Structural basis for the interaction of the chaperone Cbp3 with newly synthesized cytochrome b during mitochondrial respiratory chain assembly

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Assembly of the mitochondrial respiratory chain requires the coordinated synthesis of mitochondrial and nuclear encoded subunits, redox co-factor acquisition, and correct joining of the subunits to form functional complexes. The conserved Cbp3–Cbp6 chaperone complex binds newly synthesized cytochrome b and supports the ordered acquisition of the heme co-factors. Moreover, it functions as a translational activator by interacting with the mitoribosome. Cbp3 consists of two distinct domains: an N-terminal domain present in mitochondrial Cbp3 homologs and a highly conserved C-terminal domain comprising a ubiquinol–cytochrome c chaperone region. Here, we solved the crystal structure of this C-terminal domain from a bacterial homolog at 1.4 Å resolution, revealing a unique all-helical fold. This structure allowed mapping of the interaction sites of yeast Cbp3 with Cbp6 and cytochrome b via site-specific photo-cross-linking. We propose that mitochondrial Cbp3 homologs carry an N-terminal extension that positions the conserved C-terminal domain at the ribosomal tunnel exit for an efficient interaction with its substrate, the newly synthesized cytochrome b protein.

The inner mitochondrial membrane contains the respiratory chain complexes that, in ensemble, transfer electrons extracted from metabolites to the ultimate acceptor O₂. The bc₁ complex (complex III) plays a central role in this pathway and shuttles electrons from reduced ubiquinol to cytochrome c. The bc₁ complex consists of nine nuclear subunits and the mitochondrially encoded cytochrome b (Cytb),4 which, together with cytochrome c₁ and the Rieske iron-sulfur protein, forms the catalytic center (1). Synthesis and assembly of the bc₁ complex (see Fig. 1A) starts with the translation of the mRNA for Cytb (COB mRNA) on the mitochondrial ribosome (mitoribosome) (2, 3). The translational activators Cbp1, Cbs1, and Cbs2 help in stabilizing and binding the COB mRNA on the mitoribosome (4), and together with the Cbp3–Cbp6 complex bound in close proximity to the polypeptide tunnel exit, translation of the Cytb transcript is initiated (5). Cytb interaction with Cbp3–Cbp6 leads to a release of the complex from mitoribosomes (5) to establish the first assembly intermediate of the bc₁ complex. The incorporation of a low potential heme group at the h₁ site of Cytb triggers the binding of Cbp4 to Cytb (intermediate I) (6), which accumulates to well-detectable levels in WT cells (6, 7). Upon incorporation of the second heme group at the h₂ site, the Cbp3–Cbp6 complex is released from the fully hemylated Cytb and returns to the mitoribosome to initiate another round of COB mRNA translation (6, 7). In cases when the Cbp3–Cbp6 complex is not released from assembly intermediate I, it is unavailable to stimulate new rounds of COB mRNA translation. The Cbp3–Cbp6 complex, therefore, plays a dual role in this process because it controls both Cytb translation and assembly (7). Consequently, the respiratory deficiencies of strains lacking Cbp3 or Cbp6 are due to inefficient COB mRNA translation and bc₁ complex assembly (5). Furthermore, knockdown of the human Cbp3 homolog UQCC1 leads to loss of the human Cbp6 homolog UQCC2 and reduced levels of assembled bc₁ complex (8).

Therefore, Cbp3–Cbp6 comprise a protein complex that interacts with its substrate Cytb in a context-dependent fashion. It shares this behavior with other assembly factors of the respiratory chain (9–11). However, the understanding of how these mitochondrial respiratory chain assembly factors work is limited. The mitochondrial Cbp3 protein contains two domains: an N-terminal domain present in mitochondria and a highly conserved ubiquinol–cytochrome c chaperone domain,

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4 The abbreviations used are: Cyt, cytochrome; PDB, Protein Data Bank; pBpa, para-aminobenzoylephenylalanine; Ni-NTA, nickel–nitrilotriacetic acid; TBS, Tris-buffered saline.

