The co-chaperone Cns1 and the recruiter protein Hgh1 link Hsp90 to translation elongation via chaperoning elongation factor 2

Florian H. Schopf[1], Eva M. Huber[1], Christopher Dodt[1], Abraham Lopez[1,2], Maximilian M. Biebl[1], Daniel Andreas Rutz[1], Moritz Mühlhofer[1], Tobias Madl[1,3], Michael Sattler[1,2], Michael Groll[1] & Johannes Buchner[1]*

1 Center for Integrated Protein Science at the Department Chemie, Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany.

2 Institute of Structural Biology, Helmholtz Zentrum München, 85764 Neuherberg, Germany.

3 Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging, Molecular Biology and Biochemistry, Medical University of Graz, 8036 Graz, Austria.

* corresponding author: Johannes Buchner johannes.buchner@tum.de

Abstract

The Hsp90 chaperone machinery in eukaryotes comprises a number of distinct accessory factors. Cns1 is one of the few essential co-chaperones in yeast but its structure and function remained unknown. Here, we report the X-ray structure of the globular Cns1 fold and NMR studies on the partly disordered, essential segment of the protein. We demonstrate that Cns1 is important for maintaining translation elongation, specifically chaperoning the elongation factor eEF2. In this context, Cns1 interacts with the novel co-factor Hgh1 and forms a quaternary complex together with eEF2 and Hsp90. The *in vivo* folding and solubility of eEF2 depends on the presence of these proteins. Chaperoning of eEF2 by Cns1 is essential for yeast viability and requires a defined subset of the Hsp90 machinery as well as the identified eEF2-recruiting factor Hgh1.

Highlights

- Cns1 consists of an intrinsically disordered region (IDR), a TPR domain and a domain representing a novel fold as revealed by X-ray crystallography and NMR
- Cns1's essential function is associated with protein translation
- Cns1 regulates the folding and stability of the elongation factor eEF2
- A novel co-factor, Hgh1, is required for chaperoning eEF2
- Cns1, Hgh1 and Hsp90 form a complex with eEF2 and control eEF2 levels and activity *in vivo*

Introduction

Molecular chaperones are found in all branches of life. They bind and stabilize unfolded or partially folded proteins and support them in acquiring or maintaining their native states (Balchin et al., 2016). Therefore, they are crucial for proteostasis in general. In the cytosol of eukaryotes the Hsp90 machinery is an abundant and conserved regulator of protein conformation (Borkovich et al., 1989; Schopf et al., 2017). Although Hsp90 is promiscuous concerning client interactions, it seems more selective than Hsp70. Several classes of client proteins which depend in folding, activation and assembly on Hsp90 have been identified (McClellan et al., 2007; Taipale et al., 2012; Zhao et al., 2005). The growing list of Hsp90 clients contains many protein kinases, transcription factors and steroid hormone receptors among others (http://www.picard.ch/downloads/Hsp90interactors.pdf). Hsp90 functions as a homodimer *in vivo* (Wayne and Bolon, 2007). ATP binding and hydrolysis drive large conformational rearrangements in the chaperone. Structural and kinetic analyses revealed that during the chaperone cycle Hsp90 transits form a V-shaped open conformation to a tightly closed state via several intermediate steps (Ali et al., 2006; Dollins et al., 2007; Shiau et al., 2006).

Hsp90 function is regulated by a large cohort of helper proteins termed co-chaperones (Mayer and Le Breton, 2015), and most of them are highly conserved (Johnson, 2012). They can act either as modulators of the Hsp90 chaperone cycle or facilitate client recruitment and processing. For example, Aha1 is a strong enhancer of the Hsp90 ATPase activity and accelerates the conformational cycle (Panaretou et al., 2002; Retzlaff et al., 2010). In contrast, Sti1 keeps Hsp90 in an open conformation, thereby inhibiting its ATPase activity (Li et al., 2011; Panaretou et al., 1998; Richter et al., 2003), and, additionally, connects Hsp90 with the Hsp70 system (Johnson et al., 1998; Scheufler et al., 2000; Schmid et al., 2012b). Besides these general co-chaperones, there are also more dedicated ones, such as the essential co-chaperone Cdc37, which is a kinase-specific co-factor. Of the twelve known co-chaperones in yeast, only three, namely Cdc37, Sgt1 and Cns1, are essential (Johnson, 2012; Sahasrabudhe et al., 2017). Cdc37 is involved in kinase maturation (Brugge, 1986) and Sgt1 was reported to support kinetochore assembly (Catlett and Kaplan, 2006; Kitagawa et al., 1999). In contrast, the essential role of Cns1 is still enigmatic. Cns1 was originally discovered as a multi-copy suppressor for Hsp90 loss-of-function mutations (Nathan et al., 1999). Moreover, it was shown that the Hsp90 co-chaperones Cns1 and Cpr7 have over-lapping but largely undefined *in vivo* functions (Dolinski et al., 1998b; Marsh et al., 1998a; Tesic et al., 2003; Zuehlke and Johnson, 2012). *In vitro* studies showed that Cns1 binds with moderate affinity to both, Hsp90 and Hsp70 (Hainzl et al., 2004). Furthermore, Cns1 and Cpr7 were found to interact weakly with the 80S ribosome and *cns1* and *cpr7Δ* mutants are

sensitive to the translation inhibitor hygromycin B linking them to protein translation (Albanese et al., 2006; Tenge et al., 2015), but again how Cns1 might function in this context remained an open question.

In this study, we solved the X-ray structure of the globular folded domains of Cns1 and identified an N-terminal intrinsically disordered region (IDR) as essential for its *in vivo* function. Using NMR spectroscopy we could show that the N-terminal IDR exhibits two regions with partial helical conformation. Moreover, we determined that the essential function of Cns1 involves these helical elements, depends on Hsp90 and is associated with protein translation. Specifically, Cns1 interacts directly with the translation elongation factor eEF2 as well as the novel co-factor Hgh1. Together, these factors are crucial for eEF2 folding and stability *in vivo* thus connecting the Hsp90 chaperone machinery to protein translation.

