nature structural & molecular biology

Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies

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Folding within the crowded cellular milieu often requires assistance from molecular chaperones that prevent inappropriate interactions leading to aggregation and toxicity. The contribution of individual chaperones to folding the proteome remains elusive. Here we demonstrate that the eukaryotic chaperonin TRiC/CCT (TCP1-ring complex or chaperonin containing TCP1) has broad binding specificity *in vitro*, similar to the prokaryotic chaperonin GroEL. However, *in vivo*, TRiC substrate selection is not based solely on intrinsic determinants; instead, specificity is dictated by factors present during protein biogenesis. The identification of cellular substrates revealed that TRiC interacts with folding intermediates of a subset of structurally and functionally diverse polypeptides. Bioinformatics analysis revealed an enrichment in multidomain proteins and regions of β-strand propensity that are predicted to be slow folding and aggregation prone. Thus, TRiC may have evolved to protect complex protein topologies within its central cavity during biosynthesis and folding.

Eukaryotic cells contain several distinct chaperone families that together promote protein folding[1,2]. Misregulation of this process leads to misfolding and aggregation events that are linked to multiple pathological disorders[3,4]. It is thought that proteins differ widely in their chaperone requirements[2]. It is unclear, however, what features of a folding polypeptide, if any, determine its interaction with specific chaperones. Determining whether different chaperones evolved to meet the folding requirements of specific classes of substrates is central to understanding the logic of cellular protein folding and assembly. Addressing this possibility requires a better understanding of which types of proteins require a given chaperone. However, the cellular substrates of most eukaryotic chaperones have not yet been defined.

The essential chaperonin TRiC/CCT is distinguished from other chaperones by its unique ring-shaped architecture, which gives rise to a central cavity that serves as a folding chamber for substrate polypeptides[5,6]. It is not known why some proteins require the ring-shaped TRiC to fold whereas others can reach their native states with the assistance of simpler chaperone systems. Indeed, the cellular function of TRiC remains ill-defined and controversial. TRiC was originally proposed to be highly specialized to recognize a few cytoskeletal proteins through specific sequence elements[7]. However, the recent identification of additional TRiC substrates has called into question this original idea[8–12]. Here we have determined the principles of substrate selection by TRiC and defined the subset of cellular proteins that interact with this chaperonin in eukaryotic cells using a combination of experimental and computational analyses.

RESULTS

Principles of TRiC substrate selection

TRiC is part of a chaperone network linked to protein synthesis[13] and has been shown to facilitate folding of newly translated proteins *in vivo*[9,14]. Previous studies established that TRiC interacts transiently with a subset of cellular proteins during biogenesis[14]. We thus examined the flux of newly translated proteins through TRiC in mammalian cells using a previously established pulse-chase analysis technique, whereby newly made proteins are specifically labeled with ^{35}S-methionine during a brief pulse, and folding and maturation occurs during the chase period[14]. We isolated newly synthesized polypeptides interacting with TRiC using immunoprecipitation with antibodies against TRiC subunits β and ε (**Fig. 1a** and **Supplementary Fig. 1** online). Two-dimensional PAGE analysis showed that, soon after translation, many newly made proteins associated with TRiC. Following a period of chase, these proteins were dissociated, as expected for chaperone substrates, which should be released upon completion of folding (**Fig. 1a**, left and middle). We also observed chaperonin complex assembly during the time course of the chase, whereby the β and ε subunits associated with the remaining TRiC subunits[14] (**Fig. 1a**, middle). MS analysis of TRiC-interacting proteins

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Received 12 May; accepted 16 October; published online 16 November 2008; doi:10.1038/nsmb.1515

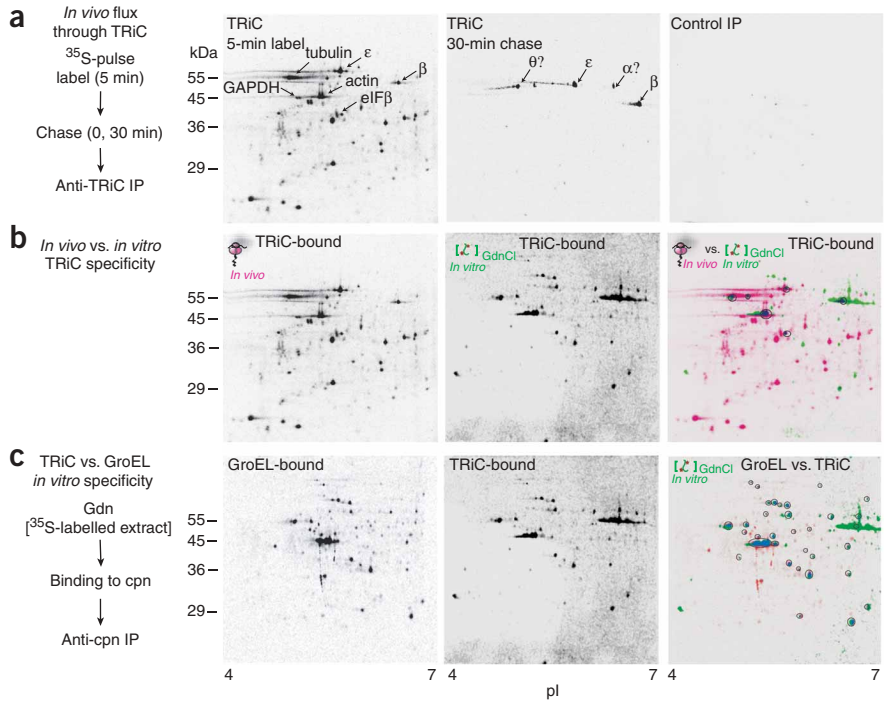

Figure 1 Principles of TRiC substrate selection in the eukaryotic cytosol. (**a**,**b**) Human fibroblast cells (TSA-201) were pulse labeled with ^{35}S-methionine for 5 min, followed by a 0 or 30 min chase. Total soluble protein was immunoprecipitated with anti-TRiC antibodies or a nonimmune antibody control, and the precipitates were separated on two-dimensional gels. (**b**) TRiC recognizes different proteins *in vitro* and *in vivo*. Two-dimensional gels of *in vitro* (green) and *in vivo* (magenta) TRiC-bound proteins were compared. The two gels were merged with overlapping spots circled and shown in blue (right). (**c**) A denatured ^{35}S-labeled cytosolic extract was diluted into buffer containing either GroEL or TRiC. Bound proteins were assessed by immunoprecipitation with anti-chaperonin (cpn) antibodies (EL or TRiC), and separated by two-dimensional gel electrophoresis. Gel images of GroEL-bound (red) and TRiC-bound (green) proteins were merged with overlapping spots circled and shown in blue (right).

