SUPPLEMENTARY FIGURES

Figure S1. Ccs1 expressed from a plasmid under the control of its endogenous promoter 
exhibits levels equal to wild-type yeast. Protein levels in cells from $CCS1$ deletion strains 
expressing Ccs1 from plasmids under the control of its endogenous promoter (pRS) or an 
overexpression promoter (pYX) were analyzed by Western blotting against the indicated 
proteins. Note that the levels of wild-type Ccs1 expressed from a pRS vector are equal to the 
levels in wild-type yeast. The Ccs1 levels in a strain expressing Ccs1 from a pYX plasmid are 
ca. 10–20 times higher compared to wild type protein levels.
Figure S2. Reproduction of the Ccs1 redox state analyses. (A)–(C) Redox state of Ccs1.

Experiments were performed as described in Figure 3. Note that the redox state of Ccs1 fluctuates independently of its expression from its endogenous promoter (pRS, (A)) or an
overexpression promoter (pYX, (B)). A fraction of Ccs1 is always present in a completely reduced state, and this fraction is increased upon Ccs1 overexpression. Conversely, Ccs1 targeted to the IMS of mitochondria bears always the disulfide bond between C27 and C64 (C). Our in vivo redox state analysis also revealed that all cysteines of Ccs1 except C27 and C64 (C17, C20, C159, C229, and C231) are present in their reduced form in the cytosol and in the IMS. This finding is in clear support for their role in copper binding. (D) Redox state of Ccs1 upon varying oxygen tension. Under low-oxygen conditions Ccs1 is more reduced than under conditions of 20% oxygen. (E) Redox state of Ccs1 in isolated mitochondria and cytosol. When analyzing the redox state of Ccs1 in isolated mitochondria or the cytosol Ccs1 appears to be more oxidized compared with the in vivo redox state analysis, which is most likely due to post-lysis oxidation (compare the steady state of isolated mitochondria with Figure 3D, lane 2, and the steady state of isolated cytosol with Figure 3C, lane 2). This indicates that the assessment of the Ccs1 redox state under these conditions has only limited informative value.
Figure S3. Characterization of Domain I of Ccs1 by circular dichroism (CD). (A) Purification of Domain I of wild-type Ccs1 and Ccs1\(^{C27S/C64S}\). Because the cellular levels of Ccs1 variants lacking either C27 or C64 were low compared with the wild-type protein (see Figure 4A), we aimed to determine the importance of the C27–C64 disulfide bond for the structure of Ccs1. We therefore prepared two purified Ccs1 Domain I variants, namely the wild type and Ccs1\(^{C27S/C64S}\). After two-step purification 10 µg of each protein was applied to SDS-PAGE. Judging by Coomassie staining both proteins were enriched to approximately 95–99 % purity. (B) Redox states of purified proteins were tested by mm-PEG24 alkylation shift assay. To this end, purified proteins were incubated with 10 mM mm-PEG24. For the reduced protein controls, proteins were treated with TCEP before mm-PEG24 was added. Samples were analyzed by SDS-PAGE and Coomassie staining. Both proteins were completely oxidized, i.e. they contained the C17–C20 disulfide. (C) CD spectra of Domain I of Ccs1 (solid line) and its Ccs1\(^{C27S/C64S}\) double mutant (dashed line). Far-UV CD spectra of both proteins were measured in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl at 20°C. The spectrum of the wild type revealed a broad trough with a minimum at about 208 nm and a pronounced shoulder at about 222 nm, which are indicative of predominantly α-helical
secondary structure (around 50%). By contrast, the strongly reduced intensity of the C27S/C64S Domain I mutant suggests that the latter has less \( \alpha \)-helical secondary structure.
SUPPLEMENTARY MATERIAL & METHODS

Expression and purification of Domain I of Ccs1: GST-tagged wild type and C27S/C64S double cysteine mutant of Domain I of Ccs1 (residues 2–74) were produced in E. coli BL21 from the pGEX-6p-1 (GE Healthcare) expression vector. Proteins were extracted by sonication in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. GST-tagged proteins were bound to Glutathion Sepharose 4B (GE Healthcare), and Domain I variants were eluted using Precission Protease. For the analysis of the redox state, the purified proteins were incubated with 10 mM mm-PEG24 (Pierce) for 1 h at room temperature in the dark. For the completely reduced control, the protein was treated with 10 mM TCEP for 15 min at room temperature or 96°C prior to mm-PEG24 treatment.

