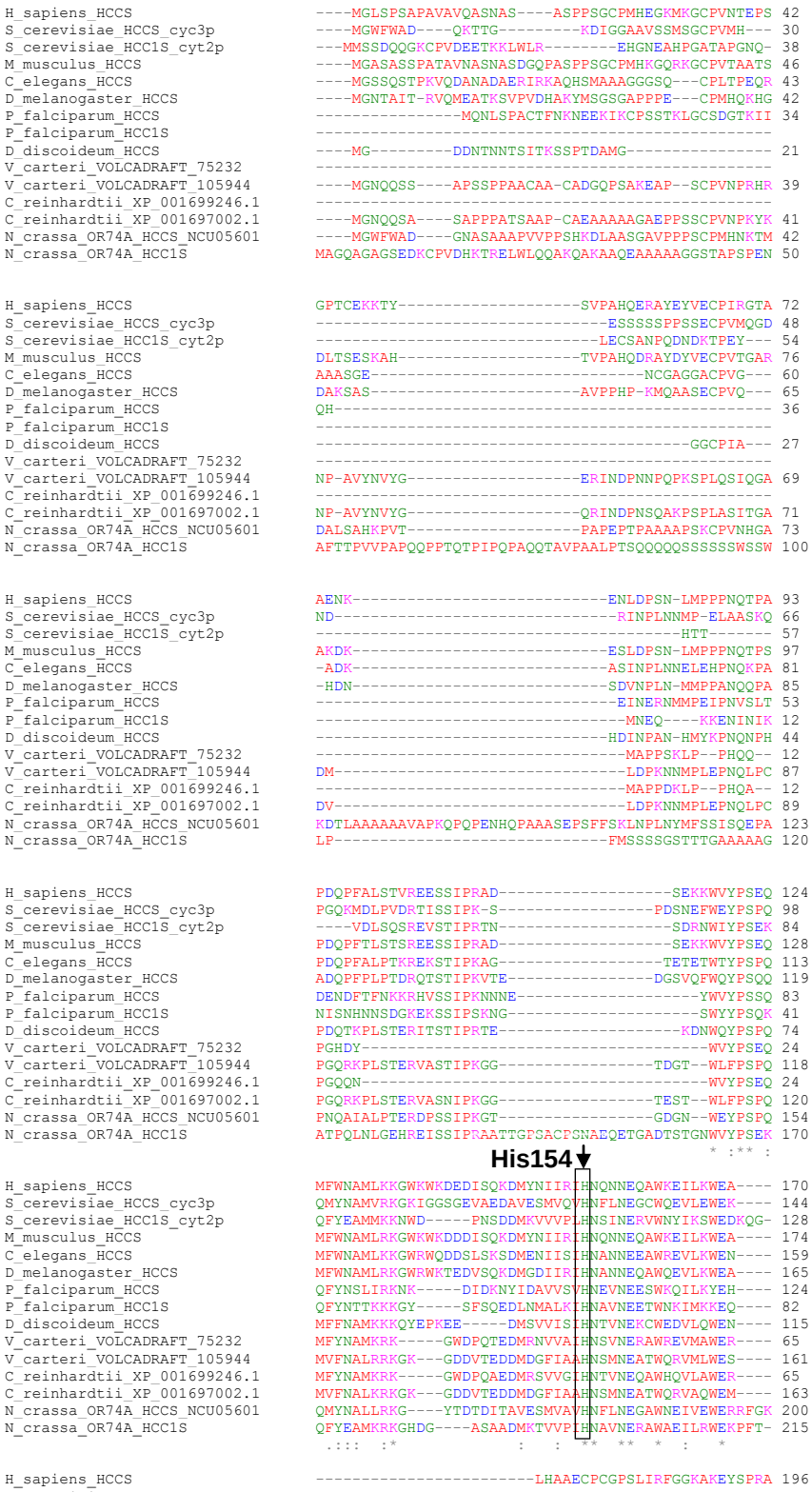


Figure S2



Sequence	Modifications	Observed	Actual Mass	Start	Stop
(K)DEDISQKDMYNIIR(I)		580.6139	1,738.82	139	152
(K)DEDISQKDmYNIIR(I)	Oxidation (+16)	585.9449	1,754.81	139	152
(K)DEDISQKDmYNIIR(I)	Oxidation (+16)	878.4138	1,754.81	139	152
(K)DEDISQKDMYNIIR(I)		870.4168	1,738.82	139	152
(K)ENLDPSNLMPPPNQTPAPDQPFALSTVREESSIPR(A)		1,282.30	3,843.87	77	111
(K)ENLDPSNLmPPPNQTPAPDQPFALSTVREESSIPR(A)	Oxidation (+16)	1,287.63	3,859.87	77	111
(R)GTAAENKENLDPSNLmPPPNQTPAPDQPFALSTVR(E)	Oxidation (+16)	1,245.28	3,732.81	70	104
(R)GTAAENKENLDPSNLmPPPNQTPAPDQPFALSTVR(E)	Oxidation (+16)	934.209	3,732.81	70	104
(R)GTAAENKENLDPSNLMPPPNQTPAPDQPFALSTVR(E)		1,239.94	3,716.81	70	104
(R)GTAAENKENLDPSNLmPPPNQTPAPDQPFALSTVREESSIPR(A)	Oxidation (+16)	1,511.40	4,531.19	70	111
(R)GTAAENKENLDPSNLMPPPNQTPAPDQPFALSTVREESSIPR(A)		1,506.07	4,515.20	70	111
(R)HDWIINR(C)		477.2509	952.4873	215	221
(R)IHNQNNEQAWK(E)		691.333	1,380.65	153	163
(R)IHNQNNEQAWKEILK(W)		622.3253	1,863.95	153	167
(R)IHNQNNEQAWKEILK(W)		932.985	1,863.96	153	167
(K)KTYSVPAHQER(A)		439.2316	1,314.67	49	59
(K)KWVYPSEQmFWNAMLK(K)	Oxidation (+16)	1,037.50	2,072.98	117	132
(K)KWVYPSEQMFWnAMLK(K)	Deamidation (+1)	1,029.99	2,057.97	117	132
(K)KWVYPSEQMFWNAMLKK(G)		729.3695	2,185.09	117	133
(K)KWVYPSEQMFWNAmLKK(G)	Oxidation (+16)	1,101.55	2,201.08	117	133
(R)SWmGYELPFDR(H)	Oxidation (+16)	708.8176	1,415.62	204	214
(R)SWMGYELPFDR(H)		700.8187	1,399.62	204	214
(R)SWmGYELPFDR(H)	Oxidation (+16)	708.8178	1,415.62	204	214
(R)SWmGYELPFDRHDWIInR(C)	Oxidation (+16), Deamidation (+1)	784.7057	2,351.10	204	221
(R)SWmGYELPFDRHDWIINR(C)	Oxidation (+16)	784.3722	2,350.09	204	221
(K)TYSVPAHQER(A)		594.2949	1,186.58	50	59
(K)WKDEDISQK(D)		574.7818	1,147.55	137	145
(K)WKDEDISQKDmYNIIR(I)	Oxidation (+16)	690.6702	2,068.99	137	152
(K)WKDEDISQKDmYNIIR(I)	Oxidation (+16)	518.2537	2,068.99	137	152
(K)WKDEDISQKDMYNIIR(I)		685.3389	2,052.99	137	152
(K)WKDEDISQKDMYNIIR(I)		514.256	2,053.00	137	152
(K)WKDEDISQKDmYNIIR(I)	Oxidation (+16)	1,035.50	2,068.99	137	152
(K)WKDEDISQKDMYNIIR(I)		1,027.50	2,052.99	137	152
(K)WVYPSEQMFWNAMLK(K)		965.4558	1,928.90	118	132
(K)WVYPSEQmFWNAmLK(K)	Oxidation (+16), Oxidation (+16)	981.4504	1,960.89	118	132
(K)WVYPSEQmFWNAmLK(K)	Oxidation (+16), Oxidation (+16)	654.6355	1,960.88	118	132
(K)WVYPSEQmFWnAmLK(K)	Oxidation (+16), Deamidation (+1), Oxidation (+16)	654.9654	1,961.87	118	132
(K)WVYPSEQmFWNAMLK(K)	Oxidation (+16)	973.4531	1,944.89	118	132
(K)WVYPSEQmFWNAmLK(K)	Oxidation (+16), Oxidation (+16)	654.6367	1,960.89	118	132
(K)WVYPSEQmFWNAmLK(K)	Oxidation (+16), Oxidation (+16)	981.4522	1,960.89	118	132
(K)WVYPSEQMFWNAmLK(K)	Oxidation (+16)	973.4536	1,944.89	118	132
(K)WVYPSEQMFWNAMLK(K)		643.9739	1,928.90	118	132
(K)WVYPSEQMFWNAmLK(K)	Oxidation (+16)	649.3039	1,944.89	118	132
(R)YVIDYYDGGEVNK(D)		767.8568	1,533.70	224	236
(R)YVIDYYDGGEVNKDYQFTILDVRPALDSLSAVWDR(M)		1,024.75	4,094.99	224	258
(K)WKDEDISQKDMYNIIR(I)		685.3384	2,052.99	137	152
(R)GTAAENKENLDPSNLmPPPNQTPAPDQPFALSTVR(E)	Oxidation (+16)	934.21	3,732.81	70	104
(R)IHNQNNEQAWK(E)		461.2256	1,380.65	153	163
(K)KTYSVPAHQER(A)		439.2311	1,314.67	49	59
(R)GTAAENKENLDPSNLMPPPNQTPAPDQPFALSTVR(E)		1,239.94	3,716.81	70	104