Results

Cns1 harbors a unique two domain structure linked by a long helix

Cns1 is an essential protein in *Saccharomyces cerevisiae* and a co-chaperone of the Hsp90 machinery (Dolinski et al., 1998a; Marsh et al., 1998b) with a unique primary sequence (Figure S1A). We therefore aimed at elucidating its three-dimensional structure. Since previous work had shown that the C-terminal domain of Cns1 alone readily crystallizes (Stanitzek, 2005), we initially cloned, expressed, purified and crystallized a deletion construct harboring residues 221-385 . The structure of the C-terminal domain was solved at 1.55 Å resolution applying anomalous phasing via seleno-methionine labelling (R_{free} 18.6%, PDB ID: 6HFM, Figure 1A, Table S1) and revealed two molecules per asymmetric unit with a root-mean-square deviation (rmsd) of 0.31 Å (Figure S1C). Additionally, we determined the crystal structure of the C-terminal domain of the human Cns1 homologue TTC4 (tetratricopeptide repeat protein 4; residue range 217-387 at 1.65 Å resolution by molecular replacement using the yeast coordinates (R_{free} 18.95%, PDB ID 6HFO, Figure 1B, Table S1). Despite low sequence conservation (18% sequence identity and 37% sequence similarity) between yeast Cns1$^{221-385}$ and human TTC4$^{217-387}$ (Figure S1A), both domains adopt the same overall fold consisting of a twisted five-stranded β-sheet surrounded by several α-helices (rmsd 1.40 Å; Figure 1C), demonstrating conservation from yeast to man. While the α-helices are variable, the central β-sheet is well conserved. It is formed by two two-stranded antiparallel β-sheets with the fifth strand acting as the bridging element (Figure 1D). A Dali search (Holm and Rosenstrom, 2010) revealed that this tertiary structure is unique and not related to any other protein topology known to date. According to the shape of its 2D projection we propose the name wheel domain for the C-terminal fold of Cns1 and TTC4 (Figure 1C).

After having solved the structure of the Cns1-C-domain, crystallization of full-length Cns1 was attempted. N-terminal truncations, protein stability improvements and extensive screening of crystallization conditions finally allowed us to determine the structure of Cns1$^{70-385}$ to 2.8 Å resolution. Patterson calculations using the coordinates of the C-terminal domain (Cns1$^{221-385}$) as a search model (R_{free} 28.8%, PDB ID 6HFT, Table S1) indicated additional electron density at the N-terminus. Iterative model building and refinement steps allowed us to place all amino acids except for the first four residues in the $2F_O$-F_C electron density map. Cns1$^{70-385}$ folds into an N-terminal tetratricopeptide repeat (TPR) domain of three units, i.e. six antiparallel α-helices (α1-α6), and the C-terminal domain connected by a straight helix (termed α7) of 44 amino acids (Figure 1E, Figure S1B). Both domains are arranged in *trans* relatively to the α7 helix (Figure S1D). Notably, the structure of the C-domain is unaltered compared to Cns1$^{221-385}$ (rmsd 0.43 Å; Figure S1E). The TPR domain of Cns1, known to interact with the C-terminal EEVD sequence of Hsp90 (Russell et al., 1999; Scheufler et al.,

2000), is structurally closely related to that of the FK506-binding protein 51 (FKBP51) involved in steroid hormone receptor signaling (Sinars et al., 2003) (Dali search: Z-score 17, rmsd 1.9 Å, identity 27%), the RNA Polymerase II-associated protein 3 (RPAP3) (Pal et al., 2014) (Z-score 16.7, rmsd 2.7, identity 28%) as well as other proteins containing TPR motifs (Figure S1F).

Although Cns1$^{70-385}$ was crystallized in the presence of 1.5 fold molar excess of MEEVD, the C-terminal Hsp90 peptide is not defined in the electron density map. We therefore mapped the binding site for the Hsp90 C-terminus by superimposing Cns1$^{70-385}$ onto the RPAP3 crystal structure in complex with the peptide SRMEEVD (PDB ID: 4CGW) (Pal et al., 2014). According to this model, the MEEVD sequence fits into a cradle-shaped groove of Cns1 formed by several basic amino acids of the TPR domain (Figure 1F, Figure S1G). Among these, the conserved residues Lys87, Lys156 and Arg160 (Figure S1A) probably provide the peptide binding site. However during crystallization the arrangement of Cns1$^{70-385}$ molecules in the lattice likely caused displacement of the MEEVD peptide due to steric hindrance with protein residues of a symmetry-related Cns1$^{70-385}$ chain (Figure S1H).

The exceptional domain arrangement of Cns1$^{70-385}$ raised the question of whether the long linker helix α7 is fixed in its orientation as previously reported for the Hsp90 co-chaperone Sti1 (Schmid et al., 2012a). Arg234 at the C-terminal end of the linker helix hydrogen bonds to residues of the wheel domain, i.e. to the main chain oxygen atoms of Phe261 and Ser263 as well as to the side chain of Glu287, and might fix the orientation of the α7 helix relative to the C-terminal domain (Figure S1I). However, since mutations of the conserved residues Arg234 and Glu287 (Figure S1A) did not provoke any phenotype in yeast, we conclude that either the N- and C- domains are held in place despite the mutations or that a fixed domain arrangement is not essential for the function of Cns1.

The N-terminal segment of Cns1 is essential for yeast viability

To determine which parts of the protein are essential for its function in yeast, we performed 5'-FOA shuffling assays (Figure 2, Figure S2A) in which we expressed Cns1 variants in a *cns1*Δ knock-out strain. In agreement with the literature (Tesic et al., 2003), yeast cells expressing C-terminal truncations of Cns1 (Cns1$^{1-220}$, Cns1$^{1-200}$, Cns1$^{1-190}$, Cns1$^{1-185}$) were viable and cells expressing solely the C-terminal domain of Cns1 (Cns1$^{169-385}$, Cns1$^{191-385}$, Cns1$^{221-385}$) were inviable (Figure 2, S2B-D). When we truncated the N-terminal segment of Cns1, Cns1$^{36-385}$ showed no obvious growth defect compared to Cns1^{wt}. However, deletion of the first 40 amino acids (Cns1$^{41-385}$) resulted in very sick cells. When more than 44 amino acids were deleted (Cns1$^{46-385}$, Cns1$^{51-385}$) the cells were inviable. Furthermore, when we depleted the C-terminal domain from a construct lacking the N-terminal 35 residues which

supports wild-type-like viability, ($Cns1^{36-205}$, $Cns1^{36-200}$ and $Cns1^{36-195}$), the cells were also very sick, indicating, that in the absence of the first 35 residues, the C-terminal domain is crucial for the *in vivo* function of the protein (Figure 2, S2B-D).
In a high throughput study it was reported that Cns1 can be replaced in yeast by its human orthologue TTC4 (Kachroo et al., 2015). In contrast, in our 5'-FOA shuffling assay, TTC4 was not able to replace Cns1 at 30°C, but cells remained viable at 23°C although they showed a severely reduced growth rate (Figure 2, S2D, J). The sequences of Cns1 and TTC4 differ mainly in the N-terminal segments (Figure S1A). A chimera consisting of the Cns1 N-terminal segment and the TTC4 TPR+C-terminal domain supported cell viability in a $Cns1^{wt}$-like manner. Thus, Cns1 is not only structurally, but also functionally conserved from yeast to man, but there are important differences in the N-terminal segment (Figure 2). Next, we wanted to know whether the N-terminal segment alone was able to support yeast growth. To our surprise, cells only expressing $Cns1^{1-82}$, a construct completely lacking the TPR domain and the C-terminal domain of Cns1, were viable, but exhibited a severe growth defect (Figure S2F).

