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Features of the Chaperone Cellular Network Revealed through Systematic Interaction Mapping

Graphical Abstract



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In Brief

Rizzolo et al. use a systematic integrative approach combining physical and genetic interaction data to construct a comprehensive chaperone network. This analysis revealed the presence of a large functional chaperone supercomplex, the NAJ complex. Furthermore, many chaperone interactors were found to form condensates.

Highlights

- Chaperone interaction map elucidated through integrated proteomic approaches
- A large functional chaperone supercomplex, named NAJ complex, is revealed
- Many chaperone interactors are found to form condensates
- The AAA+ ATPases Rvb1 and Rvb2 form condensates under nutrient starvation





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Features of the Chaperone Cellular Network Revealed through Systematic Interaction Mapping

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SUMMARY

A comprehensive view of molecular chaperone function in the cell was obtained through a systematic global integrative network approach based on physical (protein-protein) and genetic (gene-gene or epistatic) interaction mapping. This allowed us to decipher interactions involving all core chaperones (67) and cochaperones (15) of Saccharomyces cerevisiae. Our analysis revealed the presence of a large chaperone functional supercomplex, which we named the naturally joined (NAJ) chaperone complex, encompassing Hsp40, Hsp70, Hsp90, AAA+, CCT, and small Hsps. We further found that many chaperones interact with proteins that form foci or condensates under stress conditions. Using an in vitro reconstitution approach, we demonstrate condensate formation for the highly conserved AAA+ ATPases Rvb1 and Rvb2, which are part of the R2TP complex that interacts with Hsp90. This expanded view of the chaperone network in the cell clearly demonstrates the distinction between chaperones having broad versus narrow substrate specificities in protein homeostasis.

INTRODUCTION

Molecular chaperones are a highly interactive group of cellular proteins that fulfill central roles in all aspects of protein homeostasis, including protein folding, assembly, and unfolding of substrates. Chaperones are typically categorized into families based on their sequence similarity and function. Proteins of the major chaperone families in the budding yeast *Saccharomyces cerevisiae* are 2 Hsp90s, 14 Hsp70s, 22 Hsp40s, 8 CCTs, 1 Hsp60, 1 Hsp10, 6 prefoldins, 5 ATPases associated with diverse cellular activities (AAA+), 7 small heat shock proteins (sHsps), and 1 calnexin (total of 67; Table S1; Figure 1A). In addition, the Hsp70s and Hsp90s function with 4 and 11 partner proteins, respectively, that are termed cochaperones (total of 15; Table S1; Figure 1A). Despite many mechanistic and functional studies, the spectrum of cellular substrates and many of the functions mediated by these chaperones remain largely undetermined.

Given their involvement in many cellular protein homeostasis processes, it is necessary to study chaperones at a global systems level. In 2005, our group published a comprehensive physical (protein-protein) and genetic (gene-gene or epistatic) analysis of the yeast Hsp90 chaperone interaction network, showing the broad role this central chaperone has in many cellular pathways (Zhao et al., 2005). Subsequently, in 2009, we published a physical interaction atlas for 63 yeast chaperones (Gong et al., 2009). Here, genetic interaction data from synthetic genetic array (SGA) technology in yeast (Costanzo et al., 2016) was combined with protein-protein interactions (PPIs) to systematically build a comprehensive high-fidelity chaperone network from a total of 67 chaperones and 15 cochaperones. The network revealed several features of the chaperone functional distribution in the cell, and it indicated the presence of a functional chaperone supercomplex required for cellular protein homeostasis.



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Figure 1. CCo Network Based on GI Data

(A) Bar plot showing the yeast chaperones and cochaperones divided into different families. The colors used for the bars for each CCo family are the same in all the figures. On the right is a diagram showing the cellular localization of the CCos.

(B) Flow-gram showing the acquisition and analysis of the CCo GI data. CCo GIs were compiled and then GIs and GI profile correlation similarities were obtained. GIs were filtered at an intermediate threshold (|SGA score| > 0.08 and p < 0.05), and GI profile correlation similarities were computed. See also Figures S1A–S1D. (C) Heatmap clustering generated from the GI dataset is shown where the 77 CCos are organized on the x axis as both query and array and 4,583 yeast genes on the y axis. Below are three examples of GI clusters involving CCos: (i) GI cluster involving Sec63 (see also Figure S1E), (ii) GI cluster of the CCT and the prefoldin families, and (iii) GI cluster showing predominantly positive GIs between CCT and proteins involved in ribosome biogenesis (see also Figures S2 and S3). (D) Circos plot (Krzywinski et al., 2009) showing correlations in the GI profiles among CCos. CCo families are grouped and colored according to (A). Profiles were calculated for all CCo gene pairs and measured based on Pearson correlation coefficients (PCCs) from the complete GI matrix. Pairs having a PCC > 0.1 threshold are plotted. Ribbon width corresponds to the magnitude of the PCC value and the color of the ribbon corresponds to the color of the originating segment.

RESULTS

A Comprehensive High-Fidelity Genetic Interaction Chaperone and Cochaperone Network

The genome-wide genetic interaction (GI) data to build the chaperone and cochaperone (CCo) GI network are described in the Experimental Procedures, and they were acquired as part of the effort to map the full GI network in yeast (Costanzo et al., 2016). For essential genes (Giaever et al., 2002), both Decreased Abundance by mRNA Perturbation (DAmP) strains (Schuldiner et al., 2005) as well as temperature-sensitive (TS) alleles were used (Costanzo et al., 2010, 2016). The initial CCo GI raw data with 425,751 double mutants were subsequently filtered with a threshold score cutoff (|SGA score| > 0.08 and p < 0.05) as described (Costanzo et al., 2016), and they resulted in 22,443 high-confidence GIs where 13,704 were negative (SGA score < 0; i.e., double mutants with a more severe fitness defect than the expected multiplicative effect of combining the individual mutants, with the extreme case being synthetic lethality) and 8,739 were positive (SGA score > 0; i.e., double mutants with a less severe defect in fitness than expected) (Table S2; Figure 1B). The GI data included 62 chaperones and 15 cochaperones with

only 4 missing, namely, Ssa4, Ssc2, Mdj1, and Gim5 (note that Ssa4, Ssc2, and Gim5 are present in the PPI dataset; see below). CCo genes had an average of 293 Gls compared to 246 for non-CCo genes (Figure S1A). Together, the current Gl dataset contains three times the number of Gls involving a CCo gene compared to previous low- and high-throughput studies compiled from BioGrid (Breitkreutz et al., 2008). Notably, this comparison shows that our dataset includes almost double the number of essential genes in yeast compared to previous studies.