Figure S3



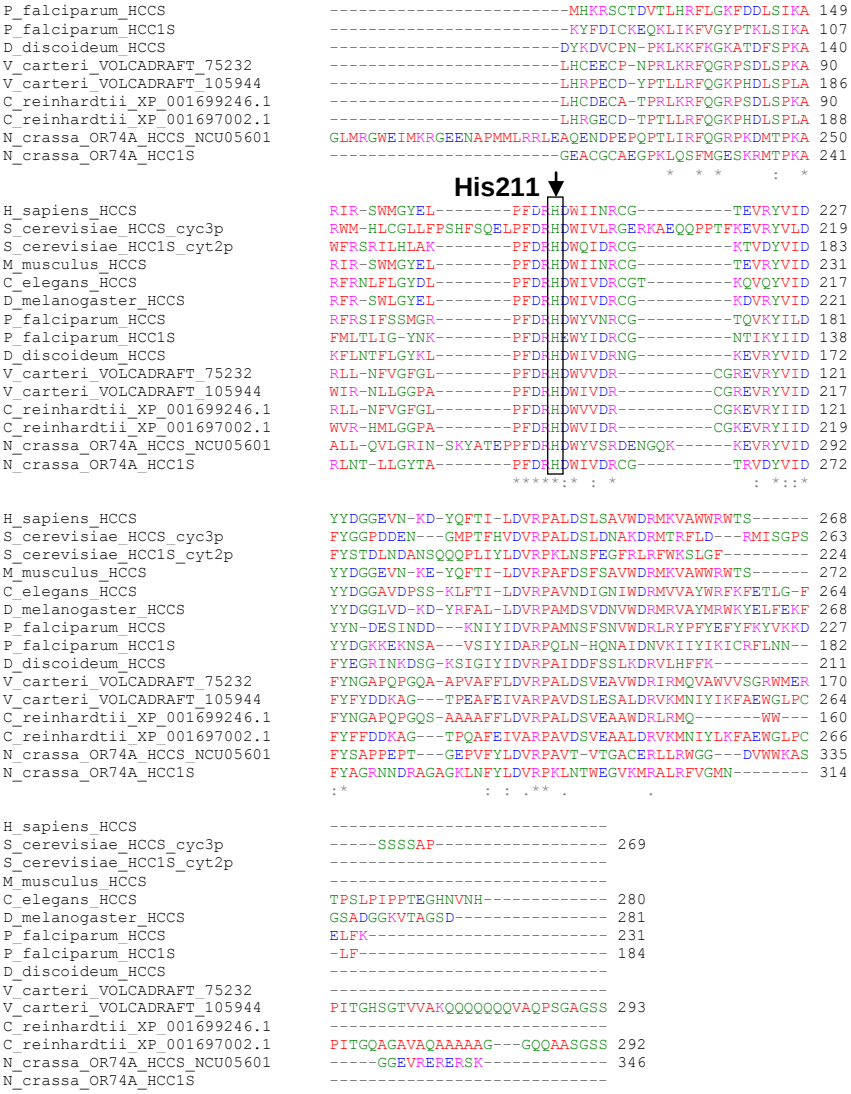

His211

Figure S4

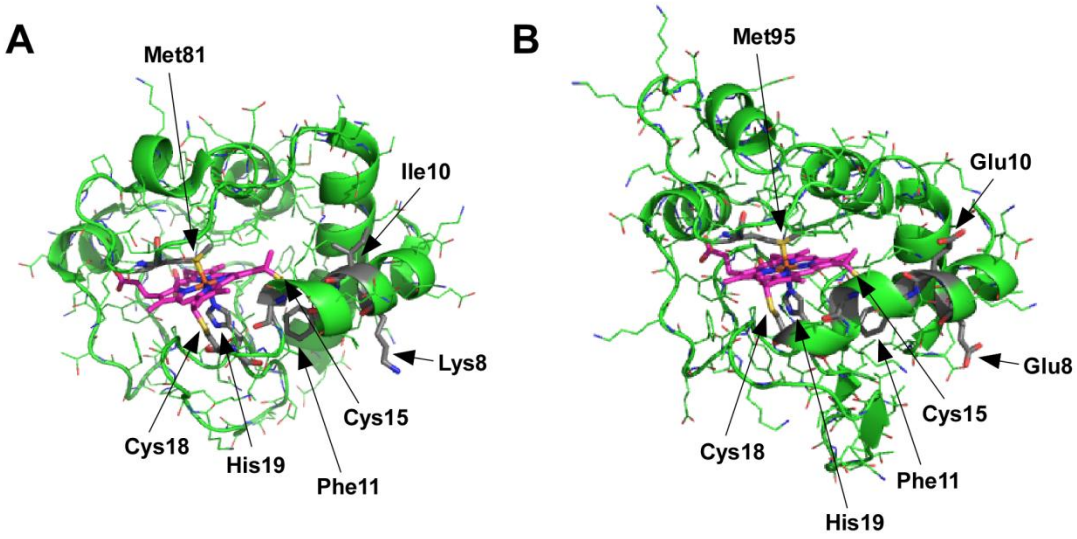

A
Met81
Ile10
Lys8
Cys15
Cys18
His19
Phe11
B
Met95
Glu10
Glu8
Cys15
Cys18
Phe11
His19