The above experiments demonstrated that the TPR domain is important for the *in vivo* function of Cns1. As Cns1 was reported to bind to both Hsp90 and Hsp70 via its TPR domain (Hainzl et al., 2004), we determined which of the interactions mediates the essential function of Cns1. To this end, we fused the N-terminal segment ($Cns1^{1-82}$) to the TPR1, TPR2A or TPR2B domains of the co-chaperone Sti1. These domains interact with Hsp90 (TPR2A, TPR2B) or Hsp70 (TPR1, TPR2B) (Scheufler et al., 2000; Schmid et al., 2012b). Proliferation assays showed that the fusion to TPR2A improved cell growth ($Cns1^{1-82-TPR2A}$) whereas $Cns1^{1-82-TPR1}$ expressing cells grew comparable to $Cns1^{1-82}$ alone and $Cns1^{1-82-TPR2B}$ gave an intermediate phenotype (Figure 2A, S2A, E, F). These data strongly suggest that Cns1's essential *in vivo* function is associated with Hsp90 and not with Hsp70. To further test this assumption, we fused the N-terminal 82 amino acids of Cns1 in frame to both Hsp90 ($Cns1^{1-82}$-Hsc82, Hsc82-$Cns1^{1-82}$) and Hsp70 (Ssa1-$Cns1^{1-82}$). Strikingly, only the fusion to Hsp90 improved cell growth compared to the $Cns1^{1-82}$ construct (Figure 2,). Thus, we conclude that the essential *in vivo* function of Cns1 is connected to Hsp90.

The sick growth phenotype of Cns1 truncations lacking both amino acids from the N-terminal and the C-terminal domain (e.g. $Cns1^{36-205}$), indicated that the C-terminal domain is important for *in vivo* function. To address this question further, we constructed mutants of Cns1 where the TPR domain is replaced by a flexible linker ($Cns1^{1-82-L-169-385}$, $Cns1^{1-82-L-191-385}$, $Cns1^{1-82-L-221-385}$). The presence of the C-terminal domain improved viability of the mutants compared to cells only expressing the N-terminal domain even in the absence of the TPR module (Figure S2A, I). We also tested if overexpression of selected Cns1 mutants has an effect on wild-

type yeast. Interestingly, only overexpression of the Cns1$^{51-385}$ mutant in wild-type yeast led to a dominant negative phenotype compared to controls underlining the functional interplay between the three domains (Figure S2H). Therefore, all three domains are important *in vivo*, although the N-terminal domain is responsible for the essential function associated with Hsp90.

The essential N-terminal segment of Cns1 is intrinsically disordered and contains two helical regions which interact with its TPR domain

To extend our structural analysis of Cns1 we performed SAXS experiments (Figure S3A-C). These data confirm that Cns1 contains two stable domains in solution as two maxima were observed. The first maximum corresponds to the radius of gyration (R_g) of the isolated domains and the second maximum is related to the distance between the centers of mass of the two domains. However, comparison of the experimental SAXS data with the SAXS data back-calculated from the crystal structure indicates that the stable domains adopt a more flexible orientation in solution (Figure S3B). Comparison of the SAXS data for selected truncation mutants, i.e. Cns1$^{1-220}$ and Cns1$^{70-220}$, suggests that the N-terminal segment of Cns1 is disordered as the pairwise distribution function of Cns1$^{1-220}$ shows a tail (80-110 Å) in addition to the single maximum characteristic for disordered regions (Figure S3B). *Ab initio* modeling confirms the basic domain structure of Cns1 in solution as obtained by crystallography but indicates flexibility in the connecting helix which allows different domain orientations (FigureS3C).

To characterize the N-terminal segment structurally, we performed NMR spectroscopy. Initially, we used the Cns1$^{1-82}$ fragment and a construct for the isolated TPR domain (Cns1$^{70-205}$). The NMR chemical shifts in ^{1}H, ^{15}N HSQC spectra of Cns1$^{1-82}$ (Figure 3A) show low dispersion and narrow linewidths, demonstrating that the N-terminal segment is intrinsically disordered. However, analysis of secondary ^{13}C chemical shifts (Wishart and Sykes, 1994) using TALOS (Shen et al., 2009) reveals two regions comprising residues 38-43 and 67-70 with helical propensity (Figure 3B). The presence of residual structure in these regions is further supported by positive {^{1}H} -^{15}N heteronuclear NOE values (Farrow et al., 1994b), while the other parts of the construct have low or negative values indicating high flexibility (Figure 3B). We confirmed the helical conformation for these regions in the presence of the TPR domain by performing a similar analysis with the Cns1$^{1-190}$ construct.

Next, we wondered if the N-terminal segment could transiently interact with the TPR domain by titrating ^{15}N-labeled Cns1$^{1-82}$ with unlabeled Cns1$^{70-205}$ and *vice versa* (Figure 3C). NMR signals of several residues in either protein show significant chemical shift perturbations (CSPs) and/or intense line broadening, indicating interaction. Notably, residues with large

CSPs in the N-terminal segment correspond to the transient helical regions, which harbor several negatively charged residues (Figure 3B, bottom).

Cns1 interacts with Hsp90 via its TPR domain

Next, we analyzed the interaction of Cns1 with Hsp90 by analytical ultracentrifugation (AUC). As many co-chaperones interact preferentially with specific conformations of Hsp90, we tested binding of Cns1 to Hsp90 in the presence of ATP, ADP or the non-hydrolyzable analogue AMP-PNP which leads to a closed conformation of Hsp90 sedimenting with a different s-value (Lorenz et al., 2014). We did not observe any difference in complex formation of Cns1 with Hsp90 (Figure 3D) suggesting that the interaction is not affected by nucleotide-induced conformational changes. We found that for stable complex formation with Hsp90, Cns1 requires the C-terminal MEEVD motif of Hsp90 (Figure 3E) confirming previous data (Hainzl et al., 2004; Tesic et al., 2003). In competition assays with Cns1 variants, we could show that the TPR domain of Cns1 is essential for the interaction as only Cns1 truncations carrying the TPR domain were able to disrupt a preformed *Cns1-Hsp90 complex (Figure S3D-F). Thus, we did not identify additional interactions between Hsp90 and Cns1 outside the TPR domain of Cns1.