CD spectroscopy: CD spectra were recorded on a Chirascan-plus from Applied Photophysics (Leatherhead, U.K.) using a digital integration time of 1 s and a data pitch of 1 nm. Proteins were dissolved at a concentration of 50 µM in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and incubated for 3 min at 20 °C prior to measurement in a 1-mm quartz cell (Hellma, Müllheim, Germany).
## TABLE S1

| Plasmid                     | Characteristics | Primer                                                                 | restriction enzymes          |
|-----------------------------|-----------------|------------------------------------------------------------------------|------------------------------|
| pYX232-                      |                 |                                                                        |                              |
| Ccs1                        | Ccs1, protein expression in yeast, TPI promotor, TRP marker           | F) GGGGATCCGATGACCACGAGACGATA CATACAGAG   | BamHI                        |
|                             |                 |                                                                        | R) GGAAGCTTCTTTGATTGGTGGCAAGGTC  | HindIII                      |
| pYX232-                      |                 |                                                                        |                              |
| Ccs1(C17S,C20S)             | Ccs1, protein expression in yeast, TPI promotor, double mutation C17S and C20S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-                      |                 |                                                                        |                              |
| Ccs1(C17S,C20S)             | Ccs1, protein expression in yeast, TPI promotor, point mutation C27S and C64S, TRP marker | F) CAACACCTTGGAAACCTCTGTAAGAGACGCATC | Quick Change primer           |
|                             |                 |                                                                        | R) GATGCGCTTTTACAGAGGATTTTCAGAGGTCATGGG |                              |
| pYX232-                      |                 |                                                                        |                              |
| Ccs1(C27S)                  | Ccs1, protein expression in yeast, TPI promotor, point mutation C27S, C64S, TRP marker | F) GCGTGAATGATATAAAGGCATCCCTAAAAATGTCCC | Quick Change primer           |
|                             |                 |                                                                        | R) GGGACATTTTTTAGGGGATGCCTTTATATCATTCACGC |                              |
| pYX232-                      |                 |                                                                        |                              |
| Ccs1(C27S,C64S)             | Ccs1, protein expression in yeast, TPI promotor, double mutation C27S and C64S, TRP marker | F) CAACACCTTGGAAACCTCTGTAAGAGACGCATC | Quick Change primer           |
|                             |                 |                                                                        | R) GATGCGCTTTTACAGAGGATTTTCAGAGGTCATGGG |                              |
| pYX232-                      |                 |                                                                        |                              |
| Ccs1(C229S,C231S)           | Ccs1, protein expression in yeast, TPI promotor, double mutation C229S and C231S, TRP marker | F) GGGGAAAATAATAAAAGGCAATGCTGCTACCGGAAAGACTG | Quick Change primer           |
|                             |                 |                                                                        | R) CAGTCCTTCCGCTAGGCGACAGACTTTGATTATTTTTCCC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1                        | presequence of cytochrome b2, TRP marker                              | F) GGGGATATGCTAAAAAACTACCTTACA   | EcoRI                        |
|                             |                 |                                                                        | R) CGGAGATTCCCTTAAGGGAACCC         | BamHI                        |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1                        | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C17S,C20S)             | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, double mutation C17S and C20S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C27S)                  | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, point mutation C27S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C27S,C64S)             | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, double mutation C27S and C64S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C229S,C231S)           | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, double mutation C229S and C231S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C159S)                 | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, point mutation C159S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C229S,C231S)           | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, double mutation C229S and C231S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C159S)                 | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, point mutation C159S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pYX232-b2                    |                 |                                                                        |                              |
| Ccs1(C229S,C231S)           | Ccs1 fused to presequence of cytochrome b2, protein expression in yeast, TPI promotor, double mutation C229S and C231S, TRP marker | F) GCCATCTCCATGCAATCGTAAATTCCGTAATGATATAAAGGC | Quick Change primer           |
|                             |                 |                                                                        | R) GCCCTTTATATCATCAGGAATTTTCAGAGGATGTCATGGGAATGGC |                              |
| pRS314-                      |                 |                                                                        |                              |
| Ccs1                        | Ccs1, protein expression in yeast with endogenous promotor, TRP marker | F) GGGGAGCTCCTAAATAATACCTTCTTTCTTGTGTTGTAATTC | SacI                         |
|                             |                 |                                                                        | R) CCGGTACAGAGACGAACGATATG        | KpnI                         |
| Vector | Description | Marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
|--------|-------------|--------|-----|------|--------|----------|-------|---------------------|
| pRS314-Ccs1(C175,C205) | Ccs1, protein expression in yeast with endogenous promoter, double mutation C175 and C205, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
| pRS314-Ccs1(C275) | Ccs1, protein expression in yeast with endogenous promoter, point mutation C275, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
| pRS314-Ccs1(C64S) | Ccs1, protein expression in yeast with endogenous promoter, point mutation C64S, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
| pRS314-Ccs1(C229S,C231S) | Ccs1, protein expression in yeast with endogenous promoter, double mutation C229S and C231S, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
| pRS314-Ccs1(C159S) | Ccs1, protein expression in yeast with endogenous promoter, point mutation C159S, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
| pRS314-Ccs1(C27S) | Ccs1, protein expression in yeast with endogenous promoter, point mutation C27S, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |
| pRS314-Ccs1(C17S,C20S) | Ccs1, protein expression in yeast with endogenous promoter, double mutation C17S and C20S, TRP marker | TRP | URA3 | BamHI | HindIII | SalI | Quick Change primer |

For more specific information, please refer to the full text in the image.