To gain insights into the functional ranking of the chaperone families, we looked at the total number and overlap of GIs among CCos (Figures S1B–S1D). Hsp70, Hsp40, and Hsp90 chaperone families tended to have the highest total number and the highest unique number of GIs (Figures S1B and S1C). Also, the Hsp70 and Hsp40 family members had the highest number of GI interactor overlap with other chaperones (Figure S1D). The Hsp70, Hsp40, Hsp90, and to a lesser extent CCT members shared many interactors (Figure S1D).

Figure 1C shows a global view of the positive and negative CCo GIs encompassing 4,583 yeast genes. CCo genes within a cluster enriched for a particular molecular pathway were mostly connected by negative GIs (Figure 1C). For example, genes encoding for proteins involved in endoplasmic reticulum (ER) translocation that form a cluster with *SEC63*, *CNE1*, *KAR2*, and *SCJ1* were highly enriched for negative GIs (Figure 1C, lower panel i). To further verify some of these hits, we performed membrane yeast two-hybrid assays (MYTH) (Snider and Stagljar, 2016) with Sec63, the essential subunit of the ER translocon containing a J domain, as a bait. Our results showed that all 6 prey proteins interacting with SEC63, indicating that a negative GI with an essential gene is a likely indicator of physical interaction. These results further validated the GI data.

Genes that encode for proteins that form part of the same complex tend to be biased toward a single type of GI (Baryshnikova et al., 2010; Costanzo et al., 2016). The type of GI depends on the essentiality of the genes involved; typically, as mentioned above, negative GIs were found with essential genes, which was the case for CCos. For example, the CCT complex, which is essential for cell viability, displayed mainly negative GIs with the prefoldin (or GIM) complex (Figure 1C, lower panel ii). This is consistent with the experimental evidence that CCT and prefoldin physically interact and function together to mainly fold and assemble actin and tubulin in addition to many other substrates (Dekker et al., 2008).

For the essential CCT complex, we observed striking positive GIs with genes whose proteins are involved in ribosome biogenesis (Figure 1C, lower panel iii). This was also observed in standard liquid growth as shown in Figure S2A. The *CCT4*-TS strain grew about 4 times slower at 30°C compared to the wild-type (WT) strain. In comparison, a *TRF5* gene knockout strain (Figure 1C, lower panel iii) exhibited a slightly slower growth profile than WT, whereas the double-mutant strain grew better than *CCT4*-TS, but not as well as *trf5* Δ or WT. TRF5 is part of the Trf4/Air2/Mtr4 polyadenylation (TRAMP) complex that has a poly(A) RNA polymerase activity and is involved in post-transcriptional quality control mechanisms (Houseley and Tollervey, 2006). The absence of TRF5 leads to production of misas-

sembled ribosomes (Woolford and Baserga, 2013), which likely causes a reduction in protein synthesis. This is indeed what we observed when comparing total protein content of cells at the same cell density for WT, single-mutant, and double-mutant strains grown at 30°C to stationary phase (Figure S2B). The total protein content was lowest for *trf5* Δ than for *trf5* Δ CCT4-TS double-mutant strain, while WT and CCT4-TS had similar higher total protein (Figure S2C). Notably, the presence of insoluble proteins was reduced in the double-mutant strain compared to CCT4-TS or *trf5* Δ single-mutant strains (Figure S2D). Hence, we interpret the presence of positive GI between the CCT complex and ribosome biogenesis genes as resulting from the fact that a CCT-TS strain exhibits accumulation of toxic misfolded proteins, which is mitigated by reducing the total number of proteins in the cell through the reduction of ribosome biogenesis.

CCos Are Highly Biased in Positive GIs with Essential Genes

Given the observations made with CCT, GIs of CCos against the essential (i.e., TS or DAmP allele strains) and nonessential genes were further evaluated. CCos were enriched in positive GIs against the essential array when compared to the rest of the tested genes (Figure S2E; p < 0.05). The positive:negative ratio against the essential array was also found to be significantly higher for CCos (Figure S2F; p < 0.05). This suggests that, despite CCos having a larger number of negative GIs in the network, they are more biased toward positive GIs with essential genes compared to other genes in yeast.

By performing gene ontology (GO) bioprocess enrichment analysis of the essential genes interacting with CCos with a log₂ positive:negative GI ratio above zero, significant cellular processes involved in these interactions were identified (Figure S3; p < 0.05). Of the 22 CCos, 17 were found to be interacting with genes that were significantly enriched in distinct bioprocesses (Figure S3). Some CCos displayed enrichments for positive GIs with diverse bioprocesses, as observed for CCTs and prefoldins. Other CCos had positive GIs with essential genes belonging to one or more bioprocesses, such as SSC1 with transcription and chromatin organization and SWA2 with proteasomal genes (discussed further below).

The CCo GI Profile Correlation Similarity Network

The GI profile of a given gene is composed of a set of positive and negative GIs with other genes in the genome. Genes whose GI profiles correlate tend to be part of the same complex or function in similar pathways (Costanzo et al., 2016). This property was used to look at the connectivity between CCos by building a GI profile correlation similarity network (Figure 1D; Table S3). The network highlights the inherent functional organization within and between the CCo families. Highly connected CCo families form part of discernible biological processes, such as the CCTs with the prefoldins or the Hsp40s with the Hsp70s (Figure 1D). We also found that the gene for the constitutive Hsp90 chaperone, HSC82, connects primarily with STI1 (known as Hop in mammalian cells), consistent with the fact that Sti1 is one of the main Hsp90 cofactors (Li et al., 2012). Interestingly, HSC82 also connects with YDJ1, the main cytoplasmic Hsp40 (Figure 1D). Indeed, there are 24 genes that overlap between



the Hsp90/Hsp90 cochaperones and Ydj1 in the GI profile correlation similarity network. This suggests that Ydj1 might be involved in the proper regulation of diverse Hsp90 clients rather than interacting promiscuously.