Since the TPR domain and the N-terminal segment of Cns1 interact intramolecularly, we probed the potential competition of this interaction with the MEEVD peptide by NMR spectroscopy. We formed complexes between $Cns1^{1-82}$ and $Cns1^{70-205}$ in which one of the partners was ^{15}N-labeled, and added the unlabeled MEEVD peptide. Strikingly, in the presence of the MEEVD peptide, some of the NMR signals of the N-terminal region moved towards the position of the free $Cns1^{1-82}$ form, while in general the intensities of the signals in the N-terminal domain increased substantially. This indicates that the intramolecular interaction in Cns1 is disrupted by the MEEVD peptide (Figure S3G). The NMR spectral changes are most significant for residues involved in the helix comprising residues 67-70. In turn, the signals of the TPR domain showed extensive CSPs and line broadening as a consequence of peptide binding, especially in some of the residues that were affected by the interaction with the N-terminal region (Figure S3H). These results indicate a competitive interaction between the N-terminal segment of Cns1 and the MEEVD peptide to the TPR domain. In summary, Hsp90 binds to the Cns1-TPR domain via its C-terminal MEEVD peptide. This interaction releases weaker intramolecular contacts between the N-terminal segment and the TPR domain of Cns1, suggesting a possible mechanism for regulating the interaction with Hsp90 and thus also the function of Cns1.

Cns1 and Cpr7 play a role in translation elongation

Previous studies reported that Cns1 has an overlapping *in vivo* function with the Hsp90 co-chaperone Cpr7 (Dolinski et al., 1998b; Marsh et al., 1998a; Tesic et al., 2003). However, which cellular process is regulated by the two co-chaperones remained enigmatic. To test whether other Hsp90 co-chaperones are involved in the Cns1/Cpr7 pathway, we constructed double mutant strains carrying either *cpr7*Δ or tet07-*CNS1* (doxycycline regulated promoter, leading to a knock down of Cns1 in the presence of doxycycline) together with deletions/knock downs of all known Hsp90 co-chaperones (Figure S4A-C). Interestingly, none of the co-chaperones additionally tested revealed a genetic interaction with *CNS1* and *CPR7*. Only the *cns1* and *cpr7* mutant strains exhibited a strong negative genetic interaction with each other. We conclude that Cns1 and Cpr7 form a unique epistasis module and that the other Hsp90 co-chaperones play, if any, only a minor role in the process. Therefore, we focused our further *in vivo* studies on the *cpr7* and *cns1* mutant strains.

As expected, *cpr7*Δ cells showed a growth defect compared to the wild-type (Figure 4A). In the absence of doxycycline, i.e. Cns1 overexpression (Figure S4D), the tet07-*CNS1* mutant strain did not show any noticeable growth defect (Figure S4A). Therefore, it is not surprising that, in the absence of doxycycline, the growth defect resulting from the deletion of the *cpr7* gene was abrogated, as *CNS1* is a multi-copy suppressor of *cpr7*Δ (Dolinski et al., 1998b; Marsh et al., 1998a). In the presence of doxycycline, the tet07-*CNS1* strain resulted in a strong growth defect. The double mutant was synthetic sick as observed before (Tesic et al., 2003).

In previous studies, the *cns1* and *cpr7* mutant strains were found to be hypersensitive to the translation inhibitor hygromycin B (Albanese et al., 2006; Tenge et al., 2015). We found that Cns1 overexpression reverts the hygromycin B hypersensitivity of the *cpr7*Δ strain (Figure 4A). To test whether the two co-chaperones are involved in protein translation, we monitored the incorporation of ^{35}S-labeled methionine in proteins. We found that compared to the wild-type, protein translation was strongly diminished in *cpr7*Δ and tet07-*CNS1* knock-down cells and that this effect was even more pronounced in the double mutant (Figure 4B, S4E).

To determine which process is affected in the mutants, we used a polysome run-off assay (Ashe et al., 2000). Under normal translation conditions, 80S ribosomes form polysomes which can be chemically frozen by addition of cycloheximide (+CHX) (Figure 4C), thus preventing run-off. In contrast, glucose deprivation for 10 min rapidly inhibits translation initiation and leads to polysome run-off from the mRNA. Strikingly, under glucose starvation conditions, polysome run-off was reduced in all three mutant strains as indicated by elevated polysome/monosome ratios (Figure 4C-G). The strongest effect was observed in the tet07-*CNS*1, *cpr7Δ* double mutant. Moreover, in the absence of doxycycline, polysome .run-off was wild-type-like in the tet07-*CNS1* strain. Cns1 overexpression in the tet07-*CNS1 cpr7*Δ strain

reversed the polysome run-off effect of *cpr7*Δ (Figure S4F). These data strongly suggest that either translation elongation or termination must be compromised in these mutants. In this regard it is important to note that Cns1 sedimented with the light fractions, indicating that its interaction with translating ribosomes is transient. In this context, it is interesting to note that Cns1 and Cpr7 are able to form mixed complexes with Hsp90 suggesting that they could act on the same client simultaneously (Figure 4H).

Cns1 interacts directly with translation factor eEF2 and the novel recruiting factor Hgh1

To define the physical interactome of Cns1, we performed tandem affinity purification with tagged Cns1 (Figure 5A). The read-out on SDS page revealed the presence of Hsp90 and Hsp70. Surprisingly, we detected two additional proteins which were identified as Hgh1 and eukaryotic elongation factor 2, eEF2 which is encoded by two identical genes, EFT1 and EFT2 in yeast. The identity of a further band at ~30 kDa could not be determined. For Hgh1, a specific function had not been defined yet. Our data now suggest that Cns1, Hgh1 and eEF2 might be part of a multi-protein complex that links the Hsp90 chaperone machine to protein translation. High throughput studies support an interaction between Cns1 and Hgh1 (Gavin et al., 2002; Schlecht et al., 2012; Tarassov et al., 2008) and between Hgh1 and eEF2 (Krogan et al., 2006; Krogan et al., 2004).