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which is also found in Cbp3 homologs in bacteria. Here, we present the crystal structure of a Cbp3 protein from the bacteria *Brucella abortus* (*BaCbp3*) at 1.4 Å resolution. The protein has a unique, all-helical fold that was solved using *ab initio* phasing. Homology modeling and biochemical analysis of Cbp3 from *Saccharomyces cerevisiae* (*ScCbp3*) allowed us to map the molecular interactions of *ScCbp3* with Cbp6 and Cytb.

**Results**

**Bioinformatics and biochemical analysis of the domain structure of yeast Cbp3**

Cbp3 is a key assembly factor of Cytb that plays an additional role in regulating translation of COB mRNA. To understand how Cbp3 operates, we aimed at determining its structure and interactions sites with other proteins. We first set out to identify conserved domains. Using ScCbp3 (UniProt no. P21560) as a representative of this structurally uncharacterized family, multiple sequence alignments were generated at different sensitivity cutoffs, using HHblits and Jackhmmer (12, 13). With the most permissive sensitivity cutoff (*E* value = 1), 649 and 740 sequences were reported in HHblits and Jackhmmer, respectively (Fig. 1B). Results from PSIPRED classify Cbp3 as an all-α fold protein, with 12 α-helices predicted (Fig. S1A). Two domains were identified: a conserved C-terminal domain spanning residues 140–290, annotated as ubiquinol–cytochrome c chaperone domain, and a variable N-terminal domain of unknown function spanning residues 61–119 (Fig. 1B). The N-terminal domain is detectable but not sufficient to stabilize Cbp6 protein levels. *EV*, empty vector; *MW*, molecular mass; *IMS*, intermembrane space; *IMM*, inner mitochondrial membrane; *MTS*, mitochondrial targeting signal.

**Figure 1. Bioinformatics and biochemical analysis of Cbp3 domain structure.** A, a schematic depicting the sequence of events, which take place during the early steps of cytochrome *b* biogenesis. See Introduction for details. *B*, multiple sequence alignments generated using UniProt sequence P21560 from *S. cerevisiae* as representative for Cbp3. The sequences in the alignment are on the y axis, and the positions of residues in the sequence are on the x axis. Black indicates alignment, whereas white indicates gaps. The conserved ubiquinol–cytochrome c chaperone domain and selected residues are indicated. *C*, growth assay of *cbp3Δ* strains expressing either full-length Cbp3 or truncated Cbp3 versions on fermentable and nonfermentable medium at 30 °C. Only the full-length Cbp3 can rescue respiration in the *cbp3Δ* strain. *D*, protein levels of Cbp3 or its truncated versions. The N-terminal domain is detectable but not sufficient to stabilize Cbp6 protein levels. *EV*, empty vector; *MW*, molecular mass; *IMS*, intermembrane space; *IMM*, inner mitochondrial membrane; *MTS*, mitochondrial targeting signal.
Expression of the truncated forms of Cbp3 did not rescue the phenotype (Fig. 1C). Previous studies showed that Cbp6 is unstable in the absence of Cbp3 (5). Therefore, we tested the protein levels of the truncated Cbp3 versions and protein levels of Cbp6. Only low amounts of the N-terminal domain were detected, whereas the conserved C-terminal domain was not stable (Fig. 1D). Furthermore, expression of the Cbp3 versions could not rescue Cbp6 protein levels.