identified only highly abundant substrates, namely the WD repeat–containing translation initiation factor-3β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in addition to the known TRiC substrates actin and tubulin (**Supplementary Table 1** online). Thus, identification of low-abundance cellular substrates of TRiC required alternative genome-wide approaches.

To better define the principles that govern TRiC substrate specificity, we next examined what determines association of cellular proteins with TRiC. In principle, chaperonin-substrate interactions may be solely determined by the presence of specific TRiC binding motifs in the substrates, such as sequence elements, that distinguish them from the rest of the proteome. A prediction of this model is that the *in vitro* substrate specificity of TRiC toward denatured cytosolic proteins will mirror that observed *in vivo*. Accordingly, we compared the subset of cellular proteins that bind TRiC upon translation *in vivo* (**Fig. 1b**, left) with those proteins binding TRiC when the same ^{35}S-labeled extract is denatured and presented to the chaperonin *in vitro* (**Fig. 1b**, middle). The sets of eukaryotic proteins interacting with TRiC *in vivo* and *in vitro* were markedly different. Whereas actin and tubulin were prominently bound in either condition, examination of the merged gels revealed less than 10% overlap between the proteins selected by TRiC *in vitro* and *in vivo* (**Fig. 1b**, right). We conclude that TRiC does not select its substrates based solely on the presence of specific sequence motifs, as was proposed from *in vitro* studies[15,16]. Instead, TRiC substrate selection in the cell is strongly dependent on the context of translation, where both co-translational folding events and cooperating chaperone systems may affect the conformation of *de novo* folding intermediates[11,17,18].

TRiC was proposed to be a highly specific chaperone, in contrast to the prokaryotic chaperonin GroEL, which shows broad specificity[7]. To further define the substrate-recognition principles of TRiC, we next determined whether TRiC recognizes a more restricted range of proteins when compared to GroEL[19]. To compare the cellular proteins recognized directly by TRiC and GroEL, we presented denatured ^{35}S-labeled cytosolic proteins to purified GroEL or TRiC and then immunoprecipitated the chaperonin-substrate complexes (**Fig. 1c** and **Supplementary Fig. 2** online). A large fraction of cellular proteins, including actin and tubulin, was recognized by both chaperonins. Comparison of the GroEL and TRiC-bound protein spectra revealed that the recognition specificities of these chaperonins were markedly similar, with more than 80% overlap between the protein sets recognized by either chaperonin (**Fig. 1c**, right). Given the well-established affinity of GroEL for hydrophobic substrate determinants, this result indicates that hydrophobicity is a strong component of TRiC substrate recognition, consistent with previous findings[8,9,20,21]. Notably, the similar binding specificity of both chaperonins suggests that TRiC retains the capacity to recognize a wide breadth of proteins and is not intrinsically a highly specific chaperonin. We conclude that TRiC possesses broad recognition specificities, yet in the cell it interacts with only a defined set of substrates.

Genomic screen for TRiC substrates during biosynthesis

Our finding that TRiC substrate selection in the cell is determined in the context of protein biosynthesis raises the question of what features distinguish proteins recognized by TRiC from the wide spectrum of potential interactors during biogenesis. Accordingly, we adapted a genome-wide approach that allowed us to identify physiologically relevant TRiC substrates by detecting chaperone interactions in the context of translation.

We used a screening approach that monitored which proteins interact with TRiC during translation of cDNA expression pools in a cell-free mammalian translation system[22]. This approach, termed small pool expression cloning (SPEC), allowed us to detect TRiC-substrate interactions in a physiologically relevant context (**Fig. 2a**). Notably, SPEC presents several unique advantages for the identification of physiological chaperone substrates. First, these translation lysates contain the full complement of chaperones and translation components required to fold most cytosolic proteins to an active state[23]. TRiC-substrate interactions are thus examined in the context of protein biosynthesis, whereby co-translational folding can occur in the presence of physiological levels of upstream chaperones and folding cofactors. Second, proteins are translated at low levels, in the picomolar

