

# Mia40-dependent oxidation of cysteines in domain I of Ccs1 controls its distribution between mitochondria and the cytosol

Christine Klöppel<sup>a,\*</sup>, Yutaka Suzuki<sup>a,\*</sup>, Kerstin Kojer<sup>a</sup>, Carmelina Petrunaro<sup>a</sup>, Sebastian Longen<sup>a</sup>, Sebastian Fiedler<sup>b</sup>, Sandro Keller<sup>b</sup>, and Jan Riemer<sup>a</sup>

<sup>a</sup>Cell Biology and <sup>b</sup>Molecular Biophysics, University of Kaiserslautern, 67663 Kaiserslautern, Germany

**ABSTRACT** Superoxide dismutase 1 (Sod1) is an important antioxidative enzyme that converts superoxide anions to hydrogen peroxide and water. Active Sod1 is a homodimer containing one zinc ion, one copper ion, and one disulfide bond per subunit. Maturation of Sod1 depends on its copper chaperone (Ccs1). Sod1 and Ccs1 are dually localized proteins that reside in the cytosol and in the intermembrane space of mitochondria. The import of Ccs1 into mitochondria depends on the mitochondrial disulfide relay system. However, the exact mechanism of this import process has been unclear. In this study we detail the import and folding pathway of Ccs1 and characterize its interaction with the oxidoreductase of the mitochondrial disulfide relay Mia40. We identify cysteines at positions 27 and 64 in domain I of Ccs1 as critical for mitochondrial import and interaction with Mia40. On interaction with Mia40, these cysteines form a structural disulfide bond that stabilizes the overall fold of domain I. Although the cysteines are essential for the accumulation of functional Ccs1 in mitochondria, they are dispensable for the enzymatic activity of cytosolic Ccs1. We propose a model in which the Mia40-mediated oxidative folding of domain I controls the cellular distribution of Ccs1 and, consequently, active Sod1.

## Monitoring Editor

Thomas D. Fox  
Cornell University

Received: Apr 4, 2011

Revised: Aug 11, 2011

Accepted: Aug 16, 2011

## INTRODUCTION

The respiratory chain is one of the main sources of reactive oxygen species (ROS; Murphy, 2009). Consequently, mitochondria contain numerous ROS-counteracting systems. Key enzymes are two superoxide dismutases (Sods): copper–zinc Sod1, which localizes to the cytosol and the mitochondrial intermembrane space (IMS), and manganese Sod2, which is present in the mitochondrial matrix. Sod1 is a homodimeric enzyme that per subunit contains one zinc

ion, one copper ion, and one disulfide bond. The incorporation of the copper ion and the formation of the disulfide bond are mediated by an activating enzyme, the copper chaperone for Sod1 (Ccs1; Culotta *et al.*, 1997; Rae *et al.*, 1999). Although Ccs1 is essential for the activation of Sod1 in yeast (Culotta *et al.*, 1997), Ccs1 knockout mice retain ~10–20% Sod1 activity (Wong *et al.*, 2000).

Ccs1 is like Sod1 found in the cytosol and in the IMS (Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001). Because neither Sod1 nor Ccs1 contains mitochondrial targeting signals, it has been unclear how both proteins are imported into the IMS. Recently, it was shown in yeast and mammalian cells that the import of Ccs1 and Sod1 relies on the mitochondrial disulfide relay (Mesecke *et al.*, 2005; Kawamata and Manfredi, 2008; Reddehase *et al.*, 2009). Thereby, the overexpression or depletion of the oxidoreductase Mia40 influences mitochondrial protein levels of Ccs1 and Sod1. During the import process, Ccs1 directly interacts with Mia40 through an intermolecular disulfide bond, whereas no direct interaction of Mia40 and Sod1 was observed (Reddehase *et al.*, 2009). However, mitochondrial Sod1 amounts increase upon overexpression of mitochondria-targeted Ccs1 in a CCS1-deletion background (Field *et al.*, 2003). Thus Sod1 is presumably enriched in the IMS by specific binding to mitochondrial

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-04-0293>) on August 24, 2011.

\*These authors contributed equally to this work.

Address correspondence to: Jan Riemer ([jan.riemer@biologie.uni-kl.de](mailto:jan.riemer@biologie.uni-kl.de)).

Abbreviations used: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; Ccs1, copper chaperone for Sod1; IMS, intermembrane space; ITS, intermembrane space-targeting signal; MISS, mitochondrial IMS-sorting signal; NEM, N-ethylmaleimide; PK, proteinase K; ROS, reactive oxygen species; Sod1, copper–zinc superoxide dismutase; Sod2, manganese superoxide dismutase; TCA, trichloroacetic acid.

© 2011 Klöppel *et al.* This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (<http://creativecommons.org/licenses/by-nc-sa/3.0>).

"ASCB®," "The American Society for Cell Biology®," and "Molecular Biology of the Cell®" are registered trademarks of The American Society of Cell Biology.

Ccs1, and Ccs1 in turn is a substrate of the mitochondrial disulfide relay.

Substrates of the mitochondrial disulfide relay usually are small proteins (10–15 kDa) composed of a single domain that is formed by a simple helix–loop–helix structure (Chacinska *et al.*, 2009; Riemer *et al.*, 2009). These proteins contain four conserved cysteines that are arranged in either two CX<sub>9</sub>C or two CX<sub>3</sub>C motifs (C, cysteine; X, any other amino acid) and consequently are termed twin CX<sub>9</sub>C or twin CX<sub>3</sub>C proteins, respectively. Mia40 substrates can only translocate into the IMS in their reduced and unfolded state. On mitochondrial import the cysteines are oxidized by Mia40 and form disulfide bonds that connect the two helices (Chacinska *et al.*, 2009; Riemer *et al.*, 2009). Thus oxidative folding and import into mitochondria are coupled processes for these small substrates. In addition to its role as thiol oxidase, Mia40 has recently demonstrated to function as a molecular chaperone that induces helix formation in its substrates (Banci *et al.*, 2010).

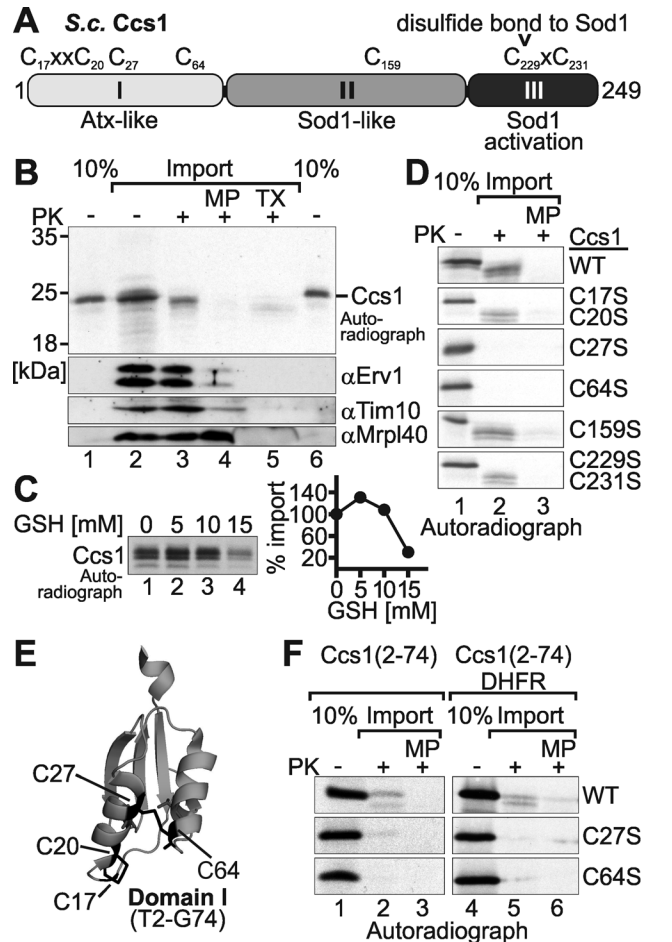
In contrast with the classic, rather small substrates of Mia40, the Ccs1 monomer has a size of 27 kDa and is composed of three domains: two copper-binding domains I and III at the amino and carboxyl termini, respectively, and a central domain II that is structurally homologous to Sod1 (Figure 1A). Whereas domain III is essential for Sod1 activation, domain I is required only under conditions of copper deprivation (Schmidt *et al.*, 1999). Of the seven cysteines of yeast Ccs1, four localize to domain I (two form the copper-binding CXXC motif), one is found in domain II, and two are in domain III in a CXC motif responsible for copper transfer to Sod1 (Figure 1A). Because its cysteines are not arranged in twin CX<sub>9</sub>C or twin CX<sub>3</sub>C motifs, it remains unclear how Ccs1 is imported, which cysteine interacts with Mia40, whether some of its cysteines are present in an oxidized state after import, and whether Mia40 assists in the folding of Ccs1.