To gain further insight into their interaction, we purified the proteins and analyzed complexes by AUC. We were able to demonstrate that Cns1, besides binding to Hsp90, is able to directly associate with eEF2 as well as with Hgh1 and that Hgh1 forms complexes with eEF2 and Cns1. Hgh1 does not bind Hsp90 (Figure 5B, C, S5). This suggests a complex pattern of assembly in which Cns1 has three interaction partners and Hgh1 has two. We also reconstituted the hetero-trimeric complex consisting of Cns1, Hgh1 and eEF2 and showed that it can be joined by Hsp90 resulting in the quaternary Cns1-Hsp90-Hgh1-eEF2 assembly (Figure 5C, S5), in agreement with our *in vivo* data (Figure 5A). Interestingly, the formation of this quaternary complex was strongly diminished, when the inviable $Cns1^{51-385}$ construct was used, underlining the importance of the N-terminal segment of Cns1 for proper association (Figure S5G).

Next, we employed H/DX experiments coupled to mass spectrometry to analyze the regions in eEF2, Hgh1 and Cns1 affected by complex formation (Figure 5 D-F, S5 H-K). In eEF2, we see changes in domain 1 and domain 3 upon binding of Cns1. Interestingly, for domain 3 which reveals the highest flexibility in eEF2 alone, we observe several segments that are stabilized against exchange in the presence of Cns1 suggesting that this domain constitutes the primary interaction site for Cns1 (Figure 5E).

When we analyzed the eEF2-Hgh1 complex, again domain 3 of eEF2 was affected. In this case, however, we see segments with higher or lower exchange compared to eEF2 alone, implicating that Hgh1 affects this domain in a complex manner. Interestingly, at early time points of the exchange the domain is more flexible than at later time points which may indicate changes in its folding and stability triggered by the interaction with Hgh1 (Fig. S5I).

For the Cns1-Hgh1 complex, it was possible to obtain information for both partners in the complex. The TPR domain of Cns1 becomes more flexible in the presence of Hgh1 while the C-terminal domain remains largely unchanged. In the largely disordered N-terminal segment of Cns1 no significant alterations in exchange were observed (Figure 5F, S5J). In Hgh1, regions with decreased exchange pointing towards sites of interaction were detected in the C-terminal part, the N-terminal region and also to some extent in the middle of the protein (Figure S5K).

AUC competition experiments of the Cns1-Hgh1 interaction suggest an interaction between Hgh1 and the C-terminal domain of Cns1, but the complex was only disrupted completely when also the TPR domain was present (Figure S5L). Interestingly, we could also detect a weak interaction between the Cns1 N-terminal segment and Hgh1 by NMR (Figure S5M), suggesting that this regions may contribute to complex formation in the presence of Hsp90 where the MEEVD peptide will release the Cns1 N-terminal segment from the TPR domain. In summary, this indicates, that all three Cns1 domain are involved in the interaction with Hgh1.

Cns1 is important for *in vivo* folding of eEF2

To test whether *in vivo* specifically eEF2 is affected by the Hsp90 components, we overexpressed proteins known to be involved in translation elongation and termination. These are eEF1A, eEF2, eEF3, eIF5A, eRF3 and eRF1. Strikingly, only upon eEF2 overexpression we observed a negative effect on the growth of *cpr7*Δ and *cns1* knock-down cells (Figure 6A, S6A), indicating that the chaperone dependence is specific for eEF2. As seen already with respect to hygromycin B sensitivity, Cns1 overexpression in the *cpr7*Δ mutant reversed the eEF2 toxicity. Deletion of *HGH1* and *CPR7* led to an increased growth defect that was also observed previously in high-throughput studies (Costanzo et al., 2010; Costanzo et al., 2016; Kuzmin et al., 2018; Rizzolo et al., 2017; Rizzolo et al., 2018). Again, Cns1 overexpression in the absence of doxycycline mitigated a negative genetic interaction between *hgh1*Δ and *cpr7*Δ. Interestingly, deletion of *HGH1* did not further enhance the growth defect of the tet07-*CNS1* strain in the presence of doxycycline, indicating that Cns1 is the limiting component in the Cns1-Hgh1 module (Figure S6B).

While the above results demonstrate the general importance of the chaperone components for eEF2 function, they do not report on their effects on eEF2 *in vivo*. To this end, we analyzed total eEF2 protein levels in different knock out or knock down strains. We observed a strong decrease of eEF2 in the *cpr7*Δ, tet07-*CNS1* or *hgh1*Δ strains (Figure 6B), suggesting that Cns1, Cpr7 and Hgh1 affect eEF2 folding and stability. To test this notion further, we analyzed the soluble vs. insoluble fraction of eEF2. The experiments revealed that in the wild-type strain eEF2 folded properly and was thus found in the supernatant. In contrast, eEF2 aggregated substantially in the absence of either Cns1 or Cpr7 and only ~20 % of eEF2 were found in the supernatant (Figure 6B). In the *hgh1Δ* strain ~ 40 % remained soluble. Taken together, our experiments demonstrate that the correct *in vivo* folding and maintenance of structural integrity of eEF2 depends strongly on Cns1, Cpr7 and Hgh1. As a control, we tested, whether the knockout of EFT2, which depletes eEF2 levels to about 50% of wild-type, also resulted in reduced protein translation (Figure S6D). Indeed, the mutant phenocopied the *cns1*, *cpr7* and *hgh1* mutants regarding ^{35}S-methionine incorporation, providing further evidence that Cns1, Cpr7 and Hgh1 maintain protein translation via chaperoning of eEF2.

Finally, we tested to what extent the *de novo* folding of eEF2 depends on Hsp90. For this purpose, we treated wild-type yeast either with DMSO as a control or with the Hsp90 inhibitor Radicicol and then induced HA-eEF2 expression. Radiciol-treated cells showed a strong reduction of *de novo* synthesized eEF2 compared to the DMSO control showing that Hsp90 has a pronounced impact on this process (Figure 6C). Together with the results on Cns1, Cpr7 and Hgh1 this demonstrates that all components of this complex affect eEF2 folding.

Discussion

In recent years, research on Hsp90 co-chaperones has shed light on their interaction with Hsp90 and their ability to modulate its chaperone cycle. In addition, some co-chaperones were reported to be responsible for the folding and activation of certain protein classes, like Cdc37 for kinases, whereas others were shown to be involved in the assembly of specific multi-protein complexes, e.g. Tah1 and Pih1 (Schopf et al., 2017). For Cns1 we lacked both insight into its structure as well as its function. Our study defines the structure of Cns1 and its role in *S. cerevisiae*. Strikingly, it links translation to Hsp90 control. Cns1 is important for the chaperoning of the translation elongation factor eEF2 in concert with Hsp90. For this function we identified Hgh1 as an important co-factor.

