

# The interaction network of the chaperonin CCT

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**The eukaryotic cytosolic chaperonin containing TCP-1 (CCT) has an important function in maintaining cellular homeostasis by assisting the folding of many proteins, including the cytoskeletal components actin and tubulin. Yet the nature of the proteins and cellular pathways dependent on CCT function has not been established globally. Here, we use proteomic and genomic approaches to define CCT interaction networks involving 136 proteins/genes that include links to the nuclear pore complex, chromatin remodelling, and protein degradation. Our study also identifies a third eukaryotic cytoskeletal system connected with CCT: the septin ring complex, which is essential for cytokinesis. CCT interactions with septins are ATP dependent, and disrupting the function of the chaperonin in yeast leads to loss of CCT-septin interaction and aberrant septin ring assembly. Our results therefore provide a rich framework for understanding the function of CCT in several essential cellular processes, including epigenetics and cell division.**

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## Introduction

The chaperonin of the eukaryotic cytosol, chaperonin containing TCP-1 (CCT) (also known as TRiC (TCP-1 ring complex)) was originally discovered and characterised because of

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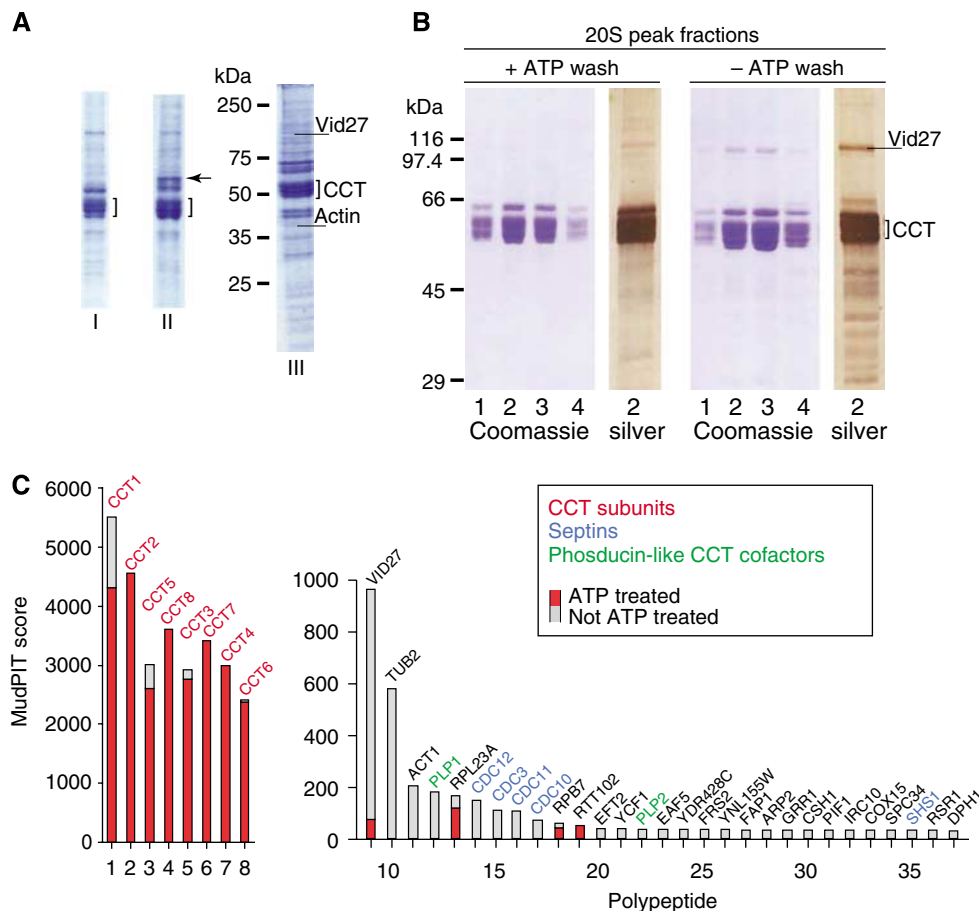
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its essential role in folding the cytoskeletal proteins actin and tubulin (reviewed in Frydman, 2001; Willison and Grantham, 2001; Cowan and Lewis, 2002). Similar to all chaperonins, CCT has a double-toroid shape with a central cavity and can, upon binding and hydrolysis of ATP, assist in the folding of non-native proteins or in the assembly of substrates with their respective partners.