Figure S5

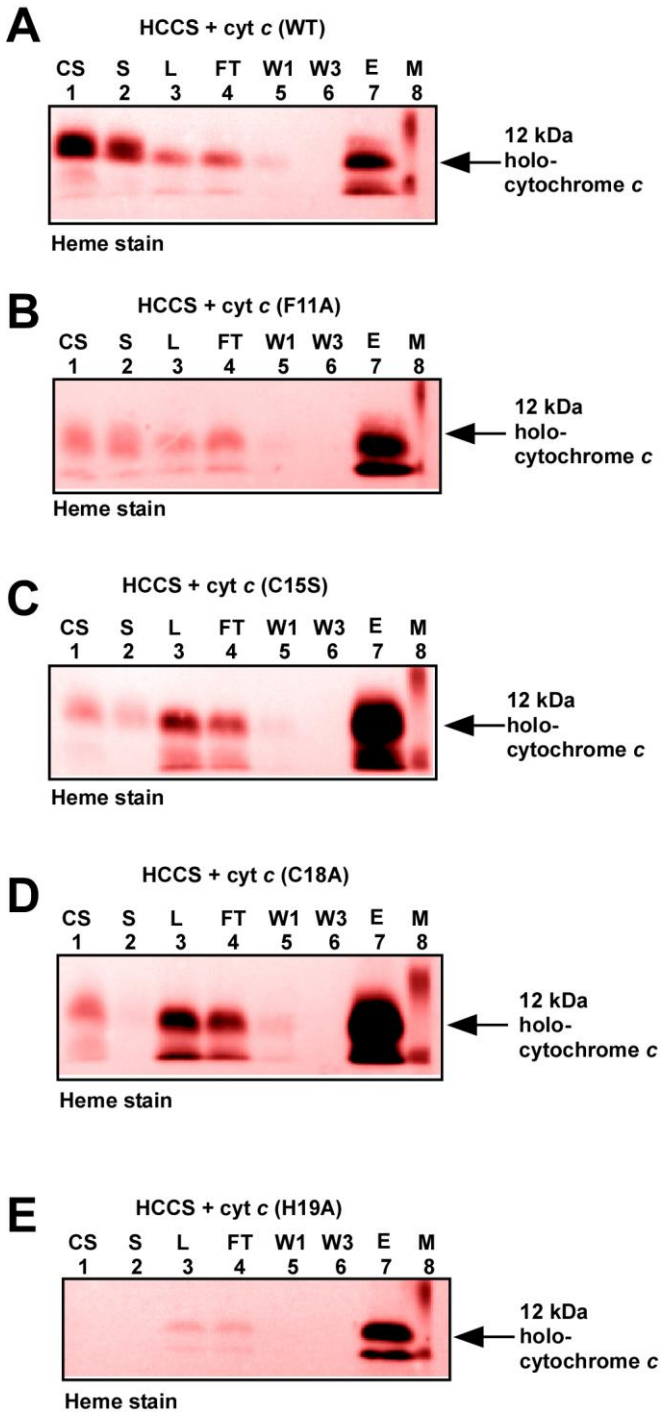

A
HCCS + cyt c (WT)
CS S L FT W1 W3 E M
1 2 3 4 5 6 7 8
12 kDa holo-cytochrome c
Heme stain
B
HCCS + cyt c (F11A)
CS S L FT W1 W3 E M
1 2 3 4 5 6 7 8
12 kDa holo-cytochrome c
Heme stain
C
HCCS + cyt c (C15S)
CS S L FT W1 W3 E M
1 2 3 4 5 6 7 8
12 kDa holo-cytochrome c
Heme stain
D
HCCS + cyt c (C18A)
CS S L FT W1 W3 E M
1 2 3 4 5 6 7 8
12 kDa holo-cytochrome c
Heme stain
E
HCCS + cyt c (H19A)
CS S L FT W1 W3 E M
1 2 3 4 5 6 7 8
12 kDa holo-cytochrome c
Heme stain

Figure S6

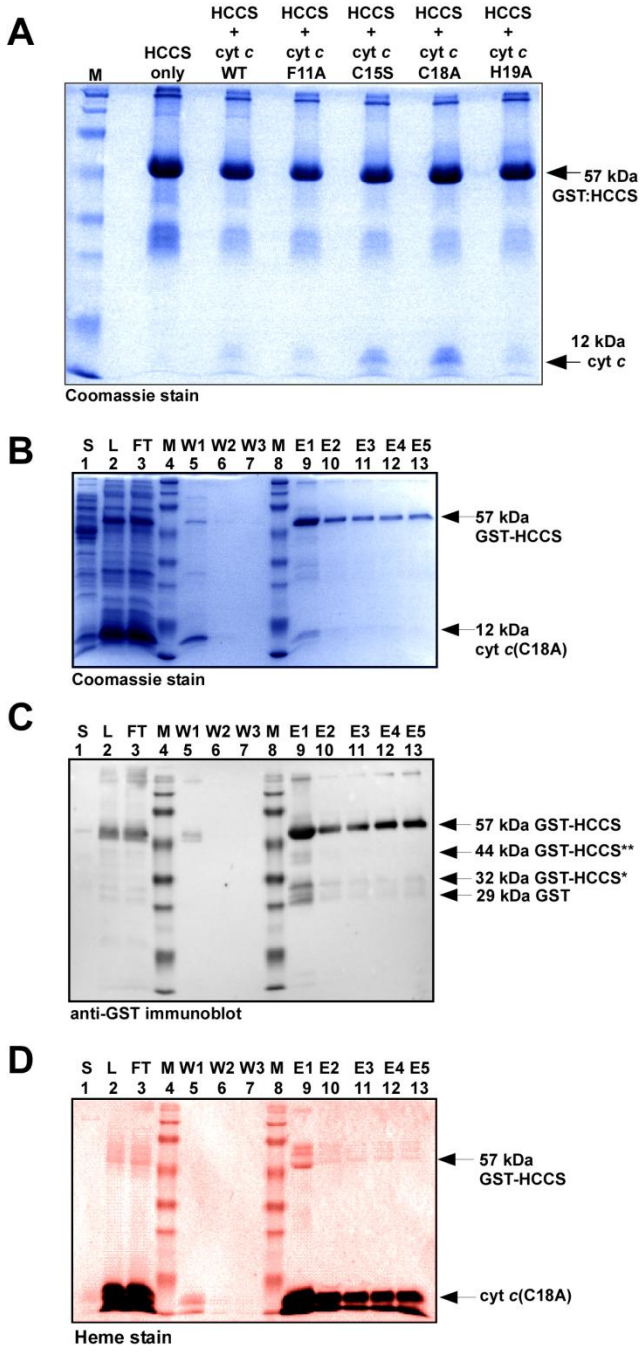

A
M
HCCS only
HCCS + cyt c WT
HCCS + cyt c F11A
HCCS + cyt c C15S
HCCS + cyt c C18A
HCCS + cyt c H19A
57 kDa GST:HCCS
12 kDa cyt c
Coomassie stain
B
S L FT M W1 W2 W3 M E1 E2 E3 E4 E5
1 2 3 4 5 6 7 8 9 10 11 12 13
57 kDa GST-HCCS
12 kDa cyt c(C18A)
Coomassie stain
C
S L FT M W1 W2 W3 M E1 E2 E3 E4 E5
1 2 3 4 5 6 7 8 9 10 11 12 13
57 kDa GST-HCCS
44 kDa GST-HCCS**
32 kDa GST-HCCS*
29 kDa GST
anti-GST immunoblot
D
S L FT M W1 W2 W3 M E1 E2 E3 E4 E5
1 2 3 4 5 6 7 8 9 10 11 12 13
57 kDa GST-HCCS
cyt c(C18A)
Heme stain

Figure S7

Peptide sequence: WT cytochrome c	Seq.	m/z (obs)	Mr (expt)	Mr (calc)
KIFIMK	9-14	390.2439	778.4732	778.4775
KIFIMK	9-14	390.2445	778.4745	778.4775
KIFIMK + ox (M)	9-14	398.2412	794.4678	794.4724
HKTGPNLHGLFGR	27-39	359.1977	1432.7615	1432.7688
HKTGPNLHGLFGR + deam (NQ)	27-39	359.4485	1433.7649	1433.7528
TGPNLHGLFGR	29-39	584.8093	1167.6040	1167.6149
TGPNLHGLFGRK	29-40	432.9085	1295.7036	1295.7099
TGPNLHGLFGRK + deam (NQ)	29-40	433.2413	1296.7020	1296.6939
KTGQAPGYSYTAANK	40-54	778.8813	1555.7480	1555.7630
KTGQAPGYSYTAANKNK	40-56	600.3039	1797.8899	1797.9009
KTGQAPGYSYTAANKNK + deam (NQ)	40-56	600.6311	1798.8716	1798.8849
TGQAPGYSYTAANK	41-54	714.8368	1427.6589	1427.6681
TGQAPGYSYTAANKNK	41-56	557.6061	1669.7966	1669.8060
GIIWGEDTLMEYLENPK + ox (M)	57-73	1012.4795	2022.9444	2022.9608
GIIWGEDTLMEYLENPK + ox (M); deam (NQ)	57-73	1012.9840	2023.9534	2023.9448
KYIPGTK	74-80	403.7397	805.4648	805.4698
MIFVGIK	81-87	404.2414	806.4681	806.4724
MIFVGIK + ox (M)	81-87	412.2377	822.4608	822.4673
MIFVGIKK	81-88	468.2883	934.5620	934.5674
MIFVGIKK + ox (M)	81-88	476.2863	950.5580	950.5623
EERADLIAYLK	90-100	660.8589	1319.7032	1319.7085
EERADLIAYLKK	90-101	483.6068	1447.7985	1447.8034
ADLIAYLK	93-100	453.7657	905.5169	905.5222
ADLIAYLKK	93-101	345.5434	1033.6084	1033.6171

MGDVEKGKKIFIMKCSQCHTVEKGGKHKTGPNLHGLFGRKAANKNKGHWGEDTLMEYLE
NPKKYIPGTKMIFVGIKKKEKATNE 76 % protein sequence coverage