Our structural analysis by X-ray crystallography and NMR together with *in vivo* experiments established both the fold of Cns1 and defined the protein's parts essential for yeast viability.

We show that Cns1 is a “three-domain” protein. It contains two folded domains and an intrinsically disordered N-terminal region. Interestingly, the C-terminal domain exhibits a novel fold, which is conserved between yeast Cns1 and human TTC4. These data prove that, TTC4 is the human homologue of Cns1. Together with our *in vivo* complementation experiments this also strongly suggests that the function of Cns1 is conserved between yeast and man. The TPR domain, which mediates the interaction with Hsp90 and the C-terminal wheel domain are connected by a long helix. The rigidity suggested by the crystal structure does not seem to be maintained in solution, as indicated by SAXS, and is also not important *in vivo* as confirmed by mutagenesis. What is important, is the N-terminal segment of Cns1. NMR spectroscopy revealed that this part is mostly disordered, but contains two segments with partial helical conformation. Interestingly, the first helix coincides with the region that conveys the essential function of Cns1 identified in our viability assays. In addition, the N-terminal tail binds to the adjacent TPR domain in a region that overlaps with the binding site for the C-terminal Hsp90 MVEED motif. This suggests an auto-inhibitory process that is coupled to Hsp90 binding potentially regulating complex assembly.

Several previous studies reported a functional overlap between Cns1 and Cpr7 *in vivo* (Marsh et al., 1998a; Tenge et al., 2015; Tesic et al., 2003; Zuehlke and Johnson, 2012), but the nature of this genetic interaction was not clear. Moreover, both proteins were reported to weakly interact with the 80S ribosome and *cns1* and *cpr7* mutants were sensitive to protein translation inhibition by hygromycin B (Albanese et al., 2006; Tenge et al., 2015). Our analysis shows that overall translation is strongly compromised in *cns1* knockdown strains reaching only 50% of the normal levels. Furthermore, we identified translation elongation as the step being affected. By over-expression of different elongation and termination factors, we determined that only eEF2 was sensitive to *CNS1* depletion or *CPR7* deletion. To proof a direct interaction between Cns1 and eEF2, we pulled out Cns1 by tandem affinity purification and identified key interaction partners. Apart from Hsp90 and Hsp70, we detected the proteins Hgh1 and eEF2. Hgh1 was a still enigmatic protein, neither its structure nor its function were known. Only in high throughput studies, an interaction between these factors had been suggested (Gavin et al., 2002; Schlecht et al., 2012; Tarassov et al., 2008). Interestingly, the analysis of the interaction of the purified proteins revealed a complex pattern: Cns1 can form complexes with eEF2 and Hgh1, in addition to its known interaction with Hsp90. In the binary Cns1-Hgh1 complex both the C-domain of Cns1 and its N-terminal part are important for a stable interaction. In this context, it is also interesting to note that for survival of yeast cells the C-terminal domain gains importance once the N-terminal segment was truncated. Together, this suggests an interplay in which the different parts of Cns1 fulfill synergistic functions. This becomes even more pronounced in the ternary complex with eEF2. Here, the N-terminal segment gains further importance and undergoes additional

interactions which stabilize the complex. Further complexity is added, when Hsp90 is present. Binding of the MEEVD sequence of Hsp90 to the Cns1 TPR domain releases contacts between the N-terminal segments of Cns1 and its TPR domain. This may result in changes in the conformation of Cns1 and affect its interaction patterns. Our *in vivo* analysis revealed that all the components are involved in eEF2 folding. Specifically, the levels of newly synthesized eEF2 depend on Hsp90 and are strongly diminished in *cns1, cpr7* and *hgh1* mutants. Besides, we could also show that eEF2 aggregated in *cns1*, *cpr7* and *hgh1* mutant strains, which adds to the decreased levels and explains the observed translation defects. It is important to note, that in our ribosome fractionation experiments, Cns1 sedimented with the light cytosolic fractions, indicating that it does not act as a co-factor of eEF2 during translation. Also the low amounts of Cns1 per cell (Ghaemmaghami et al., 2003) point to a role in a specific process. Thus, taking the different pieces of evidence together, the most likely scenario is that the Hsp90 machinery is involved in the *de novo* folding of eEF2 after it leaves the ribosome. Interestingly, eEF2 overexpression affected growth in *cpr7* and *cns1* mutants. Presumably, the accumulation of misfolded eEF2 might interfere with translation elongation. Indeed, it was reported previously that yeast cells keep their total eEF2 levels constant (Ortiz and Kinzy, 2005); interfering with the chaperones responsible for eEF2 folding might thus explain the observed phenotype. There are three possible explanations for the genetic interaction of *cns1* and *cpr7* mutants: First, Cns1 and Cpr7 show weak interaction with the ribosome. At least for Cns1 a stable interaction with the 80S ribosome is not essential since it is mediated by the C-terminal Cns1 domain (Tenge et al., 2015) which is dispensable *in vivo*. Interestingly, Cns1 and Cpr7 were found in the same complex in a yeast strain lacking the C-terminal MEEVD motif of Hsp90, but they do not interact directly (Tesic et al., 2003). Thus, the two co-chaperones might find their *in vivo* target independently of Hsp90. Second, Cpr7 could be involved in processes upstream of or parallel to eEF2 folding which impair translation elongation and lead indirectly to eEF2 aggregation. This would explain the genetic interaction. Interestingly, *cpr7* mutants show a negative interaction with genes involved in the synthesis of diphtamid which modifies His699 in eEF2 (Costanzo et al., 2010; Costanzo et al., 2016; Kuzmin et al., 2018). This modification is an example for a parallel/upstream pathway. Third, our *in vitro* approach might be missing an adapter protein comparable to Hgh1 to stabilize or facilitate the interaction of Cpr7 with eEF2.

Our results put previous observations from high-throughput studies on connections between Hgh1, Cns1, eEF2 and Hsp90 (Alford and Brandman, 2018; Brandman et al., 2012; McClellan et al., 2007) into context. Interestingly, the factors were also shown to be involved in stress granule formation (Cherkasov et al., 2015), a hallmark of impaired translation elongation. Our study now revealed that Cns1 and Hgh1 both directly interact with eEF2 and

are crucial for its proper folding and activation *in vivo*, explaining the previously observed phenotypes. Importantly, these interactions are also conserved in human cells where TTC4 was reported to interact with human Hgh1, Hsp90 and human eEF2 (Crevel et al., 2008; Huttlin et al., 2017; Huttlin et al., 2015; Kristensen et al., 2012; Taipale et al., 2014). The CCT/TRiC chaperone system seems also to be involved in eEF2 folding, as human Hgh1 was reported to interact with CCT/TRiC (Hein et al., 2015), and yeast eEF2 is a client of CCT/TRiC (Russmann et al., 2012). Interestingly, Bracher, Hartl and colleagues now found that Hgh1 also acts as a recruiting factor for CCT/TRiC fostering the interaction of eEF2 with this chaperone complex (Mönkemeyer, co-submitted manuscript). These findings suggest that Hgh1 recruits both the CCT/TRiC and the Hsp90 chaperone systems for eEF2 folding. Whether these chaperone machineries act in parallel or in a sequential manner remains to be seen.