In this study, we aimed to understand the process of mitochondrial import and folding of Ccs1 in mechanistic detail. We identified two cysteines (Cys-27 and Cys-64) in domain I that are required for mitochondrial import of Ccs1—but not for its role in Sod1 maturation—by the use of cysteine mutants. We demonstrate that C27 and C64 can be directly oxidized by Mia40 and *in vivo* form a stable disulfide bond in mitochondria, whereas they are partially reduced in the cytosol. We propose that the formation of the C27–C64 disulfide by Mia40 competes with a slower, nonenzymatic oxidation in the cytosol and thus allows the import of a fraction of Ccs1 into the IMS before folding in the cytosol can take place.

## RESULTS

### Cys-27 and Cys-64 in domain I are required for mitochondrial import of Ccs1

The import of Ccs1 into mitochondria depends on the disulfide relay of the IMS (Mesecke *et al.*, 2005; Reddehase *et al.*, 2009). For the twin CX<sub>9</sub>C and twin CX<sub>3</sub>C substrates of Mia40 the cysteines are critical for efficient import. We therefore decided to identify the cysteine(s) in Ccs1 that influence its localization by analyzing the mitochondrial import of Ccs1 cysteine-to-serine mutants. First, we established a protocol to monitor the mitochondrial import of wild-type Ccs1 (Figure 1B). To this end, we incubated radioactively labeled Ccs1 with isolated mitochondria. At the end of the import reaction the sample was split into four parts and analyzed either directly or after treatment with proteinase K (PK). The latter treatment either was applied to intact mitochondria or was preceded by hypoosmotic swelling of mitochondria to generate mitoplasts (mitochondria lacking their outer membrane) or by mitochondrial lysis with Triton X-100. A band migrating at the expected size of Ccs1



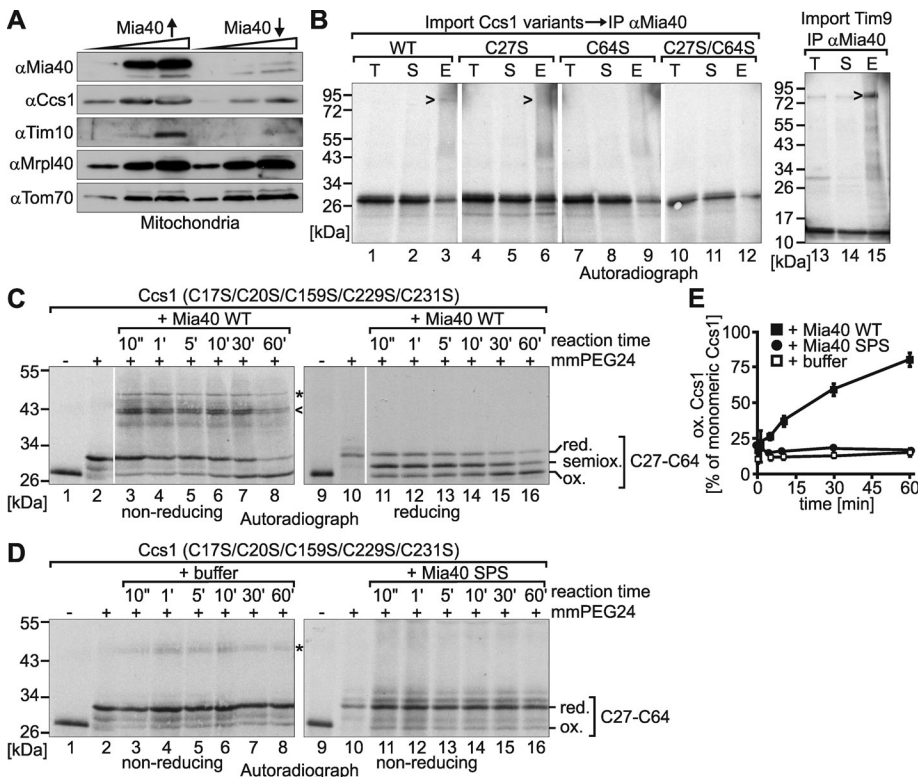
**FIGURE 1:** Cys-27 and Cys-64 are required for import of Ccs1 into isolated mitochondria. (A) Domain organization of yeast Ccs1. The three domains and the sequence positions of the seven cysteines of Ccs1 are depicted. (B) Import of Ccs1 into isolated mitochondria. *In vitro* translated radioactive Ccs1 (lanes 1 and 6, 10% control) was incubated with 50 μg of mitochondria isolated from Mia40-overexpressing strains. Subsequently, mitochondria were either analyzed directly (lane 2), treated with 100 μg/μl PK to remove nonimported proteins (lane 3), or turned into mitoplasts (MP) by hypotonic swelling and then treated with PK (lane 4) to remove all IMS proteins. One sample was also completely lysed with Triton X-100 (TX) and incubated with PK to test Ccs1 for protease resistance (lane 5). All samples were analyzed by reducing SDS–PAGE and autoradiography. The treatments with PK on mitochondria and mitoplasts were verified by immunoblotting against Erv1, Tim10 (both IMS), and Mrpl40 (matrix). (C) Glutathione dependence of Ccs1 import. Same as B, except that *in vitro* translated Ccs1 was incubated with different amounts of reduced glutathione during import. Experiments were quantified with ImageJ. (D) Import of Ccs1 cysteine mutants into isolated mitochondria. Same as B, except that *in vitro* translated Ccs1 cysteine mutants were incubated with mitochondria. Only whole mitochondria and mitoplasts were analyzed after PK digest. (E) Structure of domain I of Ccs1 (Lamb *et al.*, 1999). This domain encompasses residues 2–74 and contains four cysteines (C17, C20, C27, and C64). Note that the C27–C64 disulfide connects two antiparallel α-helices, resulting in a structure of domain I that is similar to the structure of twin CX<sub>9</sub>C or twin CX<sub>3</sub>C substrates. (F) Import of Ccs1-domain I cysteine mutants and DHFR fusion variants into isolated mitochondria. Same as D, except that *in vitro* translated variants of domain I- or domain I-DHFR fusion proteins were incubated with mitochondria.

was detected when intact mitochondria were treated with PK, indicating import of Ccs1 into mitochondria (Figure 1B, lane 3). On hypoosmotic swelling and subsequent PK treatment this signal disappeared, implying import of Ccs1 into the IMS (Figure 1B, lane 4). Notably, in some experiments we observed that the band representing Ccs1 migrated slightly faster on SDS-PAGE after PK treatment compared with untreated samples. In these cases a small part of the Ccs1 protein might still be exposed to the exterior after the import reaction. This exposed part would then be truncated upon digestion by PK. However, the main part of the protein was inaccessible to protease. The import of twin CX<sub>3</sub>C and twin CX<sub>9</sub>C substrates of Mia40 exhibits a marked dependence on reductants such as re-

duced glutathione (GSH; Bien *et al.*, 2010). When testing the import of Ccs1 we found a similar dependence of Ccs1 import (Figure 1C). At low concentrations of GSH (5 mM) import of Ccs1 proceeded faster than import in the absence of reductant. However, at higher concentrations of GSH (15 mM) Ccs1 import was strongly decreased.