**B. abortus Cbp3 as a structural template for mitochondrial Cbp3**

Our bioinformatic analysis showed that Cbp3 homologs from α-proteobacteria are most closely related to ScCbp3. We recombinantly expressed and purified the Cbp3 homolog of *B. abortus*, an α-proteobacterium, which has 22% sequence identity. The structure was solved using *ab initio* phasing and refined at 1.4 Å resolution (Tab. 1). Structure determination was facilitated by the predicted high α-helical content, which revealed a compact helical bundle with a unique fold (Fig. S1B). A search of the PDB using PDBeFOLD (14) confirmed that the Cbp3 fold was not previously described, the top hits had root-mean-square deviation values above 4 Å and only superimposed a few helices. Residues 1–181 were modeled in the high-resolution density map, with only three C-terminal residues missing from the structure (residues 181–184; Fig. 2). Of note, two magnesium ions were bound to *Ba*Cbp3, which likely originated from the crystallization condition (Fig. 2, A, C, and D). The core of *Ba*Cbp3 consists of nine helices with the long N-terminal helix protruding from the rest of the protein (Fig. 1B and Fig. S1C). Although *Ba*Cbp3 is monomeric, strong hydrophobic interactions were observed between the aliphatic residues of the N-terminal helix (Fig. 2B) and a shallow pocket of the symmetry-related molecule on the opposite side, formed by the base of helices 4–6 (Fig. S1, D and G). The core of the protein is formed by a deep hydrophobic pocket, which is rich in aromatic residues (Phe-33, Tyr-34, Tyr-115, and Tyr-151), including a central histidine (His-53). This pocket is highly conserved in *Sc*Cbp3 (Phe-143, Tyr-144, Tyr-228, Tyr-265, and His-163; Fig. 3C).

Interestingly, the two magnesium ions are located in a large cavity with a negatively charged surface (Fig. 2, B–D). The two ions present a typical octahedral coordination (Fig. 2, C and D, and Fig. S1, E and F). On one site, Mg\(^{2+}\) coordination is mediated by Asp-82, Ser-86, and Glu-89 interactions with water molecules. On the second site, Glu-74 directly interacts with Mg\(^{2+}\), which is further stabilized by water-mediated interactions with Glu-14, Asp-77, and Glu-78. Although the presence of Mg\(^{2+}\) suggests a possible metal-binding motif, no physiological function could be associated with magnesium. Likewise, residues Asp-82 (*S. cerevisiae* Asp-193) and Glu-89 (*S. cerevisiae* Glu-201) are highly conserved, whereas the other residues are variable (Fig. 3, A, C, and D).

The new fold presented by *Ba*Cbp3 was used as a template to further investigate the properties of the Cbp3 family and more particularly to determine protein contacts of Cbp3. A homology model of the yeast Cbp3 (*Sc*Cbp3) was calculated (15). The resulting model of the yeast ubiquinol–cytochrome c chaperone domain corresponded to residues 115–294 (Figs. 1A and 3, B–D), therefore missing most of the N-terminal domain, which is only conserved in mitochondrial homologs (Fig. 1B). The core of the protein is very similar to *Ba*Cbp3 with the central hydrophobic residues showing strong conservation (Fig. 3, A and C). The surface potential of the *Sc*Cbp3 model presents a similar profile to *Ba*Cbp3 (Figs. 2B and 3D), although the negatively charged cavity that holds the Mg\(^{2+}\)-binding sites in *Ba*Cbp3 is less prominent and appears substantially more shallow and hydrophobic in *Sc*Cbp3.

**Interaction sites of Cbp3 with Cbp6 and Cytb in *S. cerevisiae***

The homology model of *Sc*Cbp3 enabled us to identify surface-exposed residues that could be part of a protein–protein interaction surface with either Cbp6, Cytb, the mitochondrial ribosome, or other interaction partners. Therefore, we aimed to identify these protein contacts by site-directed photo–cross-linking (16), which detects contacts of proteins within an amino acid range. Tagged variants of Cbp3 (Cbp3His7) were con-
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Figure 3. Homology model of Cbp3 from S. cerevisiae. A, sequence alignment of Cbp3 homologs from S. cerevisiae and B. abortus. Both sequences show 28.5% identity and 44.6% similarity. The numbering reflects the residues in ScCbp3, and the coloring shows the degree of conservation. B, crystal structure of BaCbp3 (teal) superimposed with the homology model of ScCbp3, prepared with SWISS-MODEL. The N and C termini of the homology model are labeled. C, coloring as per pBp (Fig. 2C) in line with the previously described tight binding of Cbp3 and Cbp6 (5). To pinpoint an interaction surface of Cbp3 with Cbp6, we repeated the cross-link screen of all positions decorating against Cbp6. In this way, we identified four positions, Lys-148(pBpa), Arg-150(pBpa), and Lys-252(pBpa), showing a strong cross-link with Cbp6 (Fig. S2B). The identified positions were subsequently mapped on the surface of the ScCbp3 model and were consistent with a large interaction site with Cbp6 (Fig. 4F).