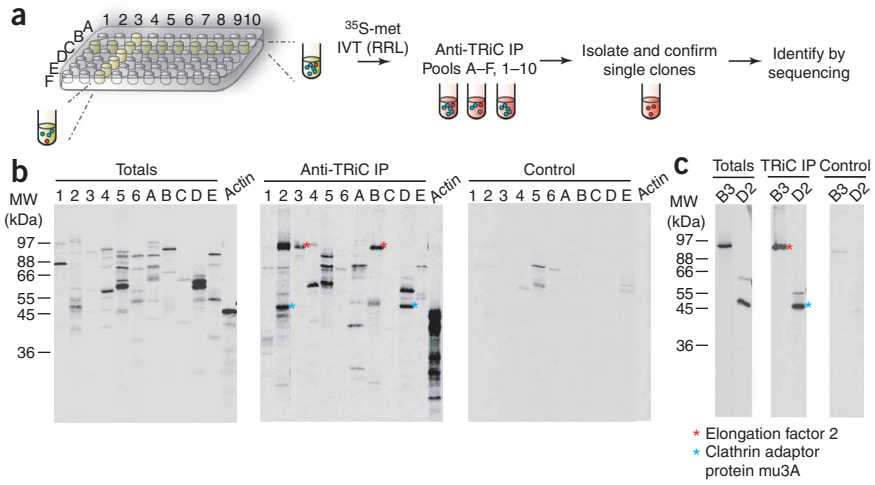

Figure 2 Screening for the TRiC interactome in the context of translation. (**a**) Small pool expression cloning screen. Bacterial cDNA clones were cultured in 96-well plates and pooled in rows and columns, from which plasmid DNA was purified. The orthogonal cDNA pools were expressed in rabbit reticulocyte lysate (RRL) in the presence of ^{35}S-methionine and immunoprecipitated with anti-TRiC antibodies. (**b**,**c**) Total expressed cDNA pools in RRL (Totals) and TRiC-bound proteins (anti-TRiC IP) were analyzed by SDS-PAGE and phosphorimaging (**b**). Immunoprecipitations with nonimmune antibodies demonstrated the specificity of the TRiC immunoprecipitations (Control). Actin expression and TRiC binding served as a positive control throughout. Red and blue asterisks indicate TRiC-interacting candidate clones present in orthogonal pools. Interactions were confirmed by expression of single candidate clones B3 and D2 in RRL, followed by anti-TRiC immunoprecipitation (**c**). cDNAs were identified by sequencing from upstream promoter sequences.

range, and thus interactions with the chaperonin can be observed without overexpression of the substrates[24]. Furthermore, the lack of bias toward detection of abundant proteins in the SPEC approach permits the identification of chaperone interactions for rare, low-abundance proteins, unlike large-scale immunoprecipitation reactions.

We carried out a high-throughput screen using single mouse cDNAs arrayed in a 96-well plate format. Orthogonal cDNA pools were made along rows and columns for each plate and analyzed by *in vitro* translation in rabbit reticulocyte lysate in the presence of ^{35}S-methionine (**Fig. 2a**). The presence of TRiC substrates encoded by cDNAs in the pools was detected by co-immunoprecipitation with the chaperonin followed by SDS-PAGE and phosphorimaging analysis (**Fig. 2b**). We isolated the cDNAs encoding candidate substrates expressed in two overlapping pools and confirmed their interaction with TRiC before using DNA sequencing to identify them (**Fig. 2c**). To simplify the screen, we subtracted the abundant housekeeping mRNAs from the cDNA library, thus increasing the proportion of lower-abundance transcripts surveyed[25]. Of note, the identification of α-tubulin among the TRiC-interacting substrates demonstrated that housekeeping proteins were still represented in the library, thus providing a good representation of transcripts in the cell (**Supplementary Table 1**).

In all, we surveyed 2,600 clones and isolated more than 100 TRiC-interacting proteins. The screen was not conducted to saturation but was concluded upon the repeated identification of several clones. From these data, we anticipate that 6–7% of all cytosolic proteins interact with TRiC. Notably, this analysis is in agreement with previous estimates from pulse-chase analysis[14] and proteomics studies[26]. Because only high-affinity interactions are detected by immunoprecipitation, the predicted number of TRiC-interacting proteins might be underestimated. Assuming that the eukaryotic cytosol contains approximately 4,500 proteins, based on estimates from the yeast *Saccharomyces cerevisiae*, our analysis predicts that on the order of 300 cytosolic proteins interact with TRiC *in vivo*.

Distinct *in vivo* kinetics of substrate flux through TRiC

Our screen revealed that a wide range of proteins interact with TRiC upon translation, most of which are conserved across eukaryotes (**Supplementary Table 1**). Accordingly, we used *S. cerevisiae* to validate the *in vivo* TRiC interactions, taking advantage of the extensive annotation of the yeast genome. Initially, we isolated candidates carrying an N-terminal GST-tag[27] by affinity purification and assessed their association with TRiC using immunoblot analysis. Notably, all of the yeast homologs of the SPEC-derived candidates surveyed bound to TRiC *in vivo*, but neither GST alone nor GST-tagged to Ypt6, a control GTP binding protein, associated with the chaperonin (**Fig. 3a**). This supports the idea that the screen identified bona fide TRiC substrates and that the interaction with TRiC is evolutionarily conserved across eukaryotic organisms.

One hallmark of chaperones that facilitate *de novo* folding is that they associate transiently with newly made proteins and dissociate during the course of polypeptide folding and maturation. As such, we examined the *in vivo* flux of SPEC-derived TRiC substrates through the chaperonin in yeast cells. Pulse-chase analysis followed by anti-TRiC immunoprecipitation indicated that, as in mammalian cells, a range of yeast proteins transiently associate with TRiC early in their biogenesis and dissociate during the chase, consistent with release upon folding (**Fig. 3b**, 15 min).

To observe the flux of single substrates through the chaperonin, we subjected TRiC immunoprecipitations to a second round of immunoprecipitation against a unique tag in the substrates (**Fig. 3c,d**). We observed transient association kinetics for the SPEC-identified substrates, indicating that these proteins transit through the chaperonin during their biogenesis (**Fig. 3d**). Notably, we observed two types of association kinetics among TRiC substrates. One class of proteins, including actin and the small purine nucleoside phosphorylase (PNP1), bound early to TRiC and rapidly dissociated (**Fig. 3d,e**). Elongation factor 2 (EFT2) also bound rapidly to TRiC, but dissociated slowly from the chaperonin (**Fig. 3d,e**). Another set of substrates showed much slower kinetics of association with TRiC. For instance, the mRNA export factor MEX67, the homotetrameric citrate synthase CIT2, the multidomain protein arginyl tRNA synthetase RRS1 (also known as ArgRS) and the WD40 G_β protein STE4 did not bind to TRiC at the earliest chase times, but instead their binding seemed to peak later in the time course (**Fig. 3d,e**). Notably, the TRiC-association kinetics were independent of substrate size because the larger EFT2 associated rapidly with TRiC whereas the smaller STE4 did not. It is tempting to speculate that the slower TRiC binding kinetics arises from the action of cooperating upstream chaperones delaying binding to TRiC during translation[13,18]. Notably, all the slower binding proteins share the common property of belonging to larger protein complexes, raising the possibility that these substrates accumulate on TRiC before their release into oligomeric assemblies. In