In summary, our results suggest a novel concept for the chaperone-dependent folding of eEF2 in yeast (Figure 7). In this context, several factors in addition to Hsp90 cooperate. Cns1 exhibits an important bridging function as it interacts with eEf2, Hsp90 and Hgh1. The interactions of Cns1, Hgh1 and Hsp90 with eEF2 are required for its stability and solubility in yeast. In the absence of these factors, eEF2 aggregates and eEF2 levels decrease leading to impaired translation elongation and loss of viability of yeast cells. The co-chaperone Cpr7 is also involved in these processes, but may work by a different mechanism. Taken together, the coordinated interaction of eEf2 with Cns1, Hgh1 and Hsp90 is required to chaperone eEF2 and support translation elongation.

Acknowledgement

This work was supported by grants from the DFG (SFB 1035 A3) to J.B./ M.S. and to M.G. (SFB1035 A2) and a fellowship from the IMPRS graduate school to F.S. E.M.H. acknowledges financial support by the Young Scholars' Program of the Bavarian Academy of Sciences and Humanities. M.M.B. was supported by a fellowship by Fonds of the chemical industry. T.M. was supported by the Austrian Science Fund (FWF: P28854 and I3792 to T.M.), the Austrian Research Promotion Agency (FFG: 864690), the Integrative Metabolism Research Center Graz, the Austrian infrastructure program 2016/2017, and BioTechMed/Graz. We thank Marcel Genge, Florian Ziegler, Florian Rührnößl, Gina Feind and Astrid König for experimental support. Timm Hassemer/Ulrich Hartl for help with the ribosome fractionation, and Maximilian Reuter/Katja Sträßer for help with tandem affinity purification. We are grateful to Elke Deuerling for reagents, Dominik Maslak (Center for industrial Biotechnology TUM) for fermentation, Marina Rodnina, Frank Peske, Namit Ranjan (MPI Göttingen) for discussions and experimental advice, Dominic Helm and Bernhard Küster (TUM) for mass spec analysis. We thank the staff of the beamlines X06SA at the Paul Scherrer Institute, Swiss Light Source, Villigen, Switzerland and ID30B at the European Synchrotron Radiation Facility, Grenoble, France for assistance during data collection.

Author contribution

F.S. M.M.B and C.D. performed *in vivo* experiments. E.M.H. and M.G. determined the crystal structure and interpreted the data. D.A.R. and C.D. performed and analyzed analytical ultracentrifugation experiments. A.L. and M.S. conducted NMR experiments. M.M. and F.S. carried out radioactive experiments. T.M. performed and analyzed SAXS experiments. F.S. E.M.H. M.G. A.L. M.S. T.M. and J.B. wrote the paper.

Figure legends

Figure 1. Crystal structures of yeast Cns1 and human TTC4

(A) Ribbon plot of the C-terminal wheel domain of yeast Cns1 (residues 221-385). The central β-sheet structure is colored in yellow, while the surrounding helices are depicted in magenta. N- and C-termini are labelled.

(B) Ribbon plot of the C-terminal domain of human TTC4 (residues 217-387). β-sheets are highlighted in green and helices in blue. Loops disordered in the crystal lattice are shown as black dotted lines. N- and C-termini are labelled.

(C) Superposition of $Cns1^{221-385}$ shown in panel A and $TTC4^{217-387}$ depicted in panel B.

(D) Topology plot of the conserved twisted β-sheet of the C-terminal domains of Cns1 and TTC4. Helices have been omitted from this plot for clarity. For an overall and a more detailed topology plot see Figure S1B.

(E) The $Cns1^{70-385}$ crystal structure shown as ribbon visualizes the two domain architecture of the protein. Color coding is according to panel A.

(F) Surface charge distributions for $Cns1^{70-385}$ at pH 7. The left illustration corresponds to the orientation shown in panel E, while the right one is rotated by 180 °. Surface colors indicate positive and negative electrostatic potentials colored from 10 kT/e (intense blue) to -10 kT/e (intense red). The blue colored pocket of the TPR domain corresponds to the binding site for the Hsp90-C-terminus (Figure S1G).

Figure 2. Cns1 is an essential Hsp90 co-chaperone in yeast

Schematic representation of Cns1 key mutants, Hsp90, TTC4 and chimeras thereof used in 5'-FOA shuffling experiments. "+"-graduation indicates cell viability starting with "++++++" for wild-type-like growth and ceases with "-" for lethality. "~" indicates cell viability at 23°C.

Figure 3. Cns1 N-terminus has residual structure and interacts with TPR domain

(A) Superposition of ^{1}H, ^{15}N HSQC spectra of the isolated N-terminal region of Cns1 ($Cns1^{1-82}$, black) free and in presence of 1.1 equivalents of unlabeled TPR domain ($Cns1^{70-205}$, red).

(B) Top: secondary ^{13}C chemical shifts ($\delta^{13}C\alpha$-$\delta^{13}C\alpha_{ref}$) - ($\delta^{13}C\beta$-$\delta^{13}C\beta_{ref}$) of $Cns1^{1-82}$ (positive values indicate helical structure, zero corresponds to random coil). Secondary elements predicted by the program TALOS+ (Shen et al., 2009) are shown on top. Mid: heteronuclear nOe values. Bottom: CSPs between $Cns1^{1-82}$ and $Cns1^{1-190}$, focusing on the 1-82 fragment. Residues that show perturbations above the median + standard deviation (dashed line) are annotated. In all plots, gray open circles correspond to residues without data.

(C) The same experiments as in (A) but this time with ^{15}N-labeled TPR (black) and 1.1

equivalents of unlabeled N-terminal segment (red).
(D) Effect of the conformational state of Hsp90 on Cns1 binding analyzed by AUC sedimentation velocity experiments (500 nM Atto488-labeled Cns1, 10 µM Hsp90 and 2 mM nucleotides as indicated).
(E) Binding of Cns1 to different fragments of Hsp90 analyzed by AUC sedimentation velocity experiments (500 nM Atto488-labeled Cns1, 5 µM Hsp90 constructs as indicated).