Previous studies showed that the Cbp3–Cbp6 complex binds to the mitoribosome in close proximity to Mrp14 (uL29m) (5). Because Mrp14 is adjacent to the tunnel exit proteins MrpL22 (uL22m), Mrp20 (uL23m), MrpL40 (uL24m), and MrpL3 (mL44), the cross-linking products of ~77 and ~86 kDa, which could represent this interaction site based on the apparent molecular masses of MrpL3, Mrp20, MrpL22, MrpL40, and MrpL4 (44, 31, 35, 34, and 37 kDa, respectively). We tested various positions facing different sides of the protein, but we could not identify any interaction site of the C-terminal domain of Cbp3 with the mitoribosome. Negative results could be explained by shortcomings in the methods, because site-directed photo–cross-linking can be sensitive to the local amino acid context, which would obstruct cross-linking. Alternatively, it is possible that the interaction between Cbp3 and the mitoribosome is instead mediated by the N-terminal domain and/or Cbp6.

Having identified the Cbp6-binding site, we explored further interaction sites with Cbp3 interactors. Cbp3 plays an important role in the assembly of the bc1 complex by stabilizing newly synthesized Cytb. Thus, we aimed to find the interaction site of Cbp3 with Cytb. To increase sensitivity, we performed in vivo labeling of mitochondrially encoded translation products with radiolabeled [35S]methionine prior to UV-cross-linking and denaturing Cbp3His7 purification. Detection of the radioactivity showed a cross-link product of 64 kDa at the position 118-119, Lys-177, Asp-188, Glu-195, and Lys-215. The cells were grown in the presence of pBpa and UV-irradiated, and the cross-link products were purified via a C-terminal His tag on Cbp3. Positions Arg-245(pBpa) and Lys-252(pBpa) showed a strong cross-link product with a 15–18-kDa partner, which we identified to be Cbp6 (Fig. 4, D and E, and Fig. S2B), in line with the previously described tight binding of Cbp3 and Cbp6 (5). The negative results could be explained by shortcomings in the methods, because site-directed photo–cross-linking can be sensitive to the local amino acid context, which would obstruct cross-linking. Alternatively, it is possible that the interaction between Cbp3 and the mitoribosome is instead mediated by the N-terminal domain and/or Cbp6. 

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Discussion

In this study, we determined the structure of the conserved ubiquinol–cytochrome \(c\) chaperone domain of \(S_{c}Cbp3\) and identified its functions in the biogenesis of the \(bc_{1}\) complex. Previous studies provided evidence that Cbp3 and Cbp6 form a complex, which is involved in the synthesis of Cyt\(b\) and the early assembly of the \(bc_{1}\) complex in yeast (7). For an efficient synthesis of Cyt\(b\), the Cbp3–Cbp6 complex interacts with the tunnel exit of the mitoribosome, where the complex binds the newly synthesized Cyt\(b\) (5). Mitochondrial Cbp3 homologs contain a highly conserved ubiquinol–cytochrome \(c\) chaperone domain at the C terminus of unknown structure, whereas a domain without annotated function is found at the N terminus. By expressing the domains of Cbp3 individually, we showed that neither domain is sufficient to restore respiration in strains lacking a full-length Cbp3. To analyze protein contacts of the conserved C terminus, we solved the structure of a Cbp3 homolog from \(B_{.}\) \(a_{.}\)\(b_{.}\) of the \(/H9251\)-proteobacteria clade, which are the evolutionary closest relatives to mitochondria.

Despite differences in the amino acid sequences, the yeast and bacterial homologs are expected to present a common overall fold of the conserved ubiquinol–cytochrome \(c\) chaperone domain. A homology model of ScCbp3 enabled us to select potential sites of interactions with components of the \(bc_{1}\) complex, the translational machinery, or other unknown interaction partners. By incorporating a photoreactive amino acid...
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Figure 5. Model of interaction sites of ScCbp3. The identified interaction sites of Cbp3 with Cbp6 and Cytb are indicated (green and red, respectively). The proposed interaction site of the N terminus of Cbp3 with proteins of the peptide tunnel exit (Mrp3, Mrp13, Mrp20, Mrp22, and Mrp40) of the mitoribosome is marked in blue. The model of Cytb is adapted from Ref. 20 (PDB code 6GIQ), whereas the model of the mitoribosome is adapted from Ref. 17 (PDB code 5MRC). PTE, polypeptide tunnel exit.