We next investigated the import of the Ccs1 cysteine mutants (Figure 1D). We generated double mutants of the two cysteine pairs that were previously shown to contribute to copper binding and transfer (C17/C20 and C229/C231; Figure 1A) and single mutants of the remaining three cysteines. The Ccs1 double-cysteine mutants Ccs1<sup>C17S/C20S</sup> and Ccs1<sup>C229S/C231S</sup>, as well as Ccs1<sup>C159S</sup>, were imported with comparable efficiency to that of wild-type Ccs1. However, Ccs1<sup>C27S</sup> and Ccs1<sup>C64S</sup> exhibited strongly diminished import, indicating that these cysteines are critical for the mitochondrial localization of Ccs1.

The cysteines at positions 27 and 64 are both situated in domain I of Ccs1 (Figure 1, A and E). A closer analysis of the structures of this domain (Lamb *et al.*, 1999, 2001) revealed that C27 and C64 face each other and, at least in one of the structures, covalently connect two antiparallel  $\alpha$ -helices (Figure 1E). Thus the structure of domain I resembles the structures of typical Mia40 substrates. We therefore asked whether domain I (corresponding to residues 2–74 of Ccs1) on its own is imported into mitochondria and whether it can serve as a targeting signal for a nonmitochondrial protein. Applying our import assay, we first demonstrated that domain I can be imported into isolated mitochondria, although with lower efficiency than full-length Ccs1 (Figure 1F, lanes 1–3). Mutation of either C27 or C64 abolished the import of domain I into mitochondria, confirming their relevance for the import process (Figure 1D). Moreover, wild-type domain I fused to the cytosolic protein dihydrofolate reductase (DHFR) allowed the import of this chimeric protein into mitochondria, whereas the C27S and C64S mutants again failed to be transported into mitochondria (Figure 1F, lanes 4–6). In summary, we found that domain I alone is sufficient for C27- and C64-dependent targeting of Ccs1 to mitochondria.



**FIGURE 2:** A disulfide bond between Cys-27 and Cys-64 of Ccs1 can be formed by Mia40 in vitro. (A) Protein levels in mitochondria isolated from strains with varying Mia40 levels. The yeast strain GalL-Mia40 was used to regulate the protein levels of Mia40. To overexpress Mia40 (Mia40<sup>↑</sup>), cells were grown in the presence of galactose. To deplete Mia40 (Mia40<sup>↓</sup>), cells were cultured in lactate medium containing 0.1% glucose for 16 h. Subsequently, mitochondria were isolated, and 10, 30, and 60  $\mu$ g of isolated mitochondria were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. (B) Interaction of Ccs1 variants with Mia40 during import into isolated mitochondria. Radiolabeled Ccs1 variants or the twin CX<sub>3</sub>C protein Tim9 were incubated with mitochondria isolated from a strain overexpressing Mia40 for 8 min at 25°C. Thiols–disulfide exchange reactions were stopped by addition of 50 mM NEM, mitochondria were lysed under denaturing conditions and diluted with Triton X-100-containing buffer, and supernatants were subjected to immunoprecipitation (IP) against Mia40. Bound material was analyzed by nonreducing SDS-PAGE and autoradiography. E, elution from beads after IP; T, total supernatant after IP. (C) In vitro oxidation of full-length Ccs1<sup>C17S/C20S/C159S/C229S/C231S</sup> by Mia40-WT. The in vitro translated radioactive Ccs1 mutant was incubated with 30  $\mu$ M purified wild-type Mia40 (Mia40-WT) for the indicated times. Subsequently, samples were TCA precipitated, treated with 10 mM mm-PEG24 for 1 h, subjected to nonreducing (lanes 1–8) or reducing (lanes 9–16) SDS-PAGE, and analyzed by autoradiography. Arrowhead, disulfide-linked complex of Mia40 and Ccs1; asterisk, background band—potentially a disulfide-linked Ccs1 dimer. (D) As in C, except that the in vitro translated radioactive Ccs1 mutant was incubated with buffer (lanes 3–8) or 30  $\mu$ M purified redox-inactive Mia40-SPS (lanes 11–16) for the indicated times. The samples were subjected to nonreducing SDS-PAGE. Asterisk, background band—potentially a disulfide-linked Ccs1 dimer. (E) Quantification of C and D. The autoradiographs of three different experiments were quantified using ImageJ.

### In vitro C27 and C64 of Ccs1 can be oxidized by Mia40

Cellular depletion of Mia40 results in lowered amounts of mitochondrial Ccs1 (Reddehase *et al.*, 2009). We verified this finding by immunoblot analyses of mitochondria isolated from strains with increased or lowered Mia40 levels (Figure 2A). Notably, the mitochondrial levels of Ccs1 did not appear to be as sensitive toward depletion of Mia40 as the levels of the twin CX<sub>3</sub>C protein Tim10.





excess DTT, modified cysteine residues with AMS. The individual size shifts of AMS-treated Ccs1 variants correlated with the number of cysteine residues (Figure 3B; see added AMS molecules, and compare with lane 8).

Next we assessed the *in vivo* redox state of the wild-type Ccs1 (Figure 3C). At steady state Ccs1 was present in two fractions: a completely reduced form (modified with seven AMS molecules) and one that migrated at an apparent size that corresponds to a modification with five AMS molecules, indicating the presence of one disulfide bond in this form (Figure 3C, lane 2). The disulfide bond in this semioxidized form of Ccs1 appears to be very stable (i.e., structural) because it could be opened only upon DTT treatment at 96°C but not at 20°C or by DTT treatment of intact cells (Figure 3C, compare lanes 3–5). Because C17/C20 and C229/C231 are localized to CXXC and CXC motifs, respectively, which usually form unstable disulfides, we reasoned that C27 and C64 might form this structural disulfide bond. We therefore determined the redox state of Ccs1<sup>C27S/C64S</sup> *in vivo* (Figure 3C, lanes 6–10). Ccs1<sup>C27S/C64S</sup> was completely reduced (Figure 3C, lane 9), indicating that the stable disulfide in the wild type was indeed formed between C27 and C64. Notably, our results demonstrate that *in vivo* all other cysteines of Ccs1 (C17, C20, C159, C229, C231) are in the reduced state, which for C17/C20 and C229/C231 is in accordance with their role in copper binding and transfer.