The GI Profile Correlation Similarity Network Provides New Insights into CCo Functions

We built a GI profile correlation similarity network composed of CCo-CCo and CCo-non-CCo correlation pairs evaluated using a Pearson correlation coefficient (PCC) threshold >0.1, yielding a total of 10,443 pairs (Table S3). The spatial analysis of functional enrichment (SAFE) program (Baryshnikova, 2016) was used to functionally annotate regions enriched for particular GO cellular bioprocesses (Figure 2A). SAFE highlights regions that are densely connected with a particular attribute, and, in this case, the network was evaluated with 4,373 biological process terms from GO (Ashburner et al., 2000). The CCo network contained 402 significantly enriched GO terms grouped into 7 regions involving 726 genes (Figure 2A).

Figure 2. The GI Profile Correlation Similarity Network of CCos

(A) The GI profile correlation similarity network, left panel, was constructed using PCCs of GI profile similarities (edges) for all CCo genes pairs (nodes). CCos are color coded as in Figure 1A. CCo gene pairs with profile similarity PCC > 0.1 were connected and plotted using a spring-embedded layout algorithm in Cytoscape. Genes that have a similar GI profile are close to each other, and genes with less similar GI profiles are positioned farther apart. Subsequently, genes were annotated using the SAFE program (Baryshnikova, 2016), right panel, identifying network regions enriched for similar GO bioprocess terms.

(B–G) Regions of the CCo similarity network significantly enriched for genes with similar GI profiles are shown for *CCT* (B), *GIM* (C), *SEC*63 (D), *YDJ1* (E), *SSB2* (F), and *HSP104* (G). See also Figure S4.

The network provides insights into the functional specialization of the CCos. In general, CCos were found to have broad specificity and to interact with a large variety of substrates. Hence, many correlated interactors tended to belong to multiple cellular processes, and they showed no significant enrichment in any specific bioprocesses in the network. However, both the CCT and prefoldin chaperones exhibited significant specialization in their functions (Figures 2B and 2C). Many of their interactors in the CCo GI profile correlation similarity network were involved in cell polarity, morphogenesis, mitosis, chromosome segregation, glycosylation, protein folding, and cell wall biogenesis (Figures 2B and 2C). This specialization

might be due to the fact that CCT and prefoldins mainly function to fold and assemble actin and tubulin (Leroux and Hartl, 2000). Most other chaperones displayed either weak or no specialization (Figures 2D–2G; Figure S4).

The Hsp90 Network

The enrichment landscape of genes correlated with Hsp90 and its cofactors, including the R2TP (consisting of Rvb1, Rvb2, Tah1, and Pih1) complex (Kakihara and Houry, 2012), is shown in Figure 3A. Most of the Hsp90 CCos were dispersed throughout the GI profile correlation similarity network, and they typically fell outside the functionally enriched regions, except for *CPR7*, *CNS1*, *PIH1*, *PPT1*, and *RVB2* (Figure 3A).

To gain further insight into the Hsp90 family interactors, in Figure 3B we show the GI profile correlation similarity network consisting of a total of 1,303 genes interacting with Hsp90 and its cofactors. GO annotations for each interacting gene obtained from the enriched regions in Figure 3A were manually curated to a more specific biological term. The order of Hsp90





ALOJTW CTT4 JPS GJT3 SPT10 DR1 CTD1 OF11 RRNJ

MRS40M TEL I MPOI

A1425 AFG2 IR065C VIK1 SGM1 PHO92 L081W EG02

PLOSOC-A MTC1 LAMS YDL121C YER013C YGR013C GTT3

LOVEC-A EROTEC ANDE BIT2 ANS1 TOF2 MR209C RCN2 AMIT

UR247WA OM14 COSt YKR015C

RRRVD HTL1 RSC6 RSC8 RSC8 RSC8 RSC8 RSC8 RSC8 RSC8 SS082

Figure 3. The Hsp90 Family and R2TP Networks

DCR2 DAK1 MVP1 ADI1

(A) SAFE analysis of the Hsp90 and R2TP on the CCo GI profile correlation similarity network is shown with the annotated bioprocess regions obtained from Figure 2A. The Hsp90 family and R2TP members are overlaid.

(B) Shown are the GI profile correlation similarities (PCC > 0.1) of interactors for Hsp90 CCos and R2TP genes grouped according to manually curated bioprocesses from (A). Ontology terms were manually curated to reflect a more specific term. Edges are color coded based on the interactor's respective bioprocess. The sizes of the nodes of Hsp90, Hsp90 cofactors, and R2TP genes reflect the number of interactors. All CCo nodes are color coded as in Figure 1A.

cochaperones based on the number of interactors from most to least is as follows: CDC37 (291 interactors), CNS1 (250), CPR7 (223), RVB2 (172), PIH1 (131), HCH1 (106), CPR6 (83), AHA1 (67), RVB1 (66), TAH1 (61), STI1 (36), PPT1 (20), and SBA1 (19) (Figure 3B).

The R2TP complex was initially discovered by our group as an Hsp90-interacting protein complex in yeast (Zhao et al., 2005), and it was found to be required for the assembly of other critical complexes (Nano and Houry, 2013). Rvb1 and Rvb2 are highly conserved AAA+ ATPases in eukaryotes and are essential for cell viability. Despite advances in characterizing the R2TP complex, there is no clear understanding of its cellular functions. We found that members of R2TP have GI profile correlation similarities with genes involved in several bioprocesses (Figure 3B), but especially between RVB2 and genes encoding for cell polarity/ morphogenesis and mitosis/chromosome segregation/DNA replication and repair (50% of its interacting genes) and between PIH1 and ribosomal-related processes (44% of its interacting genes).