(pBpa) at specific positions of Cbp3, we could investigate interactions with high precision. We identified two main protein interaction sites on ScCbp3, which involve a hydrophobic and a positively charged surface on opposite sides of Cbp3 (Fig. 5). The Cbp3–Cbp6 interface is formed by a positive protrusion formed by a long flexible loop and a more stringent shorter loop (Lys-148, Pro-150, Arg-245, and Lys-252; Fig. S1C). Unfortunately, there is no structure information available for Cbp6, but it is likely that a negatively charged pocket in Cbp6 enables binding to Cbp3. Interestingly, one position adjacent to the protrusion, Arg-151, does not cross-link to Cbp6 (Fig. S2B). Our Cbp3 model shows that this residue instead most likely interacts with other negatively charged amino acids within the protein (Glu-132 and Glu-145), maybe stabilizing the loop, and is therefore not available to interact with Cbp6. The interaction site of Cbp3 with Cytb is quite large, which fits well to the highly flexible matrix-facing loops of Cytb. These loops are overall polar or positively charged but also include a few acidic residues enabling interactions with negative amino acids (Asp-188 and Glu-195), as well as basic (Lys-185 and Lys-215) and polar residues (Gln-183).

Interestingly, Cbp3 functions as an assembly factor, as well as a translational activator in mitochondria. Because the N terminus of Cbp3 is specific to mitochondrial Cbp3 homologs, it is tempting to speculate that this domain mediates contacts with the mitoribosome and its bound Cbp6. In turn, Cbp6 is only stable in the presence of Cbp3, and a small fraction of overexpressed Cbp6 can interact with the ribosome in absence of Cbp3 (5). The interaction between Cytb and Cbp3 is only transient to stabilize the Cytb until other subunits join in the assembly intermediates (5–7). Likewise, the interaction between Cbp3–Cbp6 and the mitoribosome occurs only when Cbp3–Cbp6 is not bound to Cytb. These context-dependent interactions likely require profound flexibility in Cbp3–Cbp6 to enable conformational changes preparing the proteins for binding the different clients. It will therefore be important to reveal the structure of Cbp3–Cbp6 either in its free form, in complex with Cytb, or in complex with the mitoribosome to unravel possible conformational changes induced by substrate binding.

Experimental procedures

Bioinformatic analysis of ScCbp3

Cbp3 from S. cerevisiae was used as the reference sequence. HHblits and Jackhammer were used to search the input sequence against the latest HHblits database and Uniref100, respectively. At an E value of 1, HHblits reported 649 sequences, whereas Jackhammer gave 740. Possible contacts between pairs of amino acids in Cbp3 were predicted using Pcons2, and the resultant map displayed a clear signal between residues 140 and 290. Pfam classified this region as an ubiquinol–cytochrome c chaperone domain. We used Pconsfold and Rosetta to predict the model, whereas the quality of the model was assessed using MolProbity. The crystal structure of BaCbp3 was used as a template to produce the final homology model of ScCbp3 in SWISS-MODEL (15).