Peptide sequence: C18A cytochrome c	Seq.	m/z (obs)	Mr (expt)	Mr (calc)
KIFIMK	9-14	390.2448	778.4750	778.4775
KIFIMK + ox (M)	9-14	398.2414	794.4683	794.4724
KIFIMKCSQAHTVEK	9-23	441.4909	1761.9345	1761.9270
CSQAHTVEK	15-23	501.7349	1001.4552	1001.4600
HKTGPNLHGLFGR	27-39	359.1978	1432.7622	1432.7688
HKTGPNLHGLFGR + deam (NQ)	27-39	359.4484	1433.7644	1433.7528
TGPNLHGLFGR	29-39	584.8106	1167.6066	1167.6149
TGPNLHGLFGR + deam (NQ)	29-39	585.3031	1168.5917	1168.5989
TGPNLHGLFGRK	29-40	432.9061	1295.6964	1295.7099
TGPNLHGLFGRKTGQAPGYSYTAANK	29-54	677.3450	2705.3510	2705.3674
TGPNLHGLFGRKTGQAPGYSYTAANK + deam (NQ)	29-54	677.5961	2706.3554	2706.3514
KTGQAPGYSYTAANK	40-54	778.8821	1555.7497	1555.7630
KTGQAPGYSYTAANK + deam (NQ)	40-54	519.9261	1556.7563	1556.7471
KTGQAPGYSYTAANKNK	40-56	600.3038	1797.8895	1797.9009
KTGQAPGYSYTAANKNK + deam (NQ)	40-56	600.6306	1798.8700	1798.8849
TGQAPGYSYTAANK	41-54	714.8366	1427.6586	1427.6681
TGQAPGYSYTAANKNK	41-56	557.6059	1669.7958	1669.8060
NKGIIWGEDTLMEYLENPK + ox (M)	55-73	756.0371	2265.0895	2265.0987
NKGIIWGEDTLMEYLENPKK	55-74	793.4031	2377.1873	2377.1987
NKGIIWGEDTLMEYLENPKK + ox (M)	55-74	798.7358	2393.1854	2393.1936
NKGIIWGEDTLMEYLENPKK + ox (M); deam (NQ)	55-74	599.5536	2394.1851	2394.1777
GIIWGEDTLMEYLENPK	57-73	1004.4884	2006.9622	2006.9659
GIIWGEDTLMEYLENPK + ox (M)	57-73	1012.4834	2022.9522	2022.9608
GIIWGEDTLMEYLENPKK	57-74	712.6916	2135.0529	2135.0609
GIIWGEDTLMEYLENPKK + ox (M)	57-74	1076.5340	2151.0534	2151.0558
GIIWGEDTLMEYLENPKK + ox (M); deam (NQ)	57-74	718.3563	2152.0471	2152.0398
GIIWGEDTLMEYLENPKKYIPGTK + ox (M)	57-80	937.8090	2810.4051	2810.4200
KYIPGTK	74-80	403.7396	805.4647	805.4698
MIFVGIK	81-87	404.2420	806.4695	806.4724
MIFVGIK + ox (M)	81-87	412.2395	822.4645	822.4673
MIFVGIKK	81-88	468.2863	934.5581	934.5674
MIFVGIKK + ox (M)	81-88	476.2867	950.5588	950.5623
KEERADLIAYLK	89-100	724.9061	1447.7976	1447.8034
EERADLIAYLK	90-100	660.8576	1319.7006	1319.7085
EERADLIAYLKK	90-101	483.6065	1447.7977	1447.8034
ADLIAYLK	93-100	453.7653	905.5161	905.5222
ADLIAYLKK	93-101	517.8129	1033.6113	1033.6171
ADLIAYLKKATNE	93-105	725.3977	1448.7809	1448.7874

MGDVEKGKKIFIMKCSQAHTVEKGGKHKTGPNLHGLFGRKAANKNKGHWGEDTLMEYLE
NPKKYIPGTKMIFVGIKKKEKATNE 89 % sequence coverage

Peptide sequence: C15S cytochrome c	Seq.	m/z (obs)	Mr (expt)	Mr (calc)
KIFIMK	9-14	390.2450	778.4754	778.4775
KIFIMK + ox (M)	9-14	398.2414	794.4683	794.4724
SSQCHTVEK	15-23	509.7303	1017.4461	1017.4549
HKTGPNLHGLFGR	27-39	359.1981	1432.7633	1432.7688
HKTGPNLHGLFGR + deam (NQ)	27-39	478.9312	1433.7719	1433.7528
HKTGPNLHGLFGRK	27-40	391.2221	1560.8593	1560.8637
TGPNLHGLFGR	29-39	584.8117	1167.6089	1167.6149
TGPNLHGLFGRK	29-40	432.9092	1295.7058	1295.7099
TGPNLHGLFGRK + deam (NQ)	29-40	433.2384	1296.6933	1296.6939
TGPNLHGLFGRKTGQAPGYSYTAANK	29-54	677.3450	2705.3510	2705.3674
KTGQAPGYSYTAANK	40-54	778.8844	1555.7542	1555.7630
KTGQAPGYSYTAANKNK	40-56	600.3039	1797.8898	1797.9009
KTGQAPGYSYTAANKNK + deam (NQ)	40-56	600.6327	1798.8762	1798.8849
TGQAPGYSYTAANK	41-54	714.8344	1427.6543	1427.6681
GIIWGEDTLMEYLENPK + ox (M)	57-73	1012.4833	2022.9520	2022.9608
KYIPGTK	74-80	403.7400	805.4654	805.4698
MIFVGIK + ox (M)	81-87	412.2379	822.4612	822.4673
MIFVGIKK	81-88	468.2899	934.5652	934.5674
MIFVGIKK + ox (M)	81-88	476.2862	950.5579	950.5623
EERADLIAYLK	90-100	660.8595	1319.7044	1319.7085
EERADLIAYLKK	90-101	483.6070	1447.7993	1447.8034
ADLIAYLK	93-100	453.7666	905.5187	905.5222
ADLIAYLKK	93-101	517.8122	1033.6099	1033.6171
ADLIAYLKK	93-101	517.8149	1033.6152	1033.6171
ADLIAYLKKATNE	93-105	483.9343	1448.7811	1448.7874
ADLIAYLKKATNE + deam (NQ)	93-105	484.2661	1449.7766	1449.7715

MGDVEKGKKIFIMKSSQCHTVEKGGKHKTGPNLHGLFGRKAANKNKGHWGEDTLMEYLE
NPKKYIPGTKMIFVGIKKKEKATNE 88 % sequence coverage

Figure S8

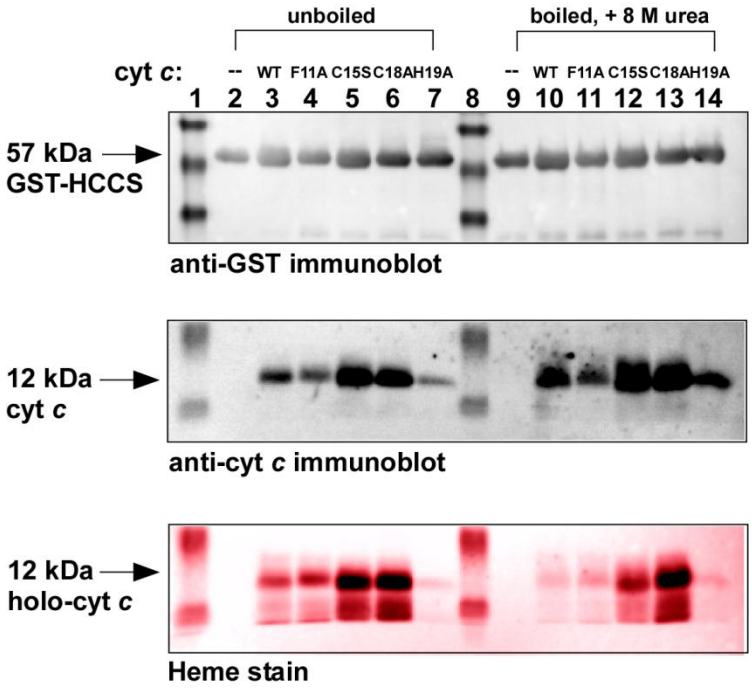

unboiled
boiled, + 8 M urea
cyt c:
-- WT F11A C15SC18AH19A -- WT F11A C15SC18AH19A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
57 kDa
GST-HCCS
anti-GST immunoblot
12 kDa
cyt c
anti-cyt c immunoblot
12 kDa
holo-cyt c
Heme stain