A High-Fidelity Physical Interaction CCo Network

To generate a network based on physical and GI data, proteins physically interacting with CCos were identified by collecting PPI pairs from several large-scale PPI screens, including ours (Gong et al., 2009), for all the CCos listed in Table S1. These studies comprised PPI data obtained by tandem-affinity purification (TAP) followed by mass spectrometry using large-scale approaches to characterize soluble multiprotein complexes



Figure 4. Building the CCo Physical Interaction Network

Schematic representation of the construction of the PPI CCo network. The first step is the compilation of CCo-containing interaction pairs (CCo-to-non-CCo or CCo-to-CCo interactions) from major studies shown as a Venn diagram. The total number of interaction pairs from each study is given in parentheses. The compiled PPIs were then scored and filtered using the indicated threshold as described in the Experimental Procedures (see also Figures S5D and S5E), resulting in 8,518 PPIs. Subsequently, Markov clustering was applied to define multiprotein complexes. The final PPI network is shown using the spring-embedded algorithm from Cytoscape. A zoom-in of the CCo supercomplex, the NAJ CCo complex, is shown with the size of each node corresponding to the degree of connectivity. All CCo nodes in the network are colored according to Figure 1A and non-CCo proteins are in gray.

(Gavin et al., 2002; Krogan et al., 2006), membrane protein interactors (Babu et al., 2012), and chaperone interactors in yeast (Gong et al., 2009), totaling 43,020 interactions (Figure 4).

The overlap between the complete GI (positive plus negative) dataset and the PPI dataset was significant ($p = 2.2 \times 10^{-16}$; Fisher's exact test), and this corresponded to about 5% of the PPIs and 9% of the GIs (positive plus negative). The highest number of interaction overlap between the 43,020 CCo PPIs and the CCo GIs was with negative GIs, followed by the GI profile correlation similarity network, and lastly positive GIs with 1,236, 795, and 774 common interactions, respectively (Figures S5A–S5C). We found that the CCos with the highest number of common interactions included the Hsp70s (Sse1/2, Ssb1/2, Ssz1, and Kar2), Hsp40s (Ydj1 and Swa2), and the Hsp90 (Hsc82) (Figures S5A–

S5C). Furthermore, in characterizing the physicochemical properties of CCo interactors, we found that the molecular weight and protein abundance of interactors in the PPI dataset were significantly greater compared to those of the GI datasets ($p = 1.2 \times 10^{-10}$ and 1.9×10^{-11} , respectively; Mann-Whitney-Wilcoxon test). This was likely due to a higher number of larger (>70 kDa) abundant proteins in the cell interacting with CCos. All other properties were similar across different datasets.

To select high-fidelity PPI interactions, several scoring methods were evaluated. The goal was to obtain the largest number of CCo-containing interaction pairs while maintaining approximately equivalent precision (selected as \geq 70%) against a reference set of PPIs derived from the CYC2014 catalog of manually curated protein complexes (Pu et al., 2009). We found



Figure 5. Overlay of the GI Profile Correlation Similarities onto MCL Clusters from PPIs

Shown is a nested network of the GI profile correlation similarities between CCo- (light blue) and non-CCo- (light gray) containing MCL clusters (complexes) described in Table S5. Positive and negative correlations are shown between complexes and only positive correlations are shown within complexes. CCos present in each complex are indicated in the bottom-right inset. Six complexes are shown in detail. The size of each node within the complexes corresponds to the number of interactors. For simplicity, complexes containing only two proteins are not shown.

Hart scoring (Hart et al., 2007) a better predictor of CCo interactions (Figures S5D and S5E). Using a Hart score threshold of 6, we obtained a finalized network containing 8,518 non-redundant pairwise associations (i.e., CCo-to-non-CCo, CCo-to-CCo, or non-CCo-to-non-CCo) (Table S4). Among these, we identified 328 associations involving a CCo with 56 of the 82 CCos captured (Figure 4; Figures S5D and S5E; Table S4). Markov clustering (MCL) (Enright et al., 2002) was then employed to organize the physical network. This resulted in a total of 370 complexes with 15 of them containing a CCo (Figure 4; Figures S5F–S5K; Table S5). Strikingly, most CCos were found to cluster together in a specific region of the proteome, specifically the families of Hsp90, Hsp70, Hsp40, AAA+, CCT, and sHsp (Figure 4; discussed further below). Such connectivity among six different CCo families has not been reported before. It should be emphasized that the MCL complexes obtained represent functional clusters and not necessarily tightly associating physical complexes.

A Combined Physical and Genetic CCo Network

Genes encoding physically interacting or co-complexed proteins tend to share many GIs in common. By overlaying interactions based on CCo GI profile correlation similarities onto the predicted protein MCL complexes obtained from the PPI network, we built a combined network highlighting CCo complexes (Figure 5). The complexes are connected based on the average inter-complex GI profile correlation similarities between the genes in each complex pair. Together, this allows the quantitative and nested representation of the GI profile correlation similarities between and within the predicted MCL complexes.

The network shown in Figure 5 displays 10 CCo-containing complexes with a direct connection to 86 non-CCo-containing

complexes. The Sec63-, Cdc37-kinase-ATP synthase-, R2TP-, Swa2-proteasome cap-, Gim1-6-, and Hsp90-Hsp70-Hsp40-AAA+-CCT-sHsp-containing complexes are highlighted in the figure. We named the Hsp90-Hsp70-Hsp40-AAA+-CCT-sHsp complex as the naturally joined (NAJ) CCo complex. The presence of such a chaperone supercomplex clearly suggests a high degree of interactor overlap and functional coordination or redundancy among these critical chaperones. The NAJ complex also highlights the tight cross-talk among the protein homeostasis machinery.