Cbp3 protein production and purification

The Cbp3 homologous sequence from B. abortus was cloned into the pET28a vector, containing a TEV-cleavable C-terminal GFP-His6 tag. The vector was transformed into BL21 (DE3) Escherichia coli strain and grown in fresh Terrific Broth medium, supplemented with kanamycin (50 μg/ml) at 180 rpm and 37 °C. Protein expression was induced when the culture was at an optical density of 0.8, by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside, whereas the incubation temperature was reduced to 16 °C and cultured for 18 h. The cells were harvested at 4 °C at 6,500 × g for 15 min using rotor JLA 8.1000. The cell pellet was resuspended in lysis buffer (300 mM NaCl, 20 mM HEPES, 3 mM DTT, complete protease inhibitor (1 tablet per 20 g of cell mass; Roche), and 1 mM MgSO4), and drops of the suspension were flash-frozen in liquid nitrogen. Frozen cells were lysed using Cryo Mill (MM400, Retsch), and the lysate was homogenized in appropriate amount of the lysis buffer. Cell debris was removed by centrifugation at 40,000 × g for 20 min at 4 °C. The supernatant was adjusted to a final concentration of 10 mM imidazole and incubated with 1 mg of Ni-NTA Superflow resin (Qiagen) per 20 mg of GFP-His6, for 4 h, at 4 °C. The slurry was transferred into a glass Econo-Column (Bio-Rad) and washed in the lysis buffer for 2 × 20 column volumes at 20 and 40 mM imidazole, respectively. Two column volumes of lysis buffer containing 250 mM imidazole was used to elute the Cbp3–GFP–His6 from the column. The eluate was dialyzed overnight in the presence of 1 mg of TEV-protease per 10 mg of fusion protein, in 3 liters of buffer (20 mM HEPES, pH 7.4, 300 mM NaCl, and 3 mM DTT). After dialyzes the sample was passed through a 5-ml bed volume Ni-NTA His-Trap column (GE Healthcare) and Cbp3 with residual residues of the TEV site after cleavage was collected in the flow through. Relative molecular mass of 10-kDa cutoff concentrators were used to concentrate the protein. The protein solution was then loaded onto a Superdex 200 10/300 gel-filtration column (GE Healthcare), which was equilibrated with buffer (300 mM NaCl, 20 mM HEPES, 3 mM DTT). From the chromato-
Working model was refined using REFMAC5 (29) and (27) followed by partial building with BUCCANEER (28). The formed with MolProbity (31). Ramachandran statistics show potential hydrogen bonds could be made. Validation was performed with PDB (6RWT).

Table 1

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| **Refinement**                                               |
| Rmerge/Rpim                                                 | 16.8/20.1 |
| B-factors                                                   | 26.8 |
| Residuals (%)                                                | 34.2 |
| Water                                                       | 42.4 |
| Root mean square deviations                                  | 0.009 |
| Bond angles (°)                                              | 1.18 |

X-ray crystallography

Crystals were obtained from a sitting drop in 20% (v/v) PEG 8000, 0.1 M Tris, pH 8.0, 0.2 M magnesium chloride hexahydrate. A drop of 200 nl of sample was mixed with equal amount of reservoir and incubated at 16 °C, where crystals grew within 24 h. The crystals were transferred briefly into a cryo-protectant solution, consisting of their respective growth condition supplemented with 20% glycerol, before freezing in liquid nitrogen.

Diffraction data were collected at station I04-1 of the Diamond Light Source (Oxon, UK), equipped with a PILATUS-6 M detector (Dectris, Switzerland). Complete data sets to a resolution of 1.42 Å were collected from a single crystal at 100 K. Complete data sets to a resolution of 1.42 Å were collected from a single crystal at 100 K. Raw data images were processed and scaled with DIALS (21) and AIMLESS (22) using the CCP4 suite 7.0 (23). The resolution cutoff chosen was based on the CC1/2 (24).

Initial phases for structure solution of BatCbp3 were obtained with ARICIMBOLDO Lite (25, 26) by setting a search mode for multiple helices of 15 residues. The resulting model consisted of seven fragments for a total of 150 polyalanine residues traced (Fig. S3). The model was manually curated, and correctly placed residues were used as a search model in PHASER (27) followed by partial building with BUCCANEER (28). The working model was refined using REFMAC5 (29) and manually adjusted with COOT (30). Water molecules were added at positions where Fo − Fe electron density peaks exceeded 3 σ, and potential hydrogen bonds could be made. Validation was performed with MolProbity (31). Ramachandran statistics show that 98% of all residues were in the most favored region, and none were in the disallowed region. Crystallographic data statistics are summarized in Table 1. The figures were made with PyMOL (Schrödinger, New York).

Plasmid construction

Cbp3 variants containing an amber stop codon (TAG) at selected sites were constructed by site-directed mutagenesis (Table S1) using a pGEM3 vector (Promega). The Cbp3 variants were later cloned into pYX142 (Novagen) using the restriction enzymes Ncol and HindIII.