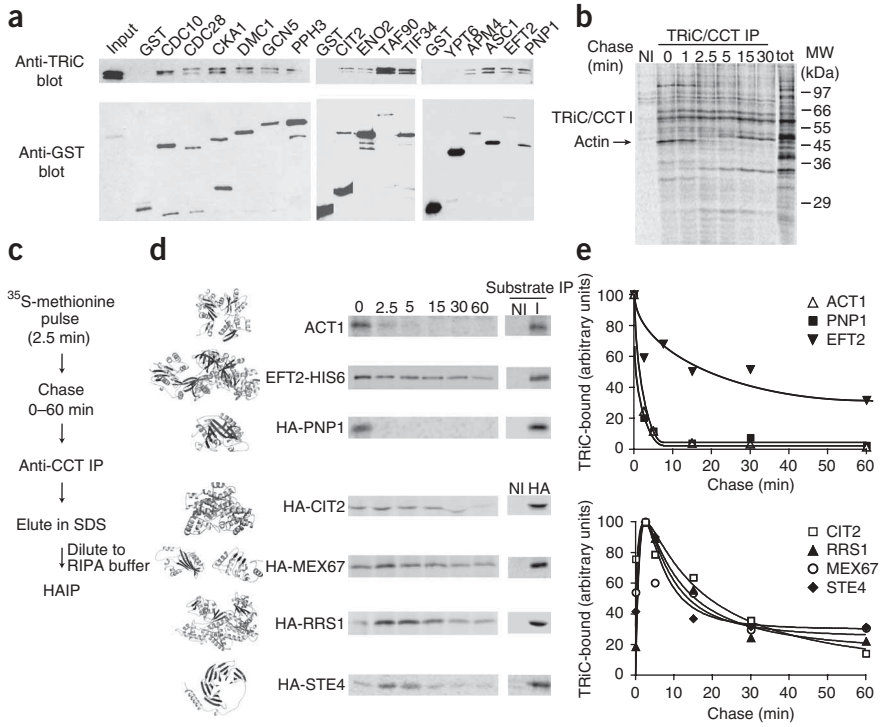

Figure 3 *In vivo* analysis of the TRiC interactome reveals distinct kinetics of substrate flux through the chaperonin. (**a**) GST fusion proteins were expressed in *S. cerevisiae* behind a galactose-inducible promoter and affinity purified with glutathione Sepharose. Purified proteins were separated by SDS-PAGE and subjected to western blot analysis with anti-TRiC or anti-GST antibodies. (**b**,**c**) The flux of newly synthesized proteins binding to TRiC was assessed by pulse-chase analysis in wild-type yeast. ^{35}S-labeled cells were harvested at the indicated time points, lysed and immunoprecipitated (IP) with anti-TRiC antibodies. NI, nonimmune; tot, total lysate. (**c**–**e**) The flux of individual proteins through TRiC was assessed by pulse-chase analysis of wild-type yeast expressing tagged substrates. TRiC-bound proteins from the pulse-chase analysis were eluted with SDS and subjected to a second immunoprecipitation with either nonimmune antibodies (NI) or antibodies recognizing tagged substrates (I). Structural models for each of the substrates were generated by multiple sequence alignment with homologous proteins using SWISS-MODEL[50]. TRiC-bound substrates isolated in the pulse-chase were quantitated with ImageQuant software (version 5.2, Amersham Biosciences) and the kinetics of their association plotted over time (**e**).

support of this idea, several WD40-containing F-box proteins translated in a cell-free system accumulated on TRiC in the absence but not in the presence of their oligomeric partner protein Skp1 (**Supplementary Fig. 3** online). We conclude that a genome-wide screen for the TRiC interactome identified bona fide TRiC substrates that flux through the chaperonin during biogenesis *in vivo*.

Physical and structural properties of the TRiC interactome

We next sought to define what distinguishes the relatively small fraction of TRiC-interacting proteins from the cytosolic proteome at large. Having identified a large set of proteins that interact with TRiC in a physiologically relevant context (herein referred to as the TRiC interactome; **Supplementary Table 1**), we applied a bioinformatics approach to identify common features among TRiC substrates that may underlie their physiological association with TRiC. Proteins from the interactome spanned a breadth of cellular processes, most of which are conserved across eukaryotic organisms. Notably, 40% of the TRiC interactome comprises essential genes, more than twice the proportion of essential genes in the yeast genome[28]. Of note, the frequency of TRiC substrates belonging to oligomeric protein complexes was also enriched in the TRiC interactome to 90%, compared to less than 50% of all yeast proteins in the MIPS complex database[29]. These two observations may be linked, as proteins that belong to oligomeric complexes are more highly connected in the protein network and, thus, are disproportionately essential[30]. We hypothesize that the eukaryotic chaperonin assists in folding proteins belonging to oligomeric assemblies and may serve as a reservoir to stabilize them against aggregation or degradation before complex formation.

Previous biochemical experiments using actin and tubulin led to the proposal that TRiC selects its substrates by specific recognition of a set of polar sequence elements[15,16]. Accordingly, we analyzed the TRiC interactome to search for the presence of specific sequence motifs that may confer TRiC binding. Sequence analysis of the TRiC interactome failed to reveal statistically significant consensus sequences, nor did we find an enrichment in previously proposed TRiC binding sequence elements[15,16]. This is consistent with the finding that TRiC is not a highly sequence-specific chaperonin (**Fig. 1c**).