Additionally, we found that complexes that are functionally specialized tend to have more connectivity in this network than those that have a broader function (Figure 5). Such is the case when comparing the Sec63-containing complex with 47 connections or the Gim1-6-containing complex with 29 connections against the NAJ and the Cdc37-containing complexes with just one and two connections, respectively (Figure 5). As expected, the Sec63-containing complex is well connected with complexes involving various ER processes as well as with the proteasome core subunit-containing complex, which likely highlights the ER-associated degradation pathway. The Gim complex shows functional specialization by connecting with proteins involved in the cytoskeleton and transcriptional regulatory processes. On the other hand, the Cdc37-containing complex interactions are less specialized, involving diverse functions ranging from several kinases both at the inter- and intra-complex level. DNA repair proteins, and interactions with several mitochondrial complexes such as the ATP synthase (Figure 5). The NAJ complex has a single edge connecting it with actin assembly and motility. Given that this is a multi-CCo complex with a very broad involvement in numerous cellular pathways, this connection reflects the most common pathway between the NAJ CCo complex and the rest of the proteins.

As shown in Figure S3, the Hsp40 protein Swa2 was found to have a very strong enrichment for positive GIs with proteasomal genes. We also see such a strong association between Swa2 and the proteasome cap in Figure 5. SWA2 interacts with 13 subunits of the proteasome (*RPN1*,5-7,10-13 and *RPT1*-4,6) and the two proteasome assembly chaperones *NAS6* and *ECM29*. As well, this complex interacts with the proteasome core particle complex. Swa2 is a multifunctional protein involved in uncoating of clathrin-coated vesicles and in interacting with ubiquitin chains. Together, this suggests that Swa2 may assist in the assembly or regulation of the proteasome. The interaction between Swa2 and the proteasome has not been reported before in the literature.

The R2TP complex clusters with members of the histone exchange complex Swr1, histone acetyltransferase complex NuA4, and members of the chromatin remodeling complex Ino80. We also found that the only gene interacting with all three *RVB1*, *RVB2*, and *PIH1* is the histone exchange ATPase *SWR1* (Figure 5). Importantly, actin is a major interactor of this complex through the Rvbs by both GI profile correlation similarity and physical interactions.

The combined network in Figure 5 clearly illustrates the different degrees of specialization of CCos. On one end, we find that the Sec63- and Gim-containing complexes have the highest connections followed by the Swa2-proteasome cap-

containing complex, and, on the other end, we find the NAJ complex with minimal connectivity.

Rvb1/2 Form Condensates in Stressed Cells

To test the predictive value of our interaction data and given the role of chaperones in preventing protein aggregation and promoting protein folding, we examined CCos that colocalize with foci-forming proteins on the CCo GI profile correlation similarity network (Figure S6). We compiled a list of 547 proteins that have previously been shown either by low- or high-throughput studies to form foci (or condensates) under heat shock or nutrient limitation (Table S6) (Bolognesi et al., 2016; Narayanaswamy et al., 2009; O'Connell et al., 2014; Shah et al., 2014; Wallace et al., 2015).

As shown in Figure S6A, these foci-forming genes are enriched (p < 0.05) in regions containing many CCo families, such as the AAA+, CCTs, prefoldins, small heat shock, Hsp40s, and notably the Hsp90-R2TP system among others. By extracting the subset of foci-forming proteins that interact with the Hsp90-R2TP system, we found that these are highly enriched in genes involved in ribosome biogenesis and translational processes (Figure S6B). Furthermore, these interactions are largely driven by the CDC37, CNS1, and CPR7 cochaperones and RVB1, RVB2, and PIH1 components of the R2TP complex (Figure S6C). Hsp82, Hsc82, and some of the Hsp90 cochaperones have been previously reported to form foci (Figure S6C; Table S6). Hence, we investigated whether subunits of the R2TP complex also form foci. No foci formation was observed for Tah1 or Pih1, but we found Rvb1/2 to form condensates under nutrient limitation conditions.

Initially, we investigated growth at 30°C in nutrient-rich conditions (YPD). Rvb levels were constant as a function of cell growth (Figure S7A) and cell cycle (Figure S7B), with Rvb1 levels being higher than Rvb2. We then generated a strain, WH12, expressing endogenously tagged *RVB1*-mRFP and *RVB2*-GFP (see the Experimental Procedures) to monitor Rvb1 and Rvb2 localization throughout the cell cycle. Rvb1 and Rvb2 predominantly colocalized to the nucleus (Figure S7C) with a nuclear-to-cytoplasmic protein (N/C) ratio around 3 to 4 (Figure S7D). Hence, neither protein expression nor protein localization was significantly changed under the conditions tested.

Next, the effect of different stresses on Rvb localization was examined (Figure 6A). Under these stress conditions, Rvb1 and Rvb2 protein levels did not significantly change, and the two proteins remained predominantly colocalized with an N/C ratio of 3 to 4 (Figure S7E, left panel), similar to that of the N/C ratio in log phase cells (Figure S7D), with a colocalization Pearson correlation coefficient of 0.81 (Figure S7E, right panel). Cells treated with water for 1 hr were very heterogeneous, exhibiting various morphologies with some undergoing apoptosis, and, thus, these cells were not quantified for N/C ratios.

In carrying out the localization studies, we noticed that a proportion of Rvb1 and Rvb2 forms condensates near the nucleus under some of the stress conditions tested (indicated by white arrows in Figure 6A), namely, stationary phase (STAT), growth in media lacking glucose (–Glc), treatment with 2-deoxy-Dglucose (DG), and growth in water. It should be noted that Rvb1 has been reported to form aggregates in cells exposed



Figure 6. Condensate Formation by Rvb1/2

(A) Colocalization of Rvb1 and Rvb2 under various stress conditions. Cells of the WH12 strain were grown to log phase and then treated with various stress conditions as follows: mock treatment, 10% ethanol (EtOH), 900 mM NaCl (NaCl), heat shock at 37° C (37° C), stationary phase (STAT), minus glucose media (–Glc), 1 mM 2-deoxy-D-glucose (DG), minus nitrogen media (–N), 0.01% methyl methanosulfonate (MMS), 10 ng/mL rapamycin (Rap), and growth in water (H₂O). White arrowheads in STAT, –Glc, DG, and water treatment point to the Rvb condensates.