To express EcYRS-Bpa (modified E. coli tyrosyl-tRNA synthetase) and tRNA (E. coli tyrosyl tRNA_CUA) for UV-cross-link experiments, EcYRS-Bpa and tRNA_CUA were amplified from pESC-EYRS-Bpa (16) and cloned into pRS403 (Nova Lifetech) using the restriction sites XbaI and SapI. The plasmid was cut within the HIS gene with NdeI for integration into the yeast genome.

To express the C-terminal domain of Cbp3, the respective domain (Cbp3_120–335 and Cbp3_145–335) was amplified by PCR and cloned with NotI and XhoI into a pYX132 plasmid containing the mitochondrial targeting sequence of Oxa1 (MTS_1–48) to ensure import. The N-terminal domain (Cbp3_1–120) was cloned with NotI and XhoI into pYX132.

Yeast strain construction

All yeast strains were isogenic to W303 (MATa leu2-3,112 trpl-1 can1-100 ural3-1 ade2-1 his3-11,15). The plasmid harboring the pBpa-RS and tRNA_CUA was integrated into the genome of a strain lacking Cbp3. In parallel this strain was transformed with pYX142 containing the respective variants of Cbp3. The obtained strains were grown on minimal medium lacking leucine (Sigma Y1376) and supplemented with 2% galactose (SGal–Leu) in the presence or absence of pBpa.

Growth assay

The cells were grown in synthetic medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, amino acids to rescue auxotrophic markers except of tryptophan) supplemented with 2% glucose (SD–Trp) to logarithmic phase and OD 0.5 was harvested. A 10-fold dilution series was prepared, and 3.5 µl were plated on synthetic medium plates supplemented with 2% glucose (fermentable; SD–Trp) or 2% glycerol (nonfermentable; SG–Trp) and incubated at 30 °C.

Analysis of protein steady-state levels

The cells were grown in synthetic medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, amino acids to rescue auxotrophic markers except of tryptophan) supplemented with 2% glucose (SD–Trp) to logarithmic phase and harvested. The cells were lysed (0.1 M NaOH, 5 min; 14,000 rpm, 2 min), and the pellet was resuspended in sample buffer to extract the proteins (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT for 3 min at 95 °C). The samples were analyzed by SDS-PAGE (16/0.2% acrylamide/bisacrylamide) followed by transfer to nitrocellulose membranes (Roth) and subsequent immunodetection.

Site-specific photo–cross-linking

The cells were grown in SGal–Leu medium to logarithmic phase and harvested (4,400 rpm for 5 min at room temperature). [25S]Methionine in vivo labeling was performed for indi-
cated samples as described in Ref. 19. The following steps were performed for labeled and nonlabeled samples. The cells were resuspended in SGal−Leu medium, transferred to a 6-/12-well plate, and irradiated at 350 nm for 60 min (Rayonet RPR200 photochemical reactor; Edwin Gaynor 897-VS, 120W, 600V). The cells were incubated in 0.1 M NaOH (5 min at room temperature) followed by lysis in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT) for direct analysis or with SDS (4% SDS, 100 mM DTT for 3 min at 95 °C) for subsequent purification. Cbp3His7 and its cross-link products was purified by Ni-NTA (Qiagen). Samples were analyzed with SDS-PAGE (16/0.2% acrylamide/bisacrylamide) followed by transfer to nitrocellulose membranes (Roth; 90 min, 100 mA temperature) followed by lysis in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM DTT) for direct analysis or with SDS (4% SDS, 100 mM DTT for 3 min at 95 °C) for subsequent purification. Cbp3His7 and its cross-link products was purified by Ni-NTA (Qiagen). Samples were analyzed with SDS-PAGE (16/0.2% acrylamide/bisacrylamide) followed by transfer to nitrocellulose membranes (Roth; 90 min, 100 mA temperature) followed by lysis in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM DTT) for direct analysis or with SDS (4% SDS, 100 mM DTT for 3 min at 95 °C) for subsequent purification.

Data availability

The atomic coordinates and structure factors (code 6RWT) have been deposited in the Protein Data Bank.


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References