Figure S9

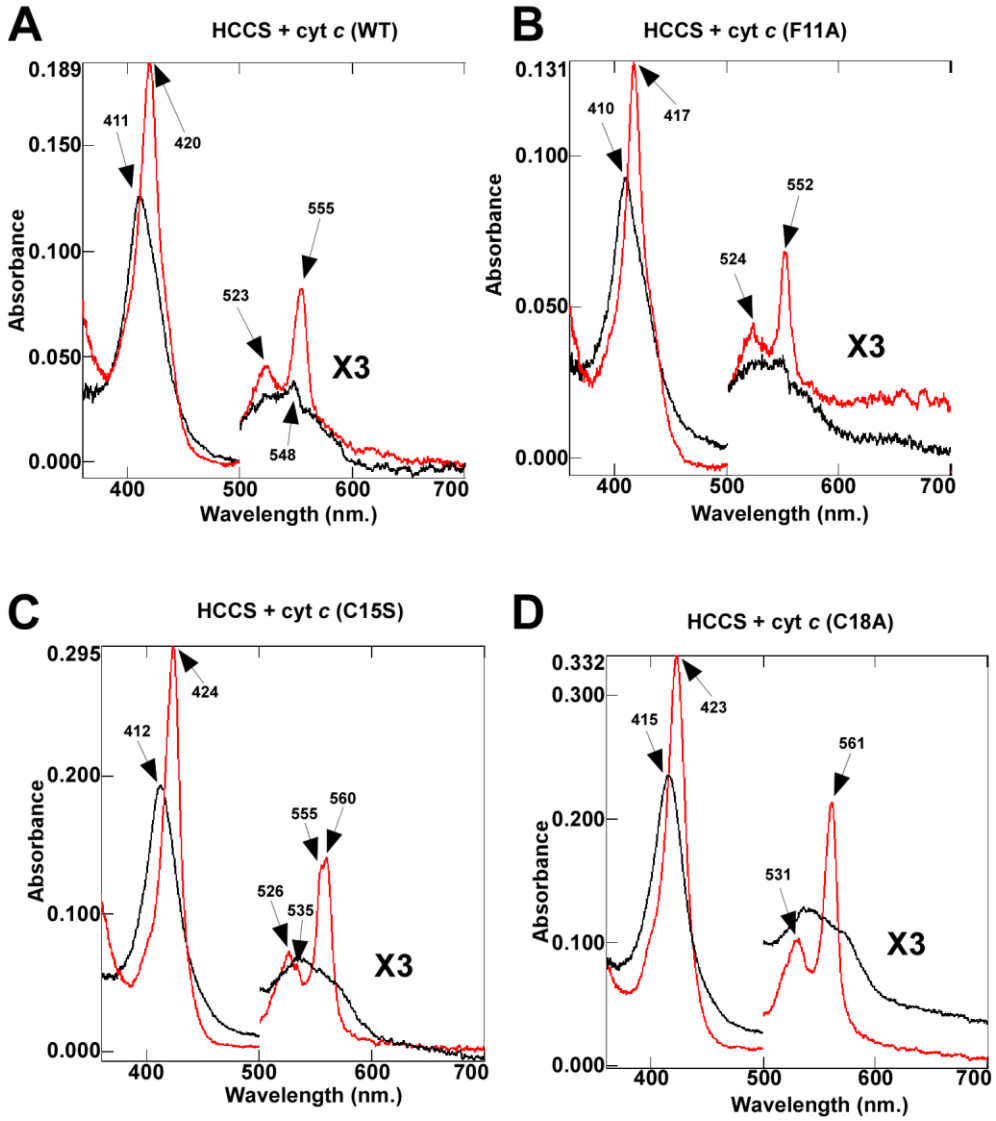

A
HCCS + cyt c (WT)
0.189
0.150
0.100
0.050
0.000
Absorbance
411
420
555
523
548
X3
400
500
600
700
Wavelength (nm.)
B
HCCS + cyt c (F11A)
0.131
0.100
0.050
0.000
Absorbance
410
417
552
524
X3
400
500
600
700
Wavelength (nm.)
C
HCCS + cyt c (C15S)
0.295
0.200
0.100
0.000
Absorbance
424
412
560
555
526
535
X3
400
500
600
700
Wavelength (nm.)
D
HCCS + cyt c (C18A)
0.332
0.300
0.200
0.100
0.000
Absorbance
423
415
561
531
X3
400
500
600
700
Wavelength (nm.)

Figure S10

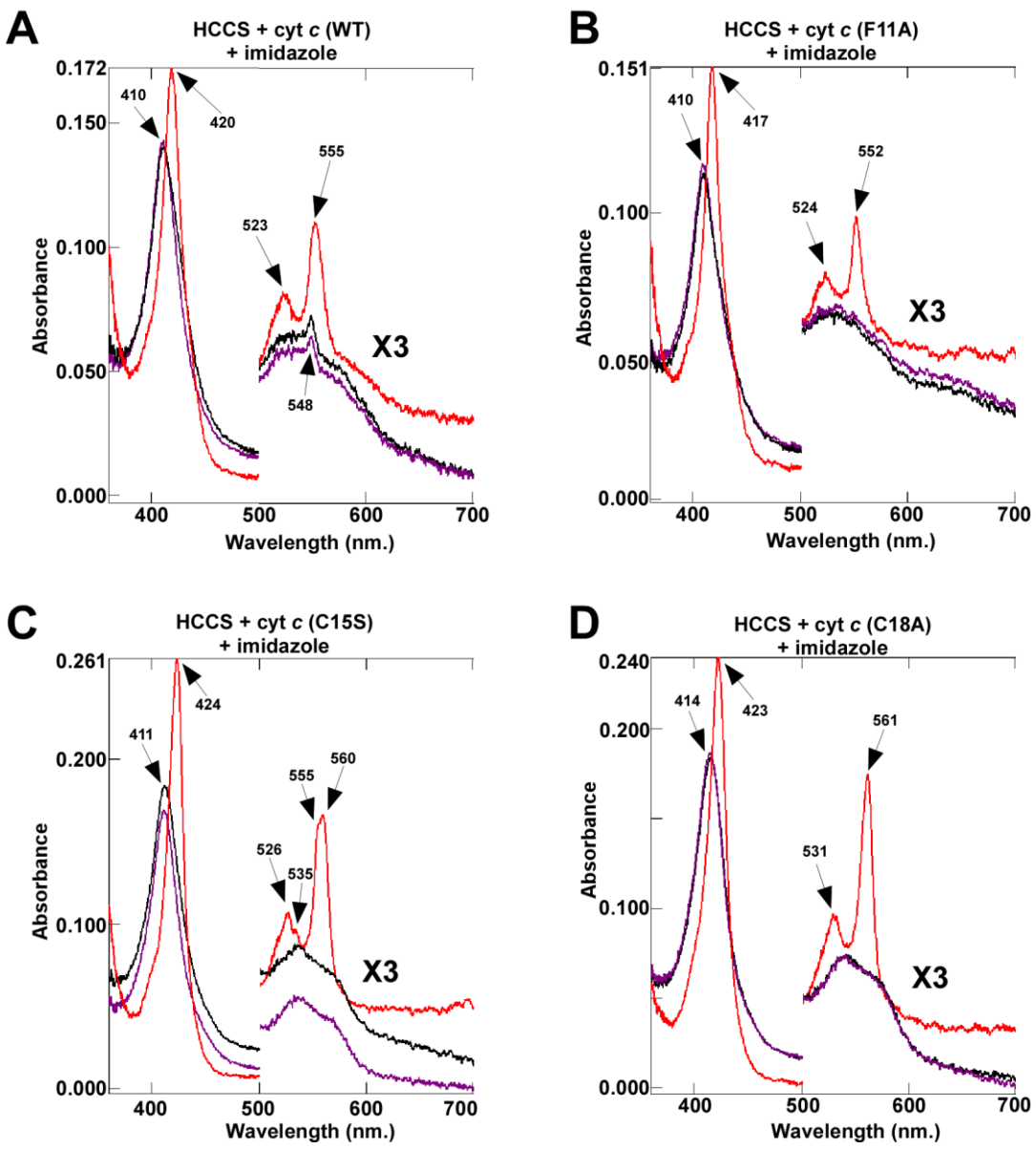
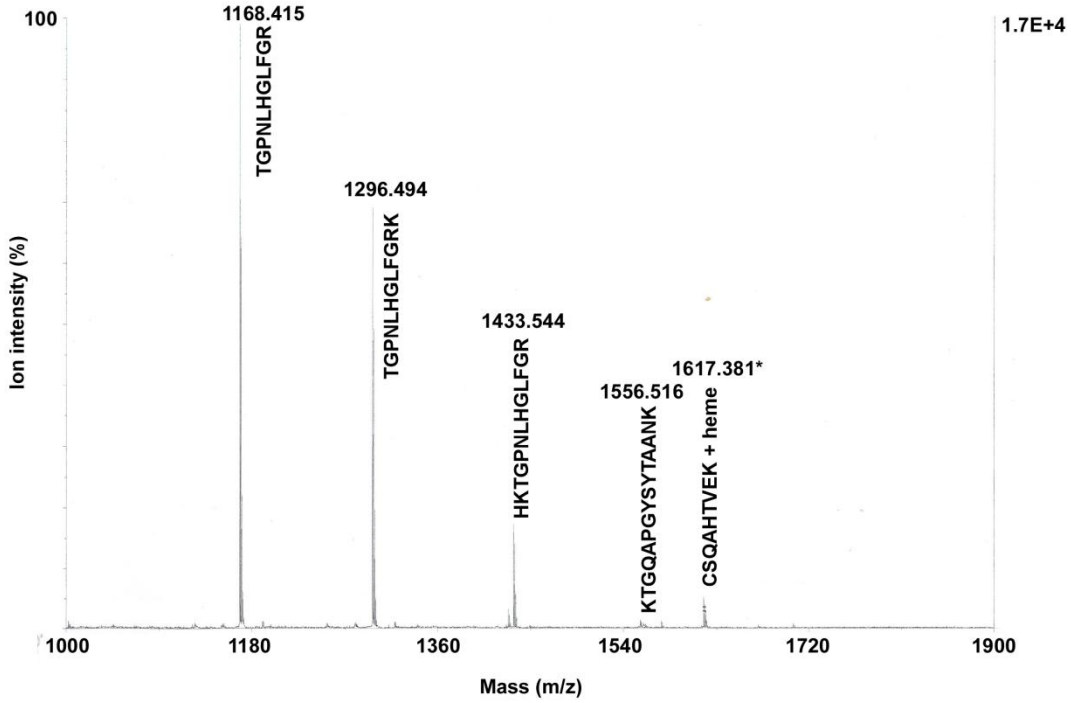