In principle, physical and/or structural properties could influence protein folding characteristics and contribute to chaperonin binding. A comparison of the size distribution of TRiC-interacting proteins to the naturally occurring distribution of cytosolic proteins in the *S. cerevisiae* proteome (**Fig. 4a**) or the murine proteome (**Supplementary Fig. 4** online) demonstrated a statistically significant enrichment for proteins ranging in size between 40 kDa and 75 kDa, in good agreement with results obtained using pulse-chase approaches[14] (**Figs. 1** and **4a**). Of note, the chaperonin chamber is predicted to accommodate polypeptides in this size range[31]. As a single folding domain is approximately 25–30 kDa[1], this finding suggests that the TRiC interactome consists mostly of multidomain proteins. Notably, our screen also identified many larger substrates, raising the possibility that some substrates are not completely encapsulated during folding. In support of this idea, the 100-kDa protein myosin is known to be an obligate substrate of TRiC[32]. Furthermore, the bacterial chaperonin GroEL similarly supports the folding of several large proteins[33].

The proteins in the TRiC interactome showed an enrichment in hydrophobic sequences (**Fig. 4b**). This finding could be explained by substrate size, as larger proteins retain correspondingly larger hydrophobic cores. Alternatively, hydrophobic interdomain contacts within substrate proteins could contribute to the higher proportion of hydrophobic sequences. Inappropriate interactions among these hydrophobic surfaces, for instance, in domain swapping, could complicate the folding of multidomain proteins[34]. Previous observations that TRiC can recognize hydrophobic determinants[8,9,20,21] (**Fig. 1c**) suggest that the chaperonin may prevent these inappropriate interactions.

Analysis of the structural propensities of the TRiC interactome revealed the most striking property shared among TRiC substrates.

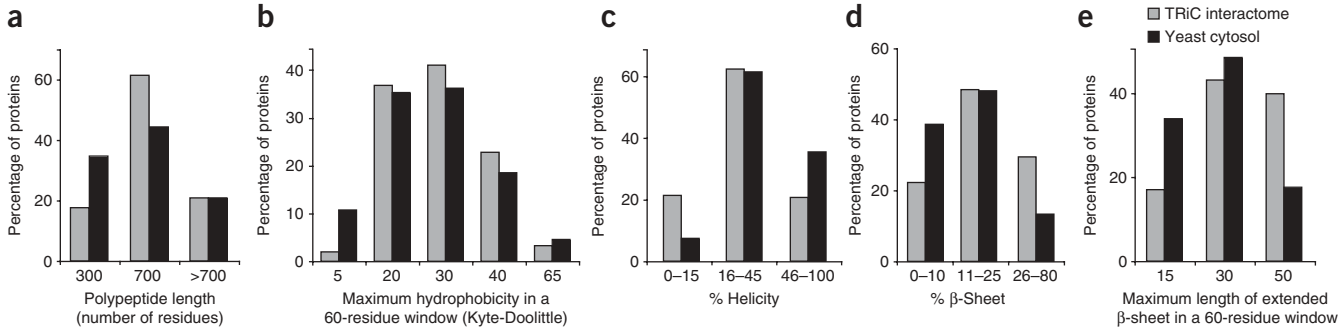

Figure 4 Physical and structural properties of the TRiC interactome. The prevalence of physical and structural properties of TRiC-interacting proteins was compared to their natural occurrence in the yeast cytosolic proteome. Protein size (number of residues) (**a**), maximal number of hydrophobic residues in a 60-residue window (**b**), percentage of helical content (**c**), percentage of β-sheet content (**d**), and maximal number of β-sheet residues in a 60-residue window (**e**) were considered. All differences between the TRiC interactome and the yeast cytosolic proteome were statistically significant, with P value $\leq 10^{-4}$, except for protein size, which had a P value of < 0.05.

Although no specific protein fold was enriched in the TRiC interactome, we observed that proteins of high β-sheet propensity and/or low α-helical content were highly enriched among TRiC substrates (**Fig. 4c**,**d**). Additional scanning-window analysis demonstrated that a high proportion of TRiC-interacting proteins contain long continuous stretches of β-sheet propensity, with a particular bias toward stretches of 35–45 amino acids (**Fig. 4e**). Thus, analysis of secondary-structure propensity identifies a clear commonality among proteins in the TRiC interactome. Notably, the enrichment in β-rich proteins suggests that TRiC substrates have complex topologies that are predicted to be slow folding and aggregation prone.

DISCUSSION

Here we combine genomic and proteomic approaches to identify the interactome of the eukaryotic chaperonin TRiC/CCT. Contrary to initial proposals that TRiC is specialized for the folding of only a small subset of eukaryotic proteins, we find that this chaperonin interacts with a broad range of polypeptides that function in many cellular processes (**Supplementary Table 1**). TRiC specificity is strongly influenced by the cellular context of translation, and a wide range of structural conformations are recognized by the chaperonin, indicating that TRiC functions to facilitate the folding of many structural protein families. A bioinformatics analysis of TRiC substrates demonstrated that the TRiC interactome is enriched in large, hydrophobic polypeptides with complex topologies, predicted to be slow folding and aggregation prone. Notably, the analysis also suggests that TRiC assists in folding subunits belonging to oligomeric protein complexes. These findings allow us to propose a model for TRiC function in the stabilization of slow-folding proteins susceptible to the formation of kinetically trapped intermediates and in the subsequent coordination of substrates into protein assemblies.

Cellular principles of TRiC substrate selection

Our studies resolve a long-standing controversy over TRiC substrate recognition and specificity. The data presented do not support the idea that TRiC selects its substrates in the cell solely on the basis of intrinsic determinants, such as specific sequence elements. Instead, TRiC recognition is strongly influenced by the context of protein biogenesis and may involve structural and physical features of the biosynthetic intermediates as well as cooperating chaperone systems (**Fig. 5**).