Because wild-type Ccs1 exists at steady state in two different redox forms we wondered whether mitochondrial Ccs1 might have a redox state different from that of cytosolic Ccs1. To perform this experiment *in vivo*, we generated yeast cells in which Ccs1 exclusively localizes to the IMS. To this end, the *CCS1*-deletion strain was transformed with plasmids encoding IMS-targeted Ccs1 variants. These plasmids encoded an extended bipartite mitochondrial targeting signal of cytochrome *b*<sub>2</sub> fused to Ccs1 (*b*<sub>2</sub>-Ccs1). This signal consists of a matrix-targeting signal followed by a hydrophobic sorting sequence ensuring transfer into the inner membrane, and an additional IMS-localized domain that prevents accidental matrix import. After import into mitochondria a part of the bipartite targeting signal is removed, and the mature *b*<sub>2</sub>-Ccs1 protein localizes to the IMS. Using the alkylation shift assay we found that all wild-type *b*<sub>2</sub>-Ccs1 molecules contained the stable C27–C64 disulfide bond (Figure 3D, lane 2). *b*<sub>2</sub>-Ccs1<sup>C27S/C64S</sup> migrated at the same position (Figure 3D, lane 9), confirming that in wild-type, IMS-localized Ccs1, C27 and C64 are oxidized (because they could not be modified with AMS). Thus our results indicate that at least a fraction of the cytosolic Ccs1 does not contain the C27–C64 disulfide (Figure 3C, lane 2).

We repeated this redox state determination multiple times and observed that the ratio between the completely reduced and the semioxidized fraction varied significantly in whole cells but not in mitochondria (Supplemental Figure S2, A–C). Such redox state changes at steady state are frequently observed also in other redox systems and might be due, for example, to differing growth states, cell densities, nutrient supplies, and oxygen tensions (Appenzeller-Herzog and Ellgaard, 2008; Appenzeller-Herzog *et al.*, 2008). Indeed, we observed differences in the redox state of Ccs1 by changes in its expression level and by varying the oxygen tension (Supplemental Figure S2, A, B, and D). When comparing the redox state of Ccs1 at endogenous and overexpressed levels (Figure 3C and Supplemental Figure S2, A and B) we found that in both cases the protein was in part semioxidized, but Ccs1 present at endogenous levels appeared to be overall in a more oxidized state. This finding might indicate that upon overexpression of Ccs1 the oxidation system in the cytosol becomes limiting. Similarly, in cells exposed to 20% oxygen a larger portion of C27 and C64 was present in the

oxidized state than in cells cultured under hypoxic conditions (Supplemental Figure S2D). Taken together, the results demonstrate that C27 and C64 form a disulfide bond in the mitochondrial fraction of Ccs1, whereas a varying fraction of cytosolic Ccs1 is completely reduced.

### Cysteine mutations in domain I affect the cellular distribution of Ccs1

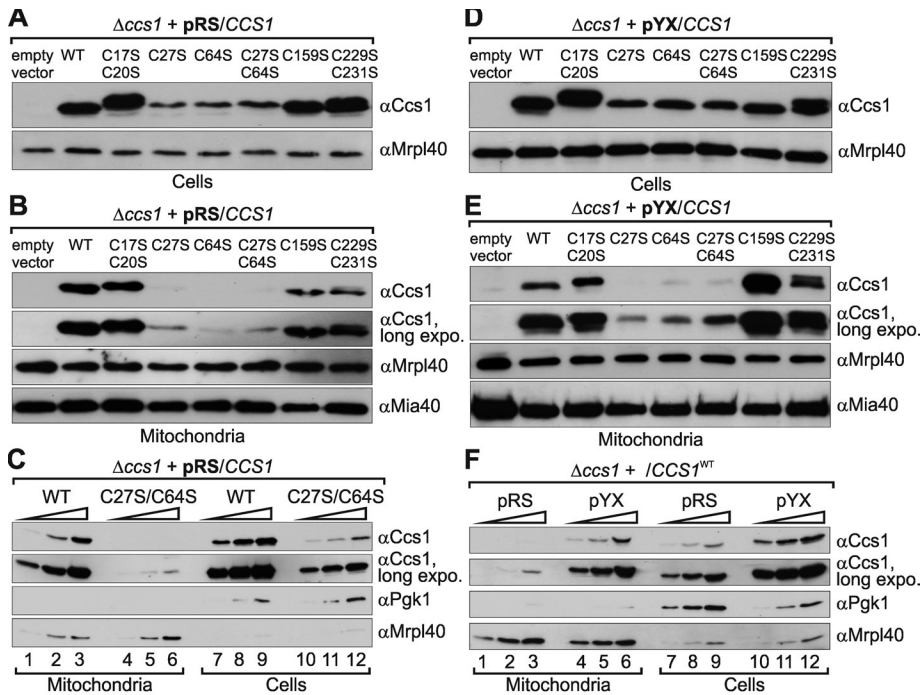
Next we assessed whether the cysteine residues at positions 27 and 64 are also required for import under *in vivo* conditions by analyzing their cellular distribution. First, we compared the expression levels of the Ccs1 cysteine mutants expressed from the endogenous *CCS1* promoter in whole cells (Figure 4A). All cells contained similar amounts of Ccs1 except for the strains expressing Ccs1<sup>C27S</sup>, Ccs1<sup>C64S</sup>, and Ccs1<sup>C27S/C64S</sup>. We assume that these three mutants are less stable compared with the wild-type Ccs1 and thus become rapidly degraded. This is also supported by circular dichroism spectra of the purified domain I of both the wild type and the C27S/C64S mutant of Ccs1, as the latter variant exhibits less secondary structure compared with the wild type (Supplemental Figure S3).

We then isolated mitochondria from all strains and treated them with low amounts of PK to remove any residual proteins still attached to the outside of the mitochondria. We confirmed with control Western blots against the IMS protein Mia40 that mitochondria remained intact during the procedure (Figure 4B, bottom). Notably, we found that mitochondria isolated from the cells expressing Ccs1<sup>C27S</sup>, Ccs1<sup>C64S</sup>, and Ccs1<sup>C27S/C64S</sup> contained only minute amounts of Ccs1 (Figure 4B). To exclude that these minute amounts of mitochondrial Ccs1<sup>C27S</sup>, Ccs1<sup>C64S</sup>, and Ccs1<sup>C27S/C64S</sup> result only from the low cellular levels of Ccs1, we compared strains expressing the wild type and Ccs1<sup>C27S/C64S</sup> in more detail (Figure 4C). We found that the strain containing Ccs1<sup>C27S/C64S</sup> harbored only ~10% of the Ccs1 found in the wild type. However, the mitochondrial Ccs1 levels in the former strain were well below 2% compared with those in the wild type, indicating that the cellular distribution of Ccs1<sup>C27S/C64S</sup> to mitochondria is indeed impaired.

To further strengthen this notion, we repeated the experiment using cells expressing the different Ccs1 variants from an overexpression plasmid. The protein levels in these cells were ~10–20 times higher than with wild-type cells (Supplemental Figure S1). Moreover, the amounts of Ccs1<sup>C27S</sup>, Ccs1<sup>C64S</sup>, and Ccs1<sup>C27S/C64S</sup> were also significantly higher in these strains than in the strains expressing Ccs1 under the control of the endogenous promoter (Supplemental Figures S1 and S4, A and D). When comparing total Ccs1 levels with mitochondrial Ccs1 amounts (Figure 4, D and E) we clearly confirmed that Ccs1<sup>C27S</sup>, Ccs1<sup>C64S</sup>, and Ccs1<sup>C27S/C64S</sup> were lacking from the mitochondrial fraction. Moreover, the distribution of Ccs1 between mitochondria and the remainder of the cell was not substantially altered compared with the distribution in cells containing endogenous levels of Ccs1 (Figure 4F; also compare Figure 4, D and E, with A and B). An exception was the C159S mutant of Ccs1, which appeared to become enriched in the mitochondrial fraction upon overexpression. Taken together, these results support that also *in vivo* C27 and C64 contribute to the accumulation of Ccs1 in mitochondria.