Figure 4. Cns1 and Cpr7 are involved in protein translation
(A) *cpr7Δ* and *cns1* knock-down mutants exhibit hygromycin B sensitivity. Ten-fold serial dilutions of the indicated yeast strains were spotted onto YPD plates containing combinations of doxycyclin (10 µg/ml) and hygromycin (25 µg/ml) as indicated
Plates were incubated at 30°C and pictures were taken after 48 h. Representative pictures out of three biological replicates.
(B) Effect of the *cpr7Δ* and *cns1* knock-down mutants on protein synthesis. The indicated yeast cultures were supplemented with a mix of ^{35}S-labeled methionine and cold methionine for 30 min and 60 min at 30 °C, respectively. Total protein extracts were speparated by SDS-PAGE. After autoradiography, signal intensities were quantified using imageJ.
(C) – (F) Polysome run-off experiments reveal decreased translation elongation in *cpr7Δ* and *cns1* knock-down mutants. Cells were grown in YPD in the presence of 10 µg/ml doxycyclin and either treated with 100 µg/ml cycloheximide (CHX) or starved for 10 min in YP medium. Total cell extracts were run on 7 - 47 % sucrose gradients and ribosome profiles were recorded at 254 nm. Westernblot analysis of ribosome fractionations. Cns1 and Rpl17A were detected as indicated.
(G) Ratio of monosomes vs. polysomes as shown in (C-F). Data from two independent biological replicates.
(H) Analysis of complex formation between Cns1, Hsp90 and Cpr7 by AUC sedimentation velocity experiments using 500 nM Atto488-labeled Cns1, 5 µM Hsp90 and Cpr7 concentrations as indicated. Normalized c(s) distributions were plotted against the apparent sedimentation coefficient (S).

Figure 5. Cns1 directly interacts with eEF2 and the novel adaptor protein Hgh1
(A) Tandem affinity purification of Cns1 indicates interaction of Cns1 with Hgh1 and eEF2 in addition to Hsp90 and Hsp70. Proteins were separated by SDS-PAGE and identified by mass spectrometry. The most prominent hits are indicated.
(B) eEF2 can directly bind Hgh1 and Cns1, but not Hsp90. AUC sedimentation velocity experiments using 500 nM Atto488-labeled eEF2 and all other indicated proteins at 2.5 µM. Normalized c(s) distributions were plotted against the apparent sedimentation coefficient (S).
(C) eEF2 is linked to Hsp90 via Hgh1 and Cns1. AUC sedimentation velocity experiments

using 500 nM Atto488-labeled eEF2 and all other indicated proteins at 2.5 µM. Normalized c(s) distributions were plotted against the apparent sedimentation coefficient (S).
(D) Left panel: Domain structure of eEF2 according to InterPro. Numbers indicate first and last residue of the domain. Coloring code of the eEF2-domains mapped onto the crystal structure of yeast elongation factor 2 (PDB ID: 1N0U). Center-left panel: eEF2 is colored according to the fractional H/D exchange at time point 600 s. B-values were mapped onto the crystal structure of yeast elongation factor 2. White to red coloring depicts increase in deuteration. Black segments were not covered in H/D exchange experiments. For clarification, large helix in domain 4 is colored in black. Center-right panel: The difference in measured H/D exchange between the eEF2-Hgh1 complex vs eEF2 at time point 600 s. Blue colors correspond to lower and red colors to higher deuteration of the complex Black segments were not covered. Right panel: Difference in H/D exchange between the eEF2-Cns1 complex and eEF2 after 600s. Coloring same as in middle panel.

(E) Left panel: Difference in H/D exchange between the eEF2-Hgh1 complex and eEF2 after 600 s. Blue colors correspond to lower and red colors to higher deuteration of the complex. Black segments were not covered in H/D exchange experiments. Insert: Zoomed view into domain 3 of eEF2. Right panel: Difference in H/D exchange between the eEF2-Cns1 complex and eEF2 after 600 s. Coloring same as in left panel. Insert: Zoomed view into domain 3 of eEF2.
(F) Left panel: fractional H/D exchange of Cns1 after 600 s mapped onto the crystal structure of $Cns1^{70-385}$ (PDB ID: 6HFT) White to red coloring depicts increase in deuteration. Black segments were not covered. Right panel: H/DX difference of Cns1 in complex with Hgh1 and Cns1 unbound. Color code same as in E.

Figure 6. Requirement of Cns1 and Hsp90 for eEF2 *de novo* folding
(A) Overexpression of eEF2 is toxic for *cpr7Δ* cells. Ten-fold serial dilutions of the indicated yeast strains were spotted onto glucose (glu) or galactose (gal)-containing -Leu plates with or without 10 µg/ml doxycyclin (dox). eEF2 was overexpressed from the GAL1 promoter, p415-GAL1 empty vector was used as control. Plates were incubated at 30°C and pictures were taken after 48 h. Representative pictures out of three biological replicates.
(B) Cns1, Cpr7 and Hgh1 are crucial for folding of eEF2 *in vivo*. Lysates from the indicated yeast strains were separated into pellet and supernatant and the distribution of eEF2 was analyzed by Western-Blot. The ratio of eEF2 in the pellet fraction and total eEF2 levels were analyzed in the *tet07-CNS1, cpr7Δ*, tet07-*CNS1/cpr7Δ* and *hgh1Δ* strains. Shown are the mean values and standard deviations from at least three independent biological replicates.
(C) eEF2 levels depend on Hsp90. *S. cerevisiae* cells were grown to log phase in the presence of 25 µM of the Hsp90 inhibitor Radicicol or DMSO as control before the

expression of HA-tagged eEF2 was induced by addition of D-galactose. Treatment with Radicicol entailed reduced levels of HA-EF2. PGK1 was used as a loading control. The data represent means and standard deviations from three independent biological replicates.

Figure 7. Model of eEF2 activation by Hgh1, Cns1 and Hsp90

Hgh1 acts as an adapter protein for both Cns1 and eEF2. Hsp90, Cns1 and Hgh1 form a multi-chaperone complex with eEF2. The formation of this complex is crucial for eEF2 activation. Cpr7 is involved in this process but seems to act on a parallel pathway. When folded properly, eEF2 can then fulfill its role in translation elongation. In the absence of components of this complex, eEF2 is instable and forms aggregates in the cell.

(Semenyuk and Svergun, 1991)

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