In vitro experiments comparing TRiC with the broad-spectrum chaperonin GroEL indicate that TRiC is not a highly specific chaperone. As these *in vitro* substrates differ substantially from the substrates binding to TRiC *in vivo*, this result implies that the TRiC-substrate interaction is dictated by the folding properties of the polypeptide in the context of translation. The substantial differences between the spectrum of cellular proteins that bind to TRiC during biosynthesis and those that TRiC selects when the same labeled proteins are presented in a denatured form highlights the importance of examining chaperone substrates in a physiologically relevant context. Furthermore, the similarities between the proteins bound by TRiC and GroEL *in vitro* suggest that the specificity of the eukaryotic chaperonin stems from the particular combination of folding kinetics and exposed recognition motifs in the co- or post-translationally generated intermediates of its cellular substrates. The lack of sequence specificity in the TRiC-substrate interaction is reinforced by the absence of sequence motifs in the *in vivo* subset that might confer TRiC binding propensity. Future studies should

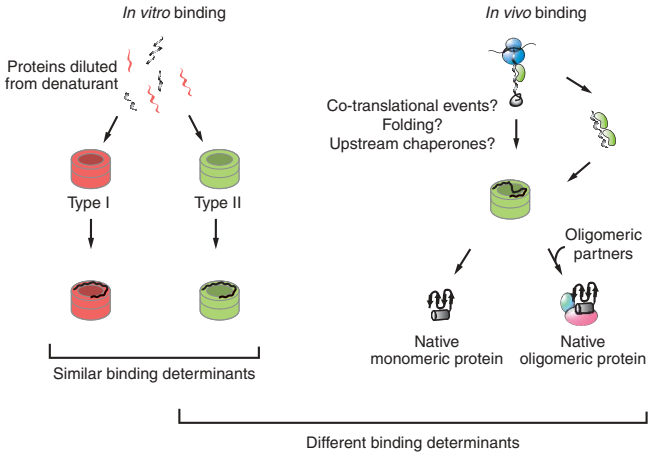

Figure 5 Function of TRiC during *de novo* protein folding. TRiC (type II) has a broad substrate binding specificity *in vitro*, similar to the type I chaperonin GroEL. However, *in vivo*, TRiC substrate selection is not based solely on intrinsic determinants, but rather specificity is dictated by factors present during protein biogenesis, such as co-translational folding or upstream chaperone systems. TRiC binds to a subset of newly synthesized polypeptides to stabilize exposed sequences during folding and prevent aggregation. The TRiC substrate set is enriched in proteins with slow-folding kinetics and aggregation propensity, such as β-domain–containing proteins. Folded substrates are finally released either in their monomeric state or in the context of oligomeric protein assemblies.

uncover the molecular determinants and the possible role of upstream cofactors in TRiC recognition.

Properties of TRiC substrates

We find that approximately 7% of the proteins we screened by genomic approaches associated with TRiC upon translation. This is consistent with our pulse-chase analysis, which indicates that approximately 5–10% of cytoplasmic proteins flux through the chaperonin. Analysis of the shared features of the interactome identified some common properties in these substrates that may illuminate the principles of substrate selection by TRiC. In particular, the enrichment of β-rich proteins among TRiC substrates provides a link between chaperonin interaction and one of the major challenges faced by the biosynthetic machinery that must facilitate cellular folding. Indeed, regions with high β-strand propensity are inherently aggregation prone and difficult to fold, such that evolutionary pressures have introduced elements of natural design to protect exposed β-edges in native protein structures[35]. Note that these design elements would not be in place until completion of folding. Thus, folding intermediates with regions of high β-sheet propensity are especially vulnerable to misfolding and aggregation. It is tempting to speculate that the chaperonin TRiC functions to stabilize these exposed β-edges against aggregation until loops or helices in the folded protein architecture emerge to protect the β-strand in the native state.

It is also possible that the enrichment in β-sheet propensity emerges from a more overarching characteristic of TRiC substrates. The observation that β-sheet propensity correlates with slow folding kinetics provides another intriguing link between our analysis of the TRiC interactome and folding in the cell. Recent studies find that complex protein topology, such as that found in β-rich proteins, is a major contributor to slow folding rates *in vitro*[36–38]. Of note, the TRiC interactome also contains all-helical proteins such as citrate synthase, raising the possibility that the enrichment in β-sheet content is diagnostic of a more general property recognized by TRiC, such as complex topology or slow folding kinetics. These features can lead to the accumulation of toxic aggregates, which have been linked to numerous amyloid diseases[4]. The idea that TRiC may have a role in preventing aggregation of amyloidogenic folding intermediates resonates with the recent identification of this chaperonin as a potent suppressor of amyloid formation by huntingtin, a polyglutamine protein that is highly aggregation prone[39–41].

Contribution of TRiC to the cellular folding network

Our data provide a clue on how the cell solves the problem of folding proteins with complex topologies, such as those with β-rich domains. During synthesis, these proteins are in a precarious position, as they must bring together many discontinuous regions to reach their native state and are thus susceptible to aggregation with neighboring proteins. Binding to the ring-shaped chaperonin TRiC would solve several problems posed by this process: first, the polyvalent binding of multiple substrate regions (for example, hydrophobic β-strands) to different subunits in a ring would sequester several aggregation-prone sequences during translation; second, the allosteric communication between subunits in one ring[42] would provide a mechanism for the concerted release of all folding elements to the central chaperonin chamber; and, finally, this chamber would provide a sequestered environment to protect slow-folding proteins until they reach the native state. Taken together, the combination of proteomic and bioinformatics analyses reported here reveal that TRiC substrates have complex topologies that are slow folding and aggregation prone, and provide a compelling rationale for the function of this oligomeric ring-shaped chaperonin in assisting cellular folding.