### Cysteine mutations in domain I do not affect the enzyme activity of Ccs1

We were also interested in the effect of cysteine mutations in Ccs1 on its activity. In yeast Ccs1 is essential for the activation of Sod1, and thus Sod1 activity can be used as a measure for the enzymatic activity of Ccs1. We applied a gel-based Sod activity assay on lysates of cells and of isolated mitochondria from yeast strains expressing



**FIGURE 4:** Mutations of Cys-27 and Cys-64 result in decreased mitochondrial levels of Ccs1. (A) Ccs1 protein levels in  $\Delta ccs1$  cells expressing different Ccs1 variants under the control of the endogenous promoter (pRS). Cells were grown to mid-log phase in S(Lac)-Trp medium, lysed, and analyzed by SDS-PAGE and Western blotting against the indicated proteins. (B) Ccs1 protein levels in mitochondria isolated from  $\Delta ccs1$  cells expressing different Ccs1 variants under the control of the endogenous promoter from a *cen* plasmid (pRS). Cells were grown to mid-log phase in S(Lac)-Trp medium and lysed, and mitochondria were isolated. To remove proteins attached to the outside of mitochondria, the mitochondrial fraction was treated with 100  $\mu$ g/ml PK. Mitochondrial fractions were analyzed by SDS-PAGE and immunoblotting using antibodies directed against Ccs1, Mrpl40 (mitochondrial matrix), and Mia40 (IMS). (C) Comparison of the cellular distribution of wild-type Ccs1 and Ccs1<sup>C27S/C64S</sup> in cells with endogenous levels of Ccs1. Experiments were performed as in A and B. Amounts of 5, 10, and 20  $\mu$ g of mitochondria and cells at OD<sub>600</sub> values of 0.05, 0.1, and 0.2 were analyzed. (D) Ccs1 protein levels in  $\Delta ccs1$  cells expressing different Ccs1 variants under the control of the triosephosphate isomerase promoter from a 2 $\mu$  plasmid (pYX). As in A, except that Ccs1 was overexpressed in  $\Delta ccs1$  cells. (E) Ccs1 protein levels in mitochondria isolated from  $\Delta ccs1$  cells expressing different Ccs1 variants under the control of the triosephosphate isomerase promoter (pYX). As in B, except that Ccs1 was overexpressed in  $\Delta ccs1$  cells. (F) Comparison of the cellular distribution of wild-type Ccs1 in cells with endogenous levels of Ccs1 and cells overexpressing Ccs1. Experiments were performed as in A and B on  $\Delta ccs1$  cells expressing wild-type Ccs1 under the control of either the endogenous promoter (pRS) or the triosephosphate isomerase promoter (pYX). Totals of 5, 10, and 20  $\mu$ g mitochondria and cells at OD<sub>600</sub> values of 0.05, 0.1, and 0.2 were analyzed.

different Ccs1 cysteine mutants in a *CCS1*-deletion background (Figure 5). When analyzing whole cells we confirmed previous findings (Schmidt *et al.*, 1999) that the mutation of C229 and C231 in Ccs1 results in the complete loss of Sod1 activity (Figure 5A). Deletion of the remaining cysteines, however, did not affect Sod1 activity in whole-cell lysates (Figure 5A). This was in strong contrast with the situation in mitochondria, in which C27 and C64 were essential for the accumulation of Sod1 activity (Figure 5B). This was due to the import defect of these Ccs1 mutants into mitochondria because fusion of the variants to a b<sub>2</sub>-targeting sequence restored mitochondrial Sod1 activity (Figures 5C).

#### Mutations in amino acids forming the potential IMS-targeting signal result in the same phenotype as the C27S and C64S mutants of Ccs1

The small twin CX<sub>3</sub>C and twin CX<sub>9</sub>C substrates of Mia40 contain an internal mitochondrial targeting signal, a so-called mitochondria

IMS-sorting signal (MISS) or intermembrane space-targeting signal (ITS; Milenkovic *et al.*, 2009; Sideris *et al.*, 2009). Such a signal has not been defined for Ccs1. Closer analysis of the Ccs1 sequence and structure revealed that amino acids I24, I57, and L61 might potentially form part of such a sequence, as they are hydrophobic residues localized to the  $\alpha$ -helix presumably interacting with Mia40 (Figure 6A). To investigate the influence of these residues on the cellular distribution, mitochondrial import, and redox state of Ccs1, we generated and analyzed I24E and L61E mutants (Figure 6, B–D). We found that these Ccs1 variants were not imported into isolated mitochondria (Figure 6B) and that they were present only at low levels in whole cells and in minute amounts in mitochondria (Figure 6C). Although they were in principle capable of activating Sod1 in whole cells, mitochondria contained lower levels of active Sod1 (Figure 6D). Thus both Ccs1 variants behaved like Ccs1<sup>C27S</sup> and Ccs1<sup>C64S</sup> and might indeed constitute a part of the MISS/ITS of Ccs1.

#### DISCUSSION

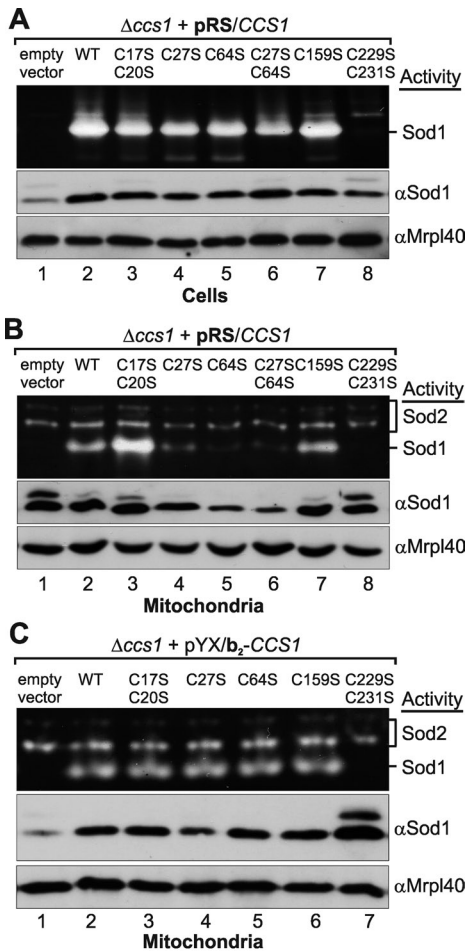
Ccs1 is dually localized to the cytosol and mitochondria. Mitochondrial import is facilitated by Mia40 (Reddehase *et al.*, 2009). However, the mechanistic details of this import process remained unclear. In this study we provide in-depth analyses of Ccs1 cellular distribution, mitochondrial import, oxidative folding by Mia40, and physiological consequences of Ccs1 mutations.

#### The oxidation of the Cys-27 and Cys-64 by Mia40 is necessary for efficient import of Ccs1 into mitochondria

We demonstrate that C27 and C64 in domain I of Ccs1 play a critical role in controlling the distribution of Ccs1 and thus the distribution of active Sod1 within the cell.