(B) Condensate formation by Rvb1 and Rvb2 upon glucose deprivation. Rvb1 and Rvb2 in WH12 strain after treatment with DG for 1 hr exhibit condensates near the nucleus. White arrow points to a condensate.

(C) Rvb1-FLAG in YK26 (*RVB1-FLAG*) strain treated with DG (upper panels) or mock treated (lower panels) was labeled with anti-FLAG primary antibodies and AlexaFluor488 secondary antibodies.

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to a high temperature of 46° C (Wallace et al., 2015), which was not a condition tested in our study.

Because the Rvb1 and Rvb2 condensates observed here appeared to be specific to glucose deprivation, we examined them in more detail (Figures 6B–6D; Movie S1). Figure S7F provides a statistical summary of the distribution of condensates in cells in stationary phase, –Glc, and DG conditions. Nearly 50% of all cells in each stress condition harbored condensates, of which most of the cells had one condensate per cell. Only 31% of cells contained condensates in the –Glc treatment, possibly due to the presence of other metabolizable nutrients in the rich media. Treatment with DG for 2 hr resulted in an increase in the number of cells harboring three condensates per cell.

We observed that condensate formation occurred for both Rvb1 and Rvb2 and that these condensates appeared in the cytoplasm, close to the nucleus (Figures 6A–6D). Figure 6B shows a closer view of Rvb1 and Rvb2 colocalized at the nucleus and in the perinuclear condensates (RFP/GFP panel). To ensure that condensate formation is not an artifact of GFP tagging, we performed indirect immunofluorescence labeling on the YK26 strain, expressing endogenous Rvb1-FLAG, in the presence of DG or mock treated (Figure 6C). Immunofluorescence labeling showed that Rvb1-FLAG also formed condensates close to the nucleus, indicating that condensate formation is not an artifact of endogenous GFP tagging.

We then explicitly determined that Rvb condensates resided in the cytoplasm using the WH4 strain. The strain expresses endogenous Rvb2-GFP and Nup49-mCherry, which is a component of the nuclear pore complex and can be used as a marker for the nuclear membrane. WH4 strain was treated with DG for 1 hr and subsequently imaged. Rvb condensates appeared on the cytoplasmic side of the nuclear membrane and localized very close to the nucleus (Figure 6D, left panel). However, treatment with DG did not affect Nup49-mCherry localization, and the distance of the condensate relative to the nuclear membrane did not change at 30 min. 1 hr. or 2 hr of DG treatment. Most of the condensates were 0.5 µm or less from the nuclear envelope (Figure 6D, right panel), and their formation depended on nuclear export by Crm1 (Figure S7G). Condensate formation was rapid and occurred within 3 min after DG addition (Figure 6E; Movie S1). These condensates were also rapidly reversible (within 10 min) upon the addition of glucose (Figure 6F; Figure S7H). The rapid nature of assembly and dissolution suggests that these condensates are not aggregates but biomolecular condensates (Rabouille and Alberti, 2017). We named these condensates Rvb bodies instantly triggered by starvation (Rbits).

Rbits did not colocalize with stress granules using Pbp1-GFP as a marker (Figure S7I) nor with P-bodies using Dhh1-mRFP as a marker (Figure S7J). Rbits also did not colocalize with protein aggregates marked by Von Hippel-Lindau factor (VHL) (Figure S7K). We also tested whether protein components in complexes containing Rvb1 and Rvb2 formed condensates that might be similar to Rbits under glucose deprivation conditions. The following proteins were assessed for condensate formation: Pih1, Nop58, and Bcd1, all of which are involved in box C/D small nucleolar ribonucleoprotein (snoRNP) assembly; Ino80, Taf14, and Nhp10, components of the Ino80 chromatin-remodeling complex; Vps71, component of the Swr1 chromatin-remodeling complex; Tti1, part of the ASTRA chromatin-remodeling complex and of the TTT complex; and Rpb1, Rpb2, Rpb3, Rpb7, Rpb8, and Rpb9 subunits of the RNA polymerase II machinery. None of these components appeared to form condensates resembling the Rbits under our tested conditions. Together these data suggest that Rvb1/2 form stress-inducible condensates independently of other proteins and stress-inducible compartments.

Condensate Formation by Rvb1/2 In Vitro Depends on pH, ATP Levels, and Crowding

To further investigate the properties of Rbits, we sought to reconstitute these condensates in vitro. Rvb1/2 complex was purified from E. coli and the proteins were labeled with a fluorophore. Yeast cells respond in many ways to stress: trehalose production is increased (Wiemken, 1990), ATP concentration drops (Weitzel et al., 1987), there is increased crowding (Mourão et al., 2014), and there is a drop in pH (Weitzel et al., 1987). Based on these observations, we tested how these parameters influence Rvb1/2 in vitro. Purified Rvb1/2 complex was diffuse in solution under neutral pH conditions (Figures 7A and 7B). However, at pH 6.0. Rvb1/2 formed round condensates of a size of about 0.1–0.3 µm. Rvb1/2 also formed condensates upon the addition of polyethylene glycol (PEG) of two different lengths to mimic a crowded cellular environment (Figures 7A and 7B). We quantified the amount of condensed material by comparing the fluorescence intensity of the condensates with the fluorescence intensity outside of the condensates. We found that condensate formation increased when using a longer PEG chain in comparison to a shorter one (Figures 7A and 7B).

It has been reported that some proteins are able to form liquid, viscoelastic, or solid compartments upon crowding (Patel et al., 2015). To probe the material properties of the Rbits, we applied fluorescence recovery after photobleaching (FRAP). When Rbits were photobleached, there was no significant fluorescence recovery in vitro (Figures 7C and 7D) or in vivo (using the WH12 strain described above; Figures 7E and 7F), suggesting that these condensates are solid.