CCT has a critical function in cellular protein folding. On the basis of immunoprecipitation experiments, a maximum estimate of 9–15% of all newly synthesised cytosolic proteins transit through CCT and thus may require its assistance for folding and/or assembly (Thulasiraman *et al*, 1999), although our own previous *in vivo* pulse-chase experiments identified fewer transiting polypeptides (~1% of total; Sternlicht *et al*, 1993; Grantham *et al*, 2006). Only a relatively small number of proteins (~30) have been shown to depend on CCT for their biogenesis; these include the cytoskeletal proteins  $\alpha$ - and  $\beta$ -actin, an actin-related protein (centractin),  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tubulin, the von Hippel–Lindau tumour suppressor, histone deacetylases (HDAC3, Set3p, and Hos2p), and cell cycle regulators (Cdc20p and Cdc55p) (Spiess *et al*, 2004). Although some CCT substrates possess seven-bladed  $\beta$ -propeller (WD40 repeat-containing) folds and have a high affinity for CCT (Valpuesta *et al*, 2002; Camasses *et al*, 2003; Passmore *et al*, 2003; Spiess *et al*, 2004; Kubota *et al*, 2006), most CCT substrates are highly diverse with respect to their functions and structures, and cannot be inferred as such based on homology alone (Valpuesta *et al*, 2005). Also of note is that some CCT-interacting proteins may not represent substrates, but instead, are cofactors or cochaperones of the chaperonin. For example, several phosphatase-like proteins interact with CCT and modulate its abilities to fold actin, tubulin, G $\beta$  and perhaps other substrates (McLaughlin *et al*, 2002; Lukov *et al*, 2006; Stirling *et al*, 2006, 2007). Another important CCT cofactor is prefoldin, a hetero-hexameric chaperone that stabilises newly made forms of actin and tubulin and transiently interacts with the chaperonin to assist in their effective delivery to the folding cavity (Geissler *et al*, 1998; Vainberg *et al*, 1998).

Previously, many putative CCT-interacting proteins were uncovered in high-throughput protein interaction screens in yeast (Gavin *et al*, 2002, 2006; Ho *et al*, 2002; Graumann *et al*, 2004; Krogan *et al*, 2006). However, for the great majority of these proteins, it remains unverified whether they are *bona fide* CCT substrates, cofactors or cochaperones. In addition, a significant number of proteins that interact with CCT may not have been identified in co-precipitation studies because of the poor accessibility of N- or C-terminal epitope tags, both of which are buried in the central cavity of the chaperonin complex (Pappenberger *et al*, 2006). Another limitation has been that synthetic-sick or -lethal genetic interaction screens have not been performed on any of the eight CCT subunit genes because they are essential for viability.

In an attempt to identify a comprehensive CCT ‘interactome’, we have taken a two-pronged approach using *Saccharomyces cerevisiae*. We used strains containing CCT



**Figure 1** Isolation of CCT substrate complexes. **(A)** Affinity purification of CCT-3CBP and CCT-6CBP on calmodulin resin. CaM-resin-purified CCT was analysed by 10% SDS-PAGE. (Lane I) CCT-3CBP complex: the eight CCT subunits migrating around the 60 kDa marker are indicated by the square bracket. (Lane II) CCT-6CBP complex: subunit Cct6p migrating at 70 kDa as a consequence of the inserted tag is indicated by an arrow. (Lane III) CCT-6CBP complex as used for in-house MALDI-MS and PMF-MS analysis. Molecular weight markers in kilodaltons are indicated on the left, Vid27p and actin are indicated as a reference (see also Supplementary Table S1). **(B)** Purification of CCT-3CBP in the presence and absence of ATP. CCT was incubated  $\pm$  5 mM ATP at room temperature during the CaM affinity purification procedure. EDTA eluates of CCT were applied to 10–40% sucrose gradients. The panels +ATP wash and –ATP wash show four adjacent sucrose gradient fractions from each 20S CCT peak electrophoresed on 10% SDS gels followed by Coomassie (fractions 1–4) or silver staining (fraction 2 from each peak). **(C)** Histograms of the CCT-3CBP-bound polypeptides plotted against their MudPIT score (Tables I). Comparisons between the ATP-treated (red bars) and untreated CCT samples (gray bars) show the proteins that are removed by 5 mM ATP treatment during the CaM chromatography step.

complexes with an exposed CBP affinity tag to isolate functional CCT complexes and identify bound proteins and we examined the genetic interaction spectrum of CCT by performing a synthetic genetic array (SGA) screen for synthetic lethal interactions with a temperature-sensitive *cct* allele. Using these integrative approaches, we identified a large number of novel CCT physical and genetic interactors that define several functional interaction networks. We have validated *in vivo* the interactions between CCT and septins, a family of GTP-binding filament-forming proteins, and shown that CCT is required for proper septin ring formation. Our data are the first to link globally CCT to specific protein complexes and cellular processes, defining its position in diverse biological networks.