Our findings indicate the following order of events (Figure 6E): After cytosolic synthesis Ccs1 can be either folded in the cytosol or translocated into mitochondria. The latter process is driven by the covalent interaction of Mia40 with immature Ccs1, which results in the formation of a stable intramolecular disulfide bond between C27 and C64 (Figures 2 and 3). The interaction with Mia40 most likely takes place through C64 (Figure 2B). IMS import signals also encompass sequence features that facilitate non-covalent binding to Mia40; in the case of Ccs1 these include the hydrophobic residues I24 and L61 (Figure 6). Without the two cysteines or the two hydrophobic residues Ccs1 is not imported into mitochondria (Figures 1, 4, and 6), and consequently IMS-localized Sod1 is not activated (Figures 5 and 6). The C27–C64 disulfide is also formed in the cytosolic form of Ccs1 even though it appears that a significant fraction of Ccs1 remains completely reduced, indicating that oxidation commences more slowly (Figure 3). It therefore seems likely that cytosolic Ccs1 oxidation occurs without the help of a dedicated oxidoreductase. On the basis of these findings we propose





**FIGURE 5:** Levels of active mitochondrial Sod1 are lower in strains expressing the C27S and C64S cysteine mutants of Ccs1. (A) Sod1 protein and activity levels in  $\Delta ccs1$  cells expressing different Ccs1 variants under the control of the endogenous promoter (pRS). Cells were grown to mid-log phase in S(Lac)-Trp medium. Subsequently, cells were either lysed in native lysis buffer and analyzed by Sod activity gels (top) or lysed in SDS lysis buffer and analyzed by SDS-PAGE and Western blotting against the indicated proteins (middle and bottom). (B) Sod1 protein and activity levels in mitochondria isolated from  $\Delta ccs1$  cells expressing different Ccs1 variants under the control of the endogenous promoter from a *cen* plasmid (pRS). Cells were grown to mid-log phase in S(Lac)-Trp medium and lysed, and mitochondria were isolated. To remove proteins attached to the outside of mitochondria, the mitochondrial fraction was treated with 100  $\mu$ g/ml PK. Mitochondrial fractions were then analyzed by Sod activity gels or by SDS-PAGE and immunoblotting using antibodies directed against Sod1, Mrpl40 (mitochondrial matrix), and Mia40 (IMS). (C) Sod1 activity and protein level in mitochondria isolated from cells expressing different  $b_2$ -Ccs1 cysteine mutants. As in B, except that Ccs1 variants were fused to a  $b_2$ -targeting sequence and expressed under the control of the triosephosphate isomerase promoter from a  $2\mu$  plasmid (pYX).

that the mitochondrial import, which is accompanied by enzyme-catalyzed oxidative folding in the IMS, competes with a slow and non-enzyme-catalyzed oxidative folding reaction of Ccs1 in the cytosol. This would ensure that a significant amount of unfolded and reduced Ccs1 remains in the import-competent state after cytosolic translation long enough to be imported into the IMS.

The import and oxidative folding of Ccs1 by Mia40 clearly show differences compared with the twin CX<sub>9</sub>C and twin CX<sub>3</sub>C substrates,

such as slower folding kinetics, lower import efficiency, and the formation of only one disulfide in a domain with a more complex structure ( $\alpha\beta\alpha$ ). The lower efficiency of mitochondrial import of Ccs1 into isolated mitochondria compared with other Mia40 substrates (Figure 1B) might stem from its slower folding kinetics (Figure 2C). Slower folding could result from a longer noncovalent interaction with Mia40. Consequently, less Mia40 is unoccupied and can serve as import receptor for Ccs1. In addition, for Ccs1 import both cysteines in domain I appear to be critical. This is in contrast to twin CX<sub>3</sub>C and twin CX<sub>9</sub>C proteins, which are imported into isolated mitochondria if only the cysteine that initially interacts with Mia40 is present (Milenkovic et al., 2007, 2009; Sideris and Tokatlidis, 2007; Sideris et al., 2009). Although we also observed a Mia40–Ccs1<sup>C27S</sup> complex, we could not detect import of this mutant into mitochondria. This might be explained by the fact that we did not include the reductant  $\beta$ -mercaptoethanol in the import buffer to detect covalent interactions between Mia40 and its substrates (Figure 2B). However,  $\beta$ -mercaptoethanol was added during the import reactions (Figure 1, except C) because reductants normally serve to accelerate the import process of Mia40 substrates (Bien et al., 2010). Furthermore, in vivo both Ccs1<sup>C27S</sup> and Ccs1<sup>C64S</sup> are almost absent from the mitochondrial fractions. To our knowledge no such in vivo analyses have been performed for cysteine mutants of twin CX<sub>3</sub>C and twin CX<sub>9</sub>C proteins. It thus remains unclear whether these proteins are depleted in mitochondria in intact cells.

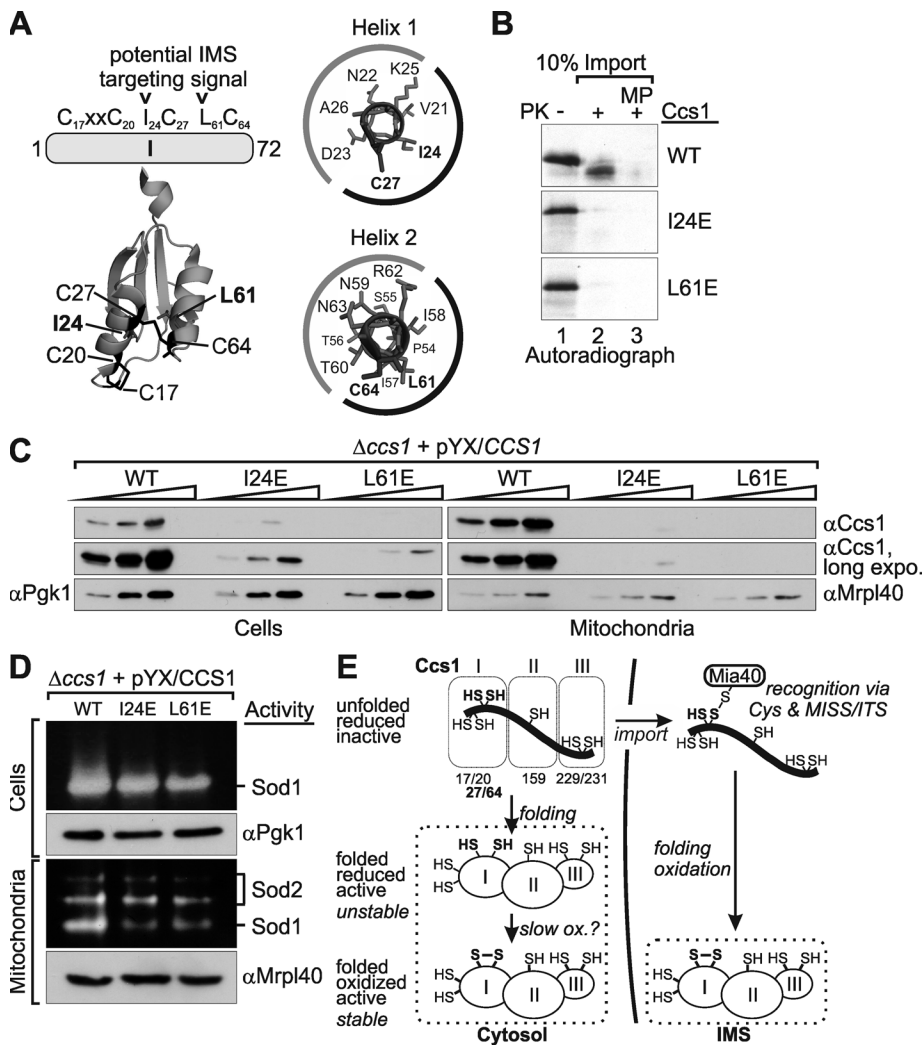
Other aspects of Mia40-dependent oxidative folding are conserved, such as the lack of import of a completely cysteine-less mutant, the formation of a stable structural disulfide connecting two antiparallel helices, and internal targeting signals (MISS/ITS) that facilitate recognition by Mia40. It was recently shown that Mia40 also can serve as a chaperone that induces the folding of one  $\alpha$ -helix in a twin CX<sub>9</sub>C substrate and subsequently forms a disulfide between two cysteines in juxtaposed helices (Banci et al., 2010). Although the overall structure of Ccs1 differs from those of the twin CX<sub>3</sub>C and twin CX<sub>9</sub>C proteins, domain I resembles these classic Mia40 substrates. This raises the possibility that Mia40, unlike chaperones of the cytosol and the endoplasmic reticulum, might be primed to induce the folding of only one specific fold in a limited subset of substrates.