A
HCCS + cyt c (WT)
+ imidazole
410
420
555
523
548
X3
Absorbance
Wavelength (nm.)
B
HCCS + cyt c (F11A)
+ imidazole
410
417
552
524
X3
Absorbance
Wavelength (nm.)
C
HCCS + cyt c (C15S)
+ imidazole
424
411
560
555
526
535
X3
Absorbance
Wavelength (nm.)
D
HCCS + cyt c (C18A)
+ imidazole
414
423
561
531
X3
Absorbance
Wavelength (nm.)

Figure S11



*Neutral monoisotopic mass calculation
CSQAHTVEK (1001.4597 Da) + heme (616.1773 Da) = 1617.637 Da

Figure S12

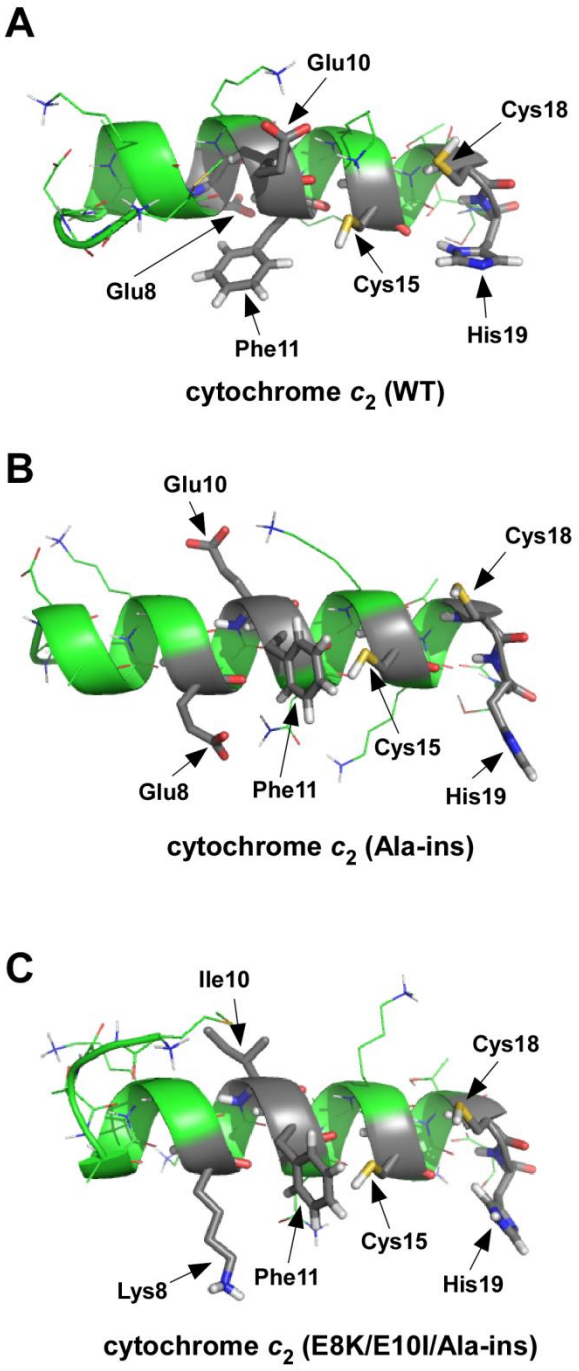

A
Glu10
Cys18
Glu8
Cys15
Phe11
His19
cytochrome c_2 (WT)
B
Glu10
Cys18
Cys15
Glu8
Phe11
His19
cytochrome c_2 (Ala-ins)
C
Ile10
Cys18
Cys15
Lys8
Phe11
His19
cytochrome c_2 (E8K/E10I/Ala-ins)

Table S1. Published studies on cytochrome *c* substrate determinants for maturation by HCCS

Reference	Compartment	HCCS	Cytochrome c	Results
Verissimo et al. (2012) Biochem Biophys Res Comm	*E. coli* cytoplasm	S. *cerevisiae*	• *S. cerevisiae* iso-2-cyt c • *R. capsulatus* cyt c2 • Iso-2-cyt c/cyt c2 chimera • Cyt c2-insK11 • Cyt c2-E9L • Cyt c2-insK11 + E9L • Cyt c2-insK11 + N12T	• *R. capsulatus* cyt c2 not matured • Iso-2-cyt c/cyt c2 chimera (and truncated chimera) fully matured • Cyt c2-insK11 matured at low level • Cyt c2-insK11 + E9L matured at high levels • Cyt c2-E9L not matured • Cyt c2-insK11 + N12T not matured
Stevens et al. (2011) FEBS Lett	*E. coli* cytoplasm	*S. cerevisiae*	• *E. caballus* cyt c • *S. cerevisiae* iso-1-cyt c • *P. denitrificans* cyt c550 • Iso-1-cyt c/cyt c550 chimera	• G6A, F10A in *E. caballus* not matured • F15A (but not G11A) in S. cerevisiae not matured • D2A, E4A, K7A, K5A, K8A matured at WT levels • *P. denitrificans* cyt c550 not matured • Chimera with *S. cerevisiae* N-term up to but not including CXXCH fused to *P. denitrificans* cyt c550 matured at WT levels
Kleingardner and Bren (2011) Metallomics	*E. coli* cytoplasm	*S. cerevisiae*	• *E. caballus* cyt c • *E. caballus* cyt c 1x-ins • *E. caballus* cyt c 3x-ins • *E. caballus* cyt c G13 ins • *E. caballus* F10A	• *E. caballus* cyt c 1x-ins not matured • Cyt c 3x-ins matured at 1/3 levels • Cyt c G13 ins matured at 1/3 levels but spectrally heterogeneous • Cyt c F10A not matured at detectable levels
Fulop et al. (2009) FEBS J	*E. coli* cytoplasm	*S. cerevisiae*	• *T. brucei* cyt c WT (AAQCH) • *T. brucei* cyt c(CAQCH) • *S. cerevisiae* iso-1-cyt c	• Both *T. brucei* cyts c matured at very low levels; 0.25 % the level of *S. cerevisiae* iso-1-cyt c
Rosell and Mauk (2002) Biochem	*E. coli* cytoplasm	*S. cerevisiae*	• *S. cerevisiae* iso-1-cyt c(C14S) • *S. cerevisiae* iso-1-cyt c(C17S)	• Iso-1-cyt c(C14S) matured at 1/10 levels of WT; spectrally distinct from WT • Iso-1-cyt c(C17S) expression 20-fold lower than C14S variant; apparently did not permit enough to conduct analysis
Silkstone et al. (2002) Biophysical Chem	*E. coli* cytoplasm	*S. cerevisiae*	• *S. cerevisiae* iso-1 cyt c(M80D/E/S/A)	• *S. cerevisiae* iso-1 cyt c Met80 variants were matured efficiently
Rumbley et al. (2002) Biochemistry	*E. coli* cytoplasm	*S. cerevisiae*	• *E. caballus* cyt c(E5G) • *E. caballus* cyt c(K9G) • *E. caballus* cyt c(V12G)	• *E. caballus* cyt c E5G, K9G, V12G variants all matured, but with increasing proximity to CXXCH, level of mis-attached heme increased (attributed to interference with formation of N-terminal helix) • Starting *E. caballus* cyt c