We then analyzed whether the condensates dissolved when the triggering condition was reversed. For the pH-induced

⁽D) WH4 strain (*RVB2-GFP* and *NUP49-mCHERRY*) was grown to log phase and treated with DG for 1 hr. Rvb2-GFP forms condensates outside the nuclear membrane (upper panels) in comparison to mock treatment (lower panels) where no condensates are observed. Right panel shows the quantification of the distance of the condensates from the nuclear membrane of 1,410 condensates in 800 cells treated with DG for 30 min, presented as a percentage of condensates relative to total condensates measured.

⁽E) Percentage of cells of WH12 strain forming Rbits after the addition of DG (see also Movie S1).

⁽F) Average percentages of cells with Rbits are represented in a bar graph obtained from images shown in Figure S7H. Experiments were repeated 3 times. Error bars represent one SD.

See also Figures S6 and S7.



Figure 7. Characterization of Rbits

(A) Condensation of purified Rvb1/2 complex was monitored at different pHs or in 10% PEG of different lengths.

(B) Condensate formation was quantified by plotting the fluorescence intensity inside against the intensity outside the condensates.

(C) The material turnover of a Rvb1/2 condensate was analyzed by FRAP.

(D) Quantification of the FRAP results for a bleached (blue) and a non-bleached condensate (green).

(E) The material turnover of Rbits formed in energy-depleted yeast cells was analyzed by FRAP.

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condensates of purified Rvb1/2, pH was increased back from 6.0 to 7.5, and this caused the condensates to dissolve (Figures 7G and 7H). Similarly, reducing crowding of PEG-induced particles by dialysis resulted in the dissolution of the Rbits (Figures 7I and 7J). In agreement with a recent report that ATP dissolves condensates (Patel et al., 2017), the presence of ATP or ADP at 10 mM prevented condensate formation at low pH (Figures 7K and 7L). A titration experiment revealed that ADP was less potent than ATP in preventing condensate formation of Rvb1/2 (Figure 7M). Trehalose, which is strongly upregulated in response to stress, did not prevent condensate formation even at high concentrations (Figures 7K and 7L).

These results suggest that, in response to energy depletion (i.e., a drop in ATP concentration), a drop in pH and an increase in crowding could trigger Rvb1/2 condensate formation in cells. This in turn would indicate that a drop in the cytosolic pH alone, independent of a metabolic response, should not induce condensate formation. To test this, we used 2,4-dinitrophenol (DNP), a protonophore, which can be used to equilibrate the pH inside the cells with that outside (Munder et al., 2016). Using this approach on the WH12 yeast cells, we indeed found that Rbits did not form at pH 6 (Figure 7N) without energy depletion. This indicates that cytosolic acidification alone is not sufficient to trigger condensation but rather multiple triggers must coincide to promote Rbits formation.

DISCUSSION

In this work, we have provided a comprehensive global survey of the protein homeostasis network of the yeast cell using genetic and physical interaction data. This analysis offered an overview of chaperone connectivity that is consistent with the extensive biochemical data available from the literature (Figure 1D); it also allowed us to identify novel features of chaperone function.

The bias of many chaperones toward positive GIs with essential genes in comparison with the rest of the genes in yeast (Figures S2E and S2F) suggests an unexpected finding of chaperones preventing or reducing cell viability if an essential gene of the cell is affected. It is possible that this feature of chaperone function may underlie a novel mechanism by which chaperones maintain protein homeostasis in the cell. While counterintuitive, we propose that chaperones maintain protein homeostasis if nonessential genes are damaged but reduce cell viability if essential genes are affected. This observation might have direct implications for protein evolution. Our interaction network provided a clear distinction between chaperones that act on multiple substrates and impact many pathways versus those that have a more defined role in the cell (Figure 2; Figure S4). Most CCos are found to be proteins with multiple functions (pleiotropic) with only a few members being more specialized. In this regard, it was very striking to find that CCT and the prefoldin complex are rather specific chaperones and act on particular cellular pathways related to cell polarity and mitosis (Figures 2B and 2C). This specificity likely arises because such chaperones act predominantly on a select set of substrates, such as actin and tubulin, rather than on a multitude of proteins. Hence, these chaperones have been optimized through evolution to become critical players in specific cellular pathways.

The procedure we used to obtain a high-fidelity physical interaction dataset (Figure 4) revealed the presence of a huge chaperone supercomplex that we named the NAJ complex (Figures 4 and 5). This complex reveals that chaperones are physically and/or functionally linked in the cell. Chaperones are always in communication with each other even when present in different cellular compartments. The presence of such a supercomplex is likely essential for maintaining cellular protein homeostasis across different organelles.

Another intriguing finding, highlighting the predictive value of our network, is the observation that many proteins that have been reported to form condensates under different growth conditions are also interactors of many CCos. Subsequently, we confirmed that Rvb1 and Rvb2 form perinuclear condensates, which we named Rbits, in response to nutrient deprivation (Figures 6 and 7; Figure S7). Although the physiological role of Rbits is currently not clear, they could possibly act as protective storage depots for the Rvb1/2 proteins in response to nutrient stress.

In conclusion, our comprehensive analysis suggests that a global integrative network approach with physical and GI data is necessary to obtain a more thorough functional overview of such versatile and modular proteins as the chaperones and their cochaperones. This study reveals new findings on chaperone functions, and it serves as a resource for gaining a better understanding of the mechanisms that govern protein homeostasis in the cell.

EXPERIMENTAL PROCEDURES

Collection and Analysis of CCo Gls

Experimental approaches used to obtain high-throughput GI data for all chaperones and cochaperones have recently been described (Costanzo et al., 2016). GIs were extracted for the following: the nonessential × nonessential

(F) Quantification of the FRAP results for a bleached (red) and a non-bleached Rbits (green).

(G) pH-induced Rvb1/2 condensates could be dissolved by re-adjusting to neutral pH.

⁽H) Quantification of the data in (G).

⁽I) PEG-induced Rvb1/2 condensates could be dissolved by dialysis against buffer containing no PEG.

⁽J) Quantification of the data in (I).