### The disulfide between C27 and C64 is not required for Ccs1 activity but seems to increase the stability of Ccs1

Our results suggest that the structural disulfide between C27 and C64 increases the stability of Ccs1. This is reflected by lower protein levels of Ccs1<sup>C27S/C64S</sup> at steady state and the lowered secondary structure content of domain I bearing the C27S/C64S mutations compared with the wild type. Ccs1 activity toward Sod1 is not affected, however, by lack of the C27–C64 disulfide. Therefore, the reduced cytosolic form of Ccs1 is still capable of activating Sod1. These results are in agreement with experiments in which a Ccs1 mutant lacking the complete domain I still was capable of activating Sod1 in whole cells (in light of our results, likely the ~95% of cytosolic Sod1; Schmidt et al., 1999). Notably, an IMS-targeted Ccs1 mutant in which C27 and C64 had been replaced by serines could activate Sod1 in mitochondria, indicating that both cysteines influence mainly the import of Ccs1 into mitochondria.

### Consequences of the dual localization of Ccs1 for the cellular antioxidative response

The dual localization of Ccs1 and Sod1 also raised the questions of whether the distribution is variable and can be regulated. Indeed, the expression of a mitochondria-targeted variant of Ccs1 in a CCS1-deletion background results in increased protein and activity levels of mitochondrial Sod1 in yeast (Field et al., 2003).



**FIGURE 6:** MISS/ITS mutants in Ccs1 behave like the C27S and C64S mutants of Ccs1. (A) Scheme of domain I of Ccs1 and location of the amino acids forming the putative MISS/ITS. Locations of amino acids forming the potential MISS/ITS are indicated in the structure of Ccs1 domain I, as well as in the helical-wheel representation of the two helices forming domain I. The black and gray labels of the wheel indicate the hydrophobic and hydrophilic faces of the helices, respectively. (B) Import of MISS/ITS mutants of Ccs1 into isolated mitochondria. The experiment was performed as indicated in Figure 1D. (C) Cellular distribution of the MISS/ITS mutants of Ccs1. The experiment was performed as indicated in Figure 4. (D) Sod activity in cells and mitochondria from *CCS1* deletion strains expressing MISS/ITS mutants of Ccs1. The experiment was performed as indicated in Figure 5. (E) Model for the mechanism of Ccs1 distribution between the cytosol and the IMS. See Discussion for details.

Likewise, growth of mammalian tissue culture cells under hypoxic conditions leads to increased amounts of Sod1 and Ccs1 in mitochondria (Kawamata and Manfredi, 2008). In yeast and in a mouse model, higher levels of mitochondrial Sod1 activity improve the capacity to withstand mitochondria-derived oxidative stress (Klöppel et al., 2010; Fischer et al., 2011).

We thus aimed to identify conditions under which the cellular distribution of Ccs1 changed. To this end, we determined the levels of Ccs1 in mitochondria and whole cells in a number of deletion strains (e.g.,  $\Delta sod1$ ,  $\Delta ctt1$ ,  $\Delta grx1\Delta grx2\Delta grx8$ , and  $\Delta glr1$ ), as well as under varying conditions of oxidative stress (e.g., treatment with paraquat, tetramethylethylenediamine; unpublished data). However, under the tested conditions the cellular Ccs1 distribution remained unchanged. Nevertheless, the lowered stability of reduced Ccs1 could constitute a way to decrease the levels of cellular Ccs1

in the absence of oxidative stress because under these conditions a higher portion of Ccs1 was reduced.

It is important to note that our findings do not exclude the possibility that regulation occurs through changes in distribution in higher eukaryotes. It has already been shown in mammalian cells that the levels of mitochondrial Ccs1 increase during hypoxia (Kawamata and Manfredi, 2008). This also raises the question of how exactly Ccs1 is imported into mammalian mitochondria. We expect that the cellular distribution of human Ccs1 is also controlled by a cysteine-dependent mechanism because overexpression of human Mia40 strongly influences mitochondrial Ccs1 amounts (Kawamata and Manfredi, 2008). However, whereas human Ccs1 domain I shares the same overall structure as its yeast counterpart, it lacks the cysteines corresponding to C27 and C64. Instead, a serine and a threonine are found at the equivalent positions and form a hydrogen bond connecting the two antiparallel  $\alpha$ -helices. It will be exciting to test which of the nine cysteines in human Ccs1 contribute to mitochondrial import of the protein.

## MATERIALS AND METHODS

### Strains and plasmids

For primers and plasmids see Supplemental Table S1. The  $\Delta ccs1$  strain was derived from the wild-type strain YPH499 (*MATa ura3-52 lys2-801\_amber ade2-101\_ochre trp1- $\Delta$ 63 his3- $\Delta$ 200 leu2- $\Delta$ 1*) by replacement of the *CCS1* open reading frame with a *URA3* cassette. Homologous recombination was verified by PCR and controlled by immunoblotting against Ccs1. The  $\Delta ccs1$  strains expressing Ccs1 variants were generated by transformation with the respective plasmids. The Gal-Mia40 strain was introduced before (Terziyska et al., 2005). Strains were grown in *S*(Lac) medium (0.85% yeast nitrogen base, 2.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 9.9% lactate, 20 mg/l adenine sulfate, 20 mg/l uracil, 100 mg/l L-leucine, 30 mg/l L-lysine, 20 mg/l L-histidine, pH 5.5) with 0.2% galactose at 30°C.

### Antibodies

The following antibodies were used: anti-Mia40 (Mesecke et al., 2005), anti-Mrp140 (Gruschke et al., 2010), anti-Pgk1 (Invitrogen, Carlsbad, CA), anti-Sod1 (Klöppel et al., 2010), anti-Tim10, and anti-Tom70 (Mesecke et al., 2005). To generate the Ccs1 antibody, full-length yeast Ccs1 was purified and used for immunization of rabbits.

### Isolation of mitochondria, cell fractionation, and Sod activity assays

These methods were performed as described (Klöppel et al., 2010), except that for the analyses of the cellular Ccs1 distribution intact isolated mitochondria were treated with 100  $\mu$ g/ml PK to remove proteins attached to the outside of mitochondria. The intactness of



the outer membrane was verified by immunoblotting against the IMS protein Mia40.

### Import of radioactively labeled proteins into isolated mitochondria and in vitro disulfide bond formation assay

These experiments were performed as described (Bien *et al.*, 2010). The import reaction was performed either in the presence of 2 mM  $\beta$ -mercaptoethanol or in the presence of varying amounts of reduced glutathione and in the absence of  $\beta$ -mercaptoethanol.