				construct carried H26N, H33N mutations
Sanders et al. (2001) Mol Micro	*E. coli* cytoplasm	*S. cerevisiae*	• *S. cerevisiae* iso-2-cyt c/*P. denitrificans* cyt c550 chimera	• Chimera (with N-term and CXXCH from iso-2-cyt c) matured
Sanders and Lill (2000) BBA	*E. coli* cytoplasm	*S. cerevisiae*	• *S. cerevisiae* iso-1-cyt c • *S. cerevisiae* iso-2-cyt c • *P. denitrificans* cyt c • *Synechocystis* sp. PCC 6803 cyt c553	• *S. cerevisiae* iso-1 and iso-2 cyt c matured at high levels • *P. denitrificans* and *Synechocystis* cyt c not matured at detectable levels
Corvest et al. (2010) Genetics	Whole *S. cerevisiae* cells	*S. cerevisiae*	• *S. cerevisiae* cyt c_1(CA[/P/T/H/L/S]CH)	• Cyt c_1(CAPCH) depends on HCCS and Cyc2p for maturation; HCC_1S maturation achieved by overexpression of HCC_1S or substitution of P with H/L/S/T
Bernard et al. (2003) JBC	Whole *S. cerevisiae* cells	Plasmid *S. cerevisiae* HCCS and HCC_1S Plasmid *H. sapiens* HCCS Plasmid *M. musculus* HCCS	• *S. cerevisiae* cyt c • *S. cerevisiae* cyt c_1 WT (CAACH) • *S. cerevisiae* cyt c_1(CAPCH) • *S. cerevisiae* cyt c_1(CADCH)	• *S. cerevisiae* HCC_1S cannot mature cyt c • *S. cerevisiae*, *H. sapiens*, and *M. musculus* HCCS can complement HCC_1S deletion for respiratory growth (i.e., they are active towards both cyt c an cyt c_1) • *S. cerevisiae* HCCS can mature CAPCH and CADCH mutants of cyt c_1
Wang et al. (1996) JBC	Whole *S. cerevisiae* cells	Chromosomal + plasmid *S. cerevisiae*	• *S. cerevisiae* iso-2-cyt c([S/A]XX[S/A]H) • *S. cerevisiae* iso-2-cyt c(F19A) • *S. cerevisiae* iso-2-cyt c(H27A) • *S. cerevisiae* iso-2-cyt c(ΔAla1-Lys14) • *S. cerevisiae* iso-2-cyt c(ΔGly15-Leu18)	• Iso-2-cyt c(S/AXXS/AH) variants not matured, but imported into mitochondria (when HCCS was overexpressed on plasmid) • Iso-2-cyt c(F19A) not matured; 10 % mitochondrial import • Iso-2-cyt c(H27A)not matured; 40 % associated with mitochondria but only 10 % internalized into mitochondria • Iso-2-cyt c(ΔAla1-Lys14) matured at low levels (10 %) • Iso-2-cyt c(ΔGly15-Leu18) not matured; 10 % mitochondrial import
Fumo et al. (1995) Gene	Whole *S. cerevisiae* cells	Chromosomal *S. cerevisiae*	• *S. cerevisiae* iso-1-cyt c(H18A)	• Iso-1-cyt c(H18A) is nonfunctional (did not support growth in medium with liquid lactate, a nonfermentable carbon source) • Iso-1-cyt c(H18A) was not detected spectrally in intact cells • In targeted random mutagenesis experiment, only His was found as a.a. 18 • Iso-1-cyt c(H18R) integrated chromosomally not support growth in lactate medium and holo-cyt c(H18R) was not

				detected in low temperature difference spectroscopy
Tanaka et al. (1990) J Biochem	Whole *S. cerevisiae* cells	Chromosomal *S. cerevisiae*	• *H. sapiens* cyt c(C14A)	• *H. sapiens* cyt c(C14A) efficiently matured and complemented CYC1 deficiency in yeast on lactate medium
Sorrell et al. (1989) JACS	Whole *S. cerevisiae* cells	Chromosomal *S. cerevisiae*	• *S. cerevisiae* iso-2-cyt c(H18R)	• Iso-2-cyt c(H18R) on a plasmid weakly complemented yeast strain lacking cytochrome c on glycerol or lactate media • Less than 1 mg/10 L culture were purified; spectrally identical to WT iso-2-cyt c • Iso-2-cyt c(H18R) has slower rate of electron transfer than WT
Tong and Margoliash (1998) JBC	Purified *S. cerevisiae* mitochondria "in vitro"	Chromosomal + plasmid *S. cerevisiae*	• *D. melanogaster* cyt c(C14S) • *D. melanogaster* cyt c(C17S) • *D. melanogaster* cyt c(C14S/C17S)	• *D. melanogaster* cyt c(C14S) matured, but at lower levels than WT • *D. melanogaster* cyt c(C17S) was matured at "trace" amounts • *D. melanogaster* cyt c(C14S/C17S) was undetectable
Veloso et al. (1983) JBC	Purified *S. cerevisiae* mitochondria "in vitro"	Chromosomal *S. cerevisiae*	• N-terminal 25 residues of *E. caballus* cyt c	• Heme was attached to *E. caballus* cyt c(N-term 25) at 25 % levels of full apocytochrome c • No maturation when Gly replaced Cys in CXXCH

Catalogue of published studies on the effects of cytochrome *c* mutations on maturation by HCCS. Studies are organized according to the biological compartment in which the study was conducted (i.e., *E. coli* cytoplasm, whole *S. cerevisiae* cells, or isolated *S. cerevisiae* mitochondria) and then in reverse chronological order. Full citations for each of the studies above are provided in the references section of the main text.

Table S2. Oligonucleotide primers, plasmids, and strains used in this research.