⁽K) Effect of the pre-addition of ATP, ADP, or trehalose on Rvb1/2 condensation.

⁽L) Quantification of the data in (K).

⁽M) Titration of ATP and ADP reveals the critical concentrations needed for the inhibition of condensate formation. Note that the left panel was chosen to be the same as the representative panel labeled as w/o in (K).

⁽N) Rbits formation in DNP-treated yeast was monitored by adding energy depletion medium or phosphate buffer at varying pHs to the cells.

Distributions and errors shown in the plots of (B), (H), (J), (L), and (M) are derived from three images of three biological replicates.

(NxN); essential × nonessential (ExN); essential × essential (ExE); *DAmP* datasets for Cct8, Cns1, Cwc23, Jac1, Sis1, and Ssz1; and the Hsp90-TS dataset (Zhao et al., 2005). An intermediate threshold (p < 0.05 and |SGA score| > 0.08) was applied to the dataset. GI profile correlation similarities were obtained by computing PCC between all chaperone query-query and query-array pairs of strains in the nonessential, essential, *DAmP*, and TS GI datasets.

Analysis of Positive and Negative GI Ratios from CCos

To calculate positive GI enrichments, positive or negative GI densities of CCos against the essential (E) and nonessential (N) arrays were computed as follows:

Positive GI density(E or N) =

 $\frac{\text{positive density of gene } X(E \text{ or } N)}{\text{Average positive density for all query genes}(E \text{ or } N)}$

The enrichment ratio of positive-to-negative GI in each dataset (i.e., E or N) was calculated as follows:

 $\textit{Positive}:\textit{Negative}(\textit{E or N}) = \frac{\textit{Positive GI density}(\textit{E or N})}{\textit{Negative GI density}(\textit{E or N})}$

Positive GI bias was calculated as follows:

Positive GI bias(E or N) = $\frac{Positive : Negative for E}{Positive : Negative for N}$

GI density was calculated by selecting a single allele per array gene. The result was averaged across 100 different randomizations. GI densities of different query strains with mutations on the same gene were averaged to obtain a single value per query gene. GI data for pairs of genes belonging to the same protein complex, obtained by merging Table S1 and a recent list of protein complexes (Costanzo et al., 2016), were removed for this analysis.

Construction of the Yeast Chaperone PPI Network

We constructed the yeast chaperone PPI network by compiling four protein interaction datasets (Gavin et al., 2002; Krogan et al., 2006; Babu et al., 2012; ChaperoneDB from Gong et al., 2009). To obtain the high-fidelity network of PPI interactions given in Table S4, the mass spectrometry (MS) datasets that included MALDI Z scores or liquid chromatography-tandem mass spectrometry (LC-MS/MS) confidence values were pre-filtered at a MALDI Z score threshold ≥ 1 and/or an LC-MS/MS confidence score $\geq 70\%$. Next, an integrated interaction score was computed by summing the hypergeometric (HG) interaction scores (Hart et al., 2007) as Gavin_HG + Babu_HG × 0.5 + Krogan_ HG × 0.5 + Gong_HG × 0.08. The final network was derived by applying an integrated score of 6, selected to maximize chaperone interaction coverage while maintaining a precision \geq 70% against the CYC2014-derived reference set (Figures S5D and S5E). The resulting integrated PPI network comprises 8,518 interactions between 2,062 proteins (Figure 4; Table S4). From these interactions, 328 involved at least one CCo capturing a total of 56 of the 82 CCos used in this study.

We compiled a gold standard reference set of chaperone complexes by using the CYC2014 catalog (Pu et al., 2009) containing 443 manually curated non-redundant yeast protein complexes. Using chaperone precision and number of chaperones as a proxy, a Hart score of 6 was chosen as threshold (Figure 4; Figures S5D and S5E). Next, given that the densely connected regions of the PPI dataset suggest associated protein units likely having a similar function (Wodak et al., 2009), we used the MCL method (Enright et al., 2002) to identify these regions from within the CCo interaction network. High-confidence 8,518 PPIs were clustered over a range of Inflation (I) parameters (1.0 to 5.2). Cluster results were benchmarked based on overall cluster properties, for example, on number of clusters and average cluster size (Figures S5F and S5G), CYC2014 (Pu et al., 2009), complex coverage through precision and homogeneity metric (Pu et al., 2007) (Figures S5H and S5I), and intra- and inter-cluster functional diversity as measured by the Shannon index of GO biological process and molecular function terms (Figure S5J). Based on the minimization of intra-cluster average functional diversity and coverage of known

CYC2014 complex members, an inflation parameter of 1.8 (Figure S5J) was chosen to generate the finalized clustering of the filtered interaction network, corresponding to 327 clusters possessing 2 or more proteins (Figure S5K; Table S5).

Construction of the GI Profile Correlation Similarity Network Overlaid onto Predicted Complexes from PPIs

Genetic profile similarity scores for all CCo GIs were overlaid onto predicted MCL clusters. Positive and negative GI profile correlation scores were computed using PCC. In Figure 5, inter-complex edges represent the average GI profile correlation similarities between the interacting members from each complex, and the intra-complex edges represent the GI profile correlation similarities between genes within each complex. Proteins in the different MCL complexes are given in Table S5.

Other experimental procedures are provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, six tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.074.

AUTHOR CONTRIBUTIONS

W.A.H. designed the study. W.A.H. and M.B. supervised the experiments and data analyses. K.R. and A.K. collected and analyzed the data. J.H., H.A.Z., and Y.K. carried out the experiments related to foci formation in vivo. E.E.B. carried out the in vitro and in vivo experiments related to foci characterization with the supervision of S.A. T.V.S. helped with strain generation. Z.M. carried out the mass spectrometry. J.S. designed and implemented the MYTH experiments with the supervision of I.S. S.P. and J.V. contributed to the bioinformatic analyses. W.W., C.P., C.L.M., M.C., and C.B. contributed the GI experiments and helped with the analyses. W.A.H. and K.R. wrote the paper with help from M.B. and all other authors.

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