### Analyses of Mia40–Ccs1 interaction after import of radioactively labeled proteins into isolated mitochondria

These experiments were performed as described (Mesecke *et al.*, 2005). During import no reductant was added.

### In vivo redox state analyses

To determine the redox state of Ccs1 in whole cells, cells were grown in selective medium to an OD<sub>600</sub> of 1.0. Cells were either left untreated or, for the in situ DTT control, incubated for 10 min at 30°C with 100 mM DTT. Then cells were harvested by centrifugation, and cell pellets were resuspended in 10% TCA. For the in vitro DTT controls, cell pellets were resuspended in 20 mM DTT and 1% Triton X-100. Cells were broken by glass bead homogenization, and the lysate was incubated for 10 min at either 20 or 96°C. At 20°C proteins are in their native conformation; however, in lysed cells the accessibility of DTT is improved compared with in situ DTT incubation. TCA was added to all samples, which then were subjected to sonification and glass bead homogenization. TCA pellets were obtained by centrifugation and were resuspended in 40  $\mu$ l AMS buffer (58 mM Tris, pH 7.0, 7% glycerol, 1.5% SDS, 0.1% bromocresol purple) containing 15 mM AMS in the case of AMS modification. Modification took place for 1 h at 20°C in the dark. Subsequently, samples were analyzed by SDS–PAGE and Western blot against Ccs1.

### ACKNOWLEDGMENTS

We thank Vera Nehr for technical assistance and Martin Ott (University of Kaiserslautern) for critical reading of the manuscript. We are grateful to Johannes Herrmann (University of Kaiserslautern) for many stimulating discussions, critical reading of the manuscript, and continuous support. Funding was obtained from the Fritz-Thyssen-Stiftung. J.R. is a European Molecular Biology Organization Long Term Fellow, and C.P. is a fellow of the Boehringer Ingelheim Fonds.

### REFERENCES

- Appenzeller-Herzog C, Ellgaard L (2008). In vivo reduction-oxidation state of protein disulfide isomerase: the two active sites independently occur in the reduced and oxidized forms. *Antioxid Redox Signal* 10, 55–64.
- Appenzeller-Herzog C, Riemer J, Christensen B, Sorensen ES, Ellgaard L (2008). A novel disulphide switch mechanism in Ero1 $\alpha$  balances ER oxidation in human cells. *EMBO J* 27, 2977–2987.
- Banci L *et al.* (2010). Molecular chaperone function of Mia40 triggers consecutive induced folding steps of the substrate in mitochondrial protein import. *Proc Natl Acad Sci USA* 107, 20190–20195.
- Bien M, Longen S, Wagener N, Chwalla I, Herrmann JM, Riemer J (2010). Mitochondrial disulfide bond formation is driven by intersubunit electron transfer in Erv1 and proofread by glutathione. *Mol Cell* 37, 516–528.
- Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009). Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138, 628–644.
- Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD (1997). The copper chaperone for superoxide dismutase. *J Biol Chem* 272, 23469–23472.
- Field LS, Furukawa Y, O'Halloran TV, Culotta VC (2003). Factors controlling the uptake of yeast copper/zinc superoxide dismutase into mitochondria. *J Biol Chem* 278, 28052–28059.
- Fischer LR, Igoudjil A, Magrane J, Li Y, Hansen JM, Manfredi G, Glass JD (2011). SOD1 targeted to the mitochondrial intermembrane space prevents motor neuropathy in the Sod1 knockout mouse. *Brain* 134, 196–209.
- Groß DP, Burgard CA, Reddehase S, Leitch JM, Culotta VC, Hell K (2011). Mitochondrial Ccs1 contains a structural disulfide bond crucial for the import of this unconventional substrate by the disulfide relay system. *Mol Biol Cell* 22, 3758–3767.
- Gruschke S, Grone K, Heublein M, Holz S, Israel L, Imhof A, Herrmann JM, Ott M (2010). Proteins at the polypeptide tunnel exit of the yeast mitochondrial ribosome. *J Biol Chem* 285, 19022–19028.
- Kawamata H, Manfredi G (2008). Different regulation of wild-type and mutant Cu,Zn superoxide dismutase localization in mammalian mitochondria. *Hum Mol Genet* 17, 3303–3317.
- Klöppel C, Michels C, Zimmer J, Herrmann JM, Riemer J (2010). In yeast redistribution of Sod1 to the mitochondrial intermembrane space provides protection against respiration derived oxidative stress. *Biochem Biophys Res Commun* 403, 114–119.
- Lamb AL, Torres AS, O'Halloran TV, Rosenzweig AC (2001). Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. *Nat Struct Biol* 8, 751–755.
- Lamb AL, Wernimont AK, Pufahl RA, Culotta VC, O'Halloran TV, Rosenzweig AC (1999). Crystal structure of the copper chaperone for superoxide dismutase. *Nat Struct Biol* 6, 724–729.
- Mesecke N, Terziyska N, Kozany C, Baumann F, Neupert W, Hell K, Herrmann JM (2005). A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. *Cell* 121, 1059–1069.
- Milenkovic D, Gabriel K, Guiard B, Schulze-Specking A, Pfanner N, Chacinska A (2007). Biogenesis of the essential Tim9–Tim10 chaperone complex of mitochondria: site-specific recognition of cysteine residues by the intermembrane space receptor Mia40. *J Biol Chem* 282, 22472–22480.
- Milenkovic D, Ramming T, Muller JM, Wenz LS, Gebert N, Schulze-Specking A, Stojanovski D, Rospert S, Chacinska A (2009). Identification of the signal directing Tim9 and Tim10 into the intermembrane space of mitochondria. *Mol Biol Cell* 20, 2530–2539.
- Murphy MP (2009). How mitochondria produce reactive oxygen species. *Biochem J* 417, 1–13.
- Okado-Matsumoto A, Fridovich I (2001). Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J Biol Chem* 276, 38388–38393.
- Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999). Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* 284, 805–808.
- Reddehase S, Grumbt B, Neupert W, Hell K (2009). The disulfide relay system of mitochondria is required for the biogenesis of mitochondrial Ccs1 and Sod1. *J Mol Biol* 385, 331–338.
- Riemer J, Bulleid N, Herrmann JM (2009). Disulfide formation in the ER and mitochondria: two solutions to a common process. *Science* 324, 1284–1287.
- Schmidt PJ, Rae TD, Pufahl RA, Hamma T, Strain J, O'Halloran TV, Culotta VC (1999). Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. *J Biol Chem* 274, 23719–23725.
- Sideris DP, Petrakis N, Katrakili N, Mikropoulou D, Gallo A, Ciofi-Baffoni S, Banci L, Bertini I, Tokatlidis K (2009). A novel intermembrane space-targeting signal docks cysteines onto Mia40 during mitochondrial oxidative folding. *J Cell Biol* 187, 1007–1022.
- Sideris DP, Tokatlidis K (2007). Oxidative folding of small Tims is mediated by site-specific docking onto Mia40 in the mitochondrial intermembrane space. *Mol Microbiol* 65, 1360–1373.
- Sturtz LA, Diekert K, Jensen LT, Lill R, Culotta VC (2001). A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. *J Biol Chem* 276, 38084–38089.
- Terziyska N, Lutz T, Kozany C, Mokranjac D, Mesecke N, Neupert W, Herrmann JM, Hell K (2005). Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions. *FEBS Lett* 579, 179–184.
- Wong PC, Waggoner D, Subramaniam JR, Tessarollo L, Bartnikas TB, Culotta VC, Price DL, Rothstein J, Gitlin JD (2000). Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. *Proc Natl Acad Sci USA* 97, 2886–2891.