Oligonucleotide	Sequence (5'-3')	Plasmid Constructed
CYCS_K6A_Fwd	ATGGGTGATGTTGAGGCGGGCAAGAAGATTTTTATTATGAAGTG	pRGK412
CYCS_K6A_Rev	CACTTCATAATAAAAATCTTCTTGCCCGCCTCAACATCACCCAT	pRGK412
CYCS_K6R_Fwd	ATGGGTGATGTTGAGCGCGGCAAGAAGATTTTTATTATGAAGTG	pRGK413
CYCS_K6R_Rev	CACTTCATAATAAAAATCTTCTTGCCGCGCTCAACATCACCCAT	pRGK413
CYCS_K6D_Fwd	ATGGGTGATGTTGAGGACGGCAAGAAGATTTTTATTATGAAGTG	pRGK414
CYCS_K6D_Rev	CACTTCATAATAAAAATCTTCTTGCCGTCCTCAACATCACCCAT	pRGK414
CYCS_F11A_Fwd	GAGAAAGGCAAGAAGATTGCGATTATGAAGTGTTCCCAGTGCC	pRGK415
CYCS_F11A_Rev	GGCACTGGGAACACTTCATAATCGCAATCTTCTTGCCTTTCTC	pRGK415
CYCS_F11Y_Fwd	GTTGAGAAAGGCAAGAAGATTTATATTATGAAGTGTTCCCAGTGC	pRGK416
CYCS_F11Y_Rev	GCACTGGGAACACTTCATAATATAAATCTTCTTGCCTTTCTCAAC	pRGK416
CYCS_C15S_Fwd	GATTTTTATTATGAAGAGCTCCCAGTGCCACACCGTTGAAAAGGG	pRGK417
CYCS_C15S_Rev	CCCTTTTCAACGGTGTGGCACTGGGAGCTCTTCATAATAAAAATC	pRGK417
CYCS_C18A_Fwd	ATGAAGTGTTCCCAGGCGCACCGTTGAAAAGGGAGG	pRGK418
CYCS_C18A_Rev	CCTCCCTTTTCAACGGTGCGCCTGGGAACACTTCAT	pRGK418
CYCS_H19A_Fwd	TATTATGAAGTGTTCCCAGTGCGCGACCGTTGAAAAGGG AGGCAA	pRGK419
CYCS_H19A_Rev	TTGCCTCCCTTTTCAACGGTCGCGCACTGGGAACACTTCATAATA	pRGK419
HCCS_H154A_Fwd	GTATAATATCATTAGAATTGCCAATCAGAATAACGAGCAGGC	pRGK420
HCCS_H154A_Rev	GCCTGCTCGTTATTCTGATTGGCAATTCTAATGATATTATAC	pRGK420
HCCS_H154G_Fwd	CATTAGAATTGGCAATCAGAATAACGAGCAGGCTTGGAAGG	pRGK421
HCCS_H154G_Rev	CCTTCCAAGCCTGCTCGTTATTCTGATTGCCAATTCTAATG	pRGK421
HCCS_H154Y_Fwd	CATTAGAATTTACAATCAGAATAACGAGCAGGC	pRGK422
HCCS_H154Y_Rev	GCCTGCTCGTTATTCTGATTGTAAATTCTAATG	pRGK422
HCCS_H211A_Fwd	GAGTTGCCTTTTGATAGGGCGGATTGGATCATAAACCGTTGC	pRGK423
HCCS_H211A_Rev	GCAACGGTTTATGATCCAATCCGCCCTATCAAAAGGCAACTC	pRGK423
HCCS_H211G_Fwd	GAGTTGCCTTTTGATAGGGGCGATTGGATCATAAACCGTTGC	pRGK424
HCCS_H211G_Rev	GCAACGGTTTATGATCCAATCGCCCCTATCAAAAGGCAACTC	pRGK424
HCCS_H211Y_Fwd	GGTATGAGTTGCCTTTTGATAGGTATGATTGGATCATAAACCG	pRGK425
HCCS_H211Y_Rev	CGGTTTATGATCCAATCATACCTATCAAAAGGCAACTCATACC	pRGK425
HCCS_H211C_Fwd	GAGTTGCCTTTTGATAGGTGCGATTGGATCATAAACCGTTG	pRGK426
HCCS_H211C_Rev	CAACGGTTTATGATCCAATCGCACCTATCAAAAGGCAACTC	pRGK426
pTXB1_HCCS_Nde1_Fwd	GGTGGTCATATGGGTTTGTCTCCATCTGC	pRGK404
pTXB1_HCCS_SapI_Rev	GGTGGTTGCTCTTCCGCATTTCGAGGTCCAACGCCACCAAGCAGC	pRGK404
pBAD_CYTC2_Nco1_Fwd	GACTCCATGGGCGACGCCGCGAAGGGCG	pRGK406
pBAD_CYTC2_XbaI_Rev	CTTCTAGATTATTTCACGACCGAGGCCAG	pRGK406
CYTC2_Ala-ins_Fwd	GGGCGAAAAAGAATTCAACGCCAAGTGCAAGACCTGCCA	pRGK407
CYTC2_Ala-ins_Rev	TGGCAGGTCTTGCACTTGGCGTTGAATTCTTTTTCGCCC	pRGK407
CYTC2_E8K_E10I_Fwd (Ala-ins)	GCGACGCCGCGAAGGGCAAAAAAATATTCAACGCCAAGTGCA	pRGK408
CYTC2_E8K_E10I_Rev (Ala-ins)	TGCACTTGGCGTTGAATATTTTTTTGCCCTTCGCGGCGTCGC	pRGK408
CYTC2_E8K_E10I_Fwd	GGCGACGCCGCGAAGGGCAAAAAAATATTCAACAAGTGCAAGAC	pRGK409
CYT_C2_E8K_E10I_Rev	GTCTTGCACTTGTTGAATATTTTTTTGCCCTTCGCGGCGTCGCC	pRGK409
CYT_C2_E8K_Fwd (Ala-ins)	GACGCCGCGAAGGGCAAAAAAGAATTCAACGC	pRGK410
CYTC2_E8K_Rev (Ala-ins)	GCGTTGAATTCTTTTTTGCCCTTCGCGGCGTC	pRGK410
CYTC2_E10I_Fwd (Ala-ins)	CGCCGCGAAGGGCGAAAAAATATTCAACGCCAAGTGC	pRGK411
CYTC2_E10I_Rev (Ala-ins)	GCACTTGGCGTTGAATATTTTTTCGCCCTTCGCGGCG	pRGK411
pGEX_HCCS_MfeI_Fwd	CTCAATTGATGGGTTTGTCTCCATCTGC	pRGK403
pET-Blue2-Down_Rev	GTTAAATTGCTAACGCAGTCA	pRGK403
pBAD_CYCS_EcoRI_Fwd	GCGGAATTCGCCATGGGTGATGTTGAG	pRGK405
pBAD_CYCS_PstI_Rev	GAGCTGCAGTTACTCATTAGTAGC	pRGK405
pET-Blue-2_HCCS_NcoI_Fwd	CCAGCCATGGGTCATCATCATCATCATCACGGTTTGTCTCCATCTGCTCC	pRGK402
pET-Blue-2_HCCS_HindIII_Rev	CGAAGCTTGTGCATAGTTTTACGAGGTCCAAC	pRGK402

Plasmid	Description	Reference
pRGK330	pBAD24-based plasmid for arabinose inducible expression	Feissner et al. 2006
pRGK389	Template for *R. capsulatus* cytochrome c_2	Richard-Fogal et al. 2012
pRGK402	Expression of N-terminal hexahistidine-tagged HCCS (pET Blue2)	This work
pRGK403	Expression of N-terminal GST-tagged HCCS (pGEX 4T-1)	This work
pRGK404	Expression of C-terminal Intein-tagged HCCS (pTXB1)	This work
pRGK405	Expression of human cytochrome c (pBAD)	This work

pRGK406	Expression of cytoplasmic *R. capsulatus* cytochrome c_2 (pBAD)	This work
pRGK407	pBAD cyt c_2(Ala-ins)	This work
pRGK408	pBAD cyt c_2(E8K/E10I/Ala-ins)	This work
pRGK409	pBAD cyt c_2(E8K/E10I)	This work
pRGK410	pBAD cyt c_2(E8K/Ala-ins)	This work
pRGK411	pBAD cyt c_2(E10I/Ala-ins)	This work
pRGK412	pBAD CYCS(K6A)	This work
pRGK413	pBAD CYCS(K6R)	This work
pRGK414	pBAD CYCS(K6D)	This work
pRGK415	pBAD CYCS(F11A)	This work
pRGK416	pBAD CYCS(F11Y)	This work
pRGK417	pBAD CYCS(C15S)	This work
pRGK418	pBAD CYCS(C18A)	This work
pRGK419	pBAD CYCS(H19A)	This work
pRGK420	pGEX HCCS(H154A)	This work
pRGK421	pGEX HCCS(H154G)	This work
pRGK422	pGEX HCCS(H154Y)	This work
pRGK423	pGEX HCCS(H211A)	This work
pRGK424	pGEX HCCS(H211G)	This work
pRGK425	pGEX HCCS(H211Y)	This work
pRGK426	pGEX HCCS(H211C)	This work

Strain	**Description**	**Reference**
RK103	Δ*ccm E. coli* strain MG1655 deleted for all *ccm* genes	Feissner et al. 2006
RK112	Δ*ccm E. coli* strain BL21 (DE3) deleted for all *ccm* genes	Richard-Fogal et al. 2012

Supporting References

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(3) Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1, 2856-60.

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