The additional finding that TRiC substrates are enriched in proteins belonging to oligomeric assemblies suggests that TRiC also functions to facilitate complex assembly. We envision two possible mechanisms for this role. In principle, the processes of TRiC-mediated folding and assembly could be directly coupled. Alternatively, TRiC could fold monomeric subunits and maintain them in an assembly-competent state until they associate with oligomeric partners. Thus, TRiC may contribute to the regulation of complex cellular processes such as signaling or cell-cycle regulation by maintaining a pool of assembly-competent but inactive protein molecules. Indeed, other chaperones such as Hsp90 have also been implicated in the regulation of signaling cascades by a similar mechanism[43].

Our data highlighting the contribution of TRiC to folding proteins involved in a wide variety of cellular functions is consistent with genetic data that link TRiC/CCT to many different cellular processes[44]. It will be interesting to examine how TRiC cooperates with other components of the cellular folding machinery. Although the few TRiC substrates characterized to date, such as actin, tubulin, Cdc20 and Cdh1 (refs. 9,45,46), show an obligate requirement for the chaperonin, it is possible that other TRiC-interacting proteins can be folded by alternative chaperone pathways. Indeed, an *in vitro* analysis of GroEL-interacting proteins revealed that only a small proportion have an absolute requirement for GroEL to fold, whereas most GroEL substrates can fold spontaneously or use alternative chaperones[47]. It is likely that TRiC substrates fall into similar categories. Future studies should determine whether some TRiC substrates can fold via alternative chaperone systems. However, even for the well-studied chaperonin GroEL, comparison of *in vitro* and *in vivo* analyses illustrates the complexity of chaperone pathways in the cell. Thus, several GroEL-interacting proteins that can fold spontaneously *in vitro* nonetheless aggregate *in vivo* upon GroEL impairment[33,47]. Clearly, much remains to be learned about the function of chaperonins and how they cooperate with other chaperones in the cellular folding network.

METHODS

Tissue culture and pulse-chase analysis of mammalian proteins. Human fibroblast TSA-201 cells used for pulse-chase analysis were cultured in complete medium at 37 °C (DMEM with 10% (v/v) FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine). Cells were starved for 15 min in starvation media (DMEM minus methionine and cysteine, supplemented with 5% (v/v) dialyzed FBS to remove amino acids; Sigma, 10 kDa MWCO), before they were labeled with 0.8 mCi ml^{-1} ^{35}S-methionine for 5 min, and then chased for 30 min in chase media (complete medium supplemented with 0.4% (w/v) cysteine and methionine). We then harvested the cells in ATP depletion buffer (ice-cold PBS with 1 mM azide, 5 mM EDTA, and 2 mM deoxyglucose), lysed them by dounce homogenization in buffer A (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, 5% (v/v) glycerol), and clarified the lysate by centrifugation. Equivalent amounts of protein were then immunoprecipitated with 2 μl anti–TCP1 β antibodies as described[14]. Briefly, lysates were incubated with antibodies for 40 min, rotated with 10 μl Protein G Sepharose for another 40 min, and immunoprecipitates were washed in TBS plus Tween20 buffer as described[14].

Two-dimensional gel analysis and mass spectrometry. Briefly, we labeled TSA-201 cells with ^{35}S-methionine for 5 min, followed by a 0 or 30 min chase in chase media as described above. Cells lysates were immunoprecipitated with anti–TCP1 β antibodies, and immunoprecipitates were resuspended in 250 μl IEF rehydration buffer (8M urea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer, pH range 4–7, 2.8 mg ml^{-1} DTT, trace bromophenol blue) with 400 μg unlabeled lysate (~15 mg ml^{-1}), before being separated on 13-cm Immobiline DryStrips, pH range 4–7 (Pharmacia Biotech), according to the manufacturer's instructions. The proteins were separated in the second dimension by 10% SDS-PAGE,

and the gels were silver stained and analyzed by phosphorimaging. We superposed the silver-stained and radiolabeled gel images to identify proteins to be analyzed further by MS. Protein spots were excised from a keratin-free gel run in a parallel sample and digested with 12.5 ng μl^{-1} trypsin at 37 °C for a period of 4 h up to overnight, and identified by LC/MS as described[48]. Data analysis was performed with the algorithm Protein Prospector (http://prospector.ucsf.edu/).

Specificity of *in vivo* versus *in vitro* binding. TSA-201 cells were labeled overnight with 10 μCi ml^{-1} ^{35}S-methionine in starvation medium supplemented with 1% (v/v) complete medium. To generate a cytosolic protein extract, the cells were harvested, lysed, and then denatured with 6 M guanidinium chloride. We assessed *in vitro* binding to the chaperonin by diluting the denatured extract (~5 mg ml^{-1}) by 100 times into 200 μl buffer B (25 mM HEPES, pH 7.4, 100 mM KCl, 5 mM EDTA, 1 mM 2-deoxyglucose, 1mM NaN_3) containing either the chaperonin GroEL (0.1 μM) or TRiC (0.25 μM). Samples were incubated for 20 min at 30 °C, followed by 20 min on ice, and then clarified by ultracentrifugation for 25 min at 20,000*g*. Bound proteins were then co-immunoprecipitated with anti-chaperonin antibodies (5 μl rabbit anti-GroEL or 2 μl rabbit anti–TCP1 β antibodies), separated simultaneously by two-dimensional gel electrophoresis, and then visualized by autoradiography.

To compare proteins bound to GroEL and TRiC, gel images were differentially colorized in Adobe Photoshop and overlaid. We carried out minor rescaling of images using actin and tubulin spots as points of reference.

Small pool expression cloning. We identified TRiC-interacting proteins by screening for TRiC coimmunoprecipitation of expressed cDNAs from a subtracted murine library. The cDNA library was generated from mRNAs that were differentially expressed in G1-S and G2-M C2C12 cells as described[25]. cDNAs were inserted into the pCS+ vector behind the SP6 promoter and transformed into bacteria. Bacterial cDNA clones were grown in a 16 × 12 array and then pooled by rows and columns, from which plasmid DNA were purified (Qiagen miniprep). cDNA pools (~250 ng) were expressed *in vitro* in a rabbit reticulocyte lysate–coupled transcription-translation system (8 μl, Promega TNT Quick Coupled Transcription/Translation Systems) in the presence of ^{35}S-methionine (0.75 μCi μl^{-1}) at 30 °C for 45 min. Translations were stopped by incubation with 10 mM EDTA, 50 μg ml^{-1} RNase A and 5 mg ml^{-1} methionine for 10 min on ice before they were immmunoprecipitated with anti–TCP1 β antibodies. TRiC-substrate complexes were separated by 12% SDS-PAGE and analyzed by phosphorimaging. Proteins of similar size present in two orthogonal pools were selected as candidate clones. Then, the interactions of individual expressed cDNAs were confirmed by *in vitro* expression followed by anti-TRiC immunoprecipitation. Confirmed clones were finally identified by SP6-primed sequencing and BLAST analysis. In total, we screened 2,600 clones, from which approximately 1,500 clones were estimated to have an insert generating a detectable translation product.

***In vivo* validation of substrates—Interaction of GST-fusion proteins.** N-terminal GST fusion proteins were expressed in yeast (strain Y258) behind a galactose-inducible promoter from the pEGH expression vector as described[27]. Cells were grown to log phase in raffinose synthetic media minus uracil before protein expression was induced with 2% (w/v) galactose for 3 h. Cells were harvested and lysed by bead beating for 10 min at 4 °C in buffer C (25 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 0.1% (v/v) TritonX-100) with 20 mM EDTA. Extracts were clarified and equivalent protein amounts were incubated with glutathione Sepharose for 1 h before washing four times with TBS 0.1% (v/v) Tween20. Purified complexes were separated by 12% SDS-PAGE and immunoblotted with anti-GST mAb (Covance, clone 4C10) or rabbit affinity-purified CCT antibodies raised against the apical domains of yeast CCT3, 5, 6 and 8.

Yeast pulse-chase analysis. Wild-type cells (strain BY4743) expressing plasmid-borne copies of hemagglutinin-tagged substrates were grown to log phase, induced via a copper-inducible promoter with 200 μM $CuSO_4$ for 30 min, and starved for 30 min in complete synthetic medium without methionine. The cells were then labeled with 100 μCi ml^{-1} ^{35}S-methionine for 2.5 min and chased with 20 mM cold methionine. At the indicated time points, aliquots were quickly chilled and harvested in 250 mM cold azide to deplete the cells of ATP and 0.5 mg ml^{-1} cycloheximide to stop protein synthesis. Lysates prepared in buffer C were clarified and TRiC-substrate complexes isolated by immunoprecipitation using 4 μl CCT-specific antibodies. CCT-interacting proteins were eluted with 1% (w/v) SDS and 5 mM DTT for 15 min at 30 °C and then diluted to the final concentration of RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) DOCA, 0.1% (w/v) SDS). Hemagglutinin-tagged substrates were re-immunoprecipitated from the eluate with 2 μl anti-hemagglutinin antibodies (clone HA.11, Babco), separated by 12% SDS-PAGE and analyzed by phosphorimaging. Isolated protein substrates were quantitated with ImageQuant software (version 5.2, Amersham Biosciences) and plotted over the time course of the chase. All substrates were N-terminally hemagglutinin tagged and cloned into the pCu426 vector under the control of a copper-inducible promoter[49], with the exception of EFT2, which was C-terminally His_6 tagged. Protein structures were retrieved from the Protein Data Bank or modeled against homologous structures by multiple sequence alignment via SWISS-MODEL[50].

Bioinformatic characterization of the TRiC interactome. We compiled a comprehensive list of TRiC-interacting proteins, including proteins identified in this study as well as other reports[9,11,12,19,32,45,46,51–55]. TRiC-interacting proteins identified in large-scale proteomics studies were also included if they bound to three or more TRiC subunits[26]. Trends found among the TRiC-interacting proteins were compared with the distribution of cytosolic proteins occurring in the yeast proteome (**Fig. 4**) or the murine proteome (**Supplementary Fig. 3**). The cytosolic protein class was estimated by removing proteins with one or more transmembrane helices from the proteome, using the prediction server TMHMM[56], resulting in a reduction of the proteome by approximately 25%, in agreement with previous predictions[57].

We predicted physical and structural properties of proteins using computer algorithms: the Kyte-Doolittle scale was used to measure hydrophobicity[58] and PSIPRED was used to determine secondary structure[59]. Scanning-window analysis determined the maximal number of hydrophobic (or β-strand) residues within a 60-residue window for each protein. *P*-values were calculated by χ^2 analysis. *P*-values calculated using the unequal variance *t*-test[60] also confirmed significant differences in the means of the distributions. Functional properties of proteins such as essentiality and complex formation were also determined. Data on protein essentiality was downloaded from the *Saccharomyces* Genome Database (http://www.yeastgenome.org). A list of yeast complexes containing at least two non-TRiC protein members was obtained from the MIPS database[29].

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

The authors thank members of the Frydman laboratory and R. Andino for comments and discussion. The cDNA library was a generous gift from W. Zhao and G. Fang (Department of Biology, Stanford University). We thank W. Harper (Department of Pathology, Harvard Medical School), T. Kinzy (Department of Molecular Genetics & Microbiology, Rutgers University), and Michael Rexach (Department of Biological Sciences, Stanford University) for gifts of plasmids and antibodies. This work was supported by grants from the US National Institutes of Health and the W.M. Keck Foundation.

AUTHOR CONTRIBUTIONS

J.F. directed the project; A.Y.Y. and J.F. designed the project, analyzed the data and wrote the manuscript; A.Y.Y. carried out all experiments; H.-T.J.L. and A.B. carried out MS analysis; and Y.X. and M.G. carried out bioinformatics data analysis and contributed to the manuscript.

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