## Results

### Purification of yeast CCT and identification of bound proteins

Previously, we showed that incorporation of a CBP tag in an exposed region of the CCT complex allows for its effective

purification using calmodulin resin chromatography (Pappenberger *et al*, 2006). To discover novel CCT-interacting proteins from *S. cerevisiae*, we developed a stringent purification protocol utilising two different versions of tagged CCT, one bearing the CBP tag in the Cct3p subunit (CCT-3CBP; Pappenberger *et al*, 2006) and another in the Cct6p subunit (CCT-6CBP; see Materials and methods).

The presence of the tag in the Cct3p subunit does not interfere with CCT function *in vivo* or *in vitro* (Pappenberger *et al*, 2006) and CCT-6CBP in haploid yeast is also viable and folding-competent *in vitro* (data not shown). A conventional 1D SDS gel protein separation and band-excision protocol, as shown for a CCT-6CBP sample in Figure 1A, identified CCT subunits, Hsc70 family members, known substrates such as Act1p, and many other proteins (Supplementary Table S1). Indeed, several proteins were recovered by both Cct3p- and Cct6p-tagged complexes, including actin, as expected, and three of five core septin subunits, proteins not previously known to interact with CCT.

To discover new CCT substrates—those proteins that need CCT for either folding or assembly—we used ATP-dependent

**Table I** CCT–3CBP interacting proteins in the absence of ATP, identified by LC-ESI-MS

Gene name	Score	Mass (Da)	Polypeptide	Copies per cell	Synthesis rate
<i>TCP1/CCT1</i>	5511	60 899	<i>Chaperonin subunit</i>	ND	0.41
<i>CCT2</i>	4409	57 510	<i>Chaperonin subunit</i>	ND	0.38
<i>CCT5</i>	3010	61 152	<i>Chaperonin subunit</i>	ND	0.81
<i>CCT8</i>	2976	61 965	<i>Chaperonin subunit</i>	2200	0.34
<i>CCT3</i>	2929	59 404	<i>Chaperonin subunit</i>	ND	0.48
<i>CCT7</i>	2924	60 211	<i>Chaperonin subunit</i>	ND	ND
<i>CCT4</i>	2554	57 967	<i>Chaperonin subunit</i>	5530	0.34
<i>CCT6</i>	2413	60 171	<i>Chaperonin subunit</i>	ND	0.42
VID27	955	89 190	WD40 (yeast/plants)	3500	0.16
TUB2	574	51 233	$\beta$ -Tubulin	ND	0.40
ACT1	201	41 906	Actin	215 000	2.30
PLP1	180	26 834	Phosducin-like protein 1	2250	0.15
RPL23A	165	14 578	60S ribosomal subunit	ND	3.39
CDC12	147	46 753	Septin	1170	0.23
CDC3	108	60 188	Septin	ND	0.22
CDC11	105	47 790	Septin	9280	0.16
CDC10	70	37 059	Septin	14 100	0.26
RPB7	58	24 330	RNA pol II subunit 16	6420	ND
RTT102	43	21 177	SWI/SNF and RSC complex	1550	0.16
EFT2	39	93 660	Diphthamide/His target	160 782	2.37
YCF1	38	172 196	ABC transporter (cadmium)	396	0.05
PLP2	36	32 887	Phosducin-like protein 2	7700	0.22
EAF5	36	32 887	NuA4 acetyltransferase	937	ND
FRS2	35	57 511	Phenylalanyl-tRNA synthetase a	ND	0.30
YDR428C	35	31 565	Serine hydrolase	ND	ND
YNL155W	34	31 953	AN1-type zinc-finger	4280	ND
FAP1	33	113 668	Transcription/binds FKBP12	589	ND
ARP2	33	44 217	Arp2/3 actin nucleation	6650	0.35
GRR1	33	133 848	F-box SCF E3 ligase	ND	0.03
CSH1	33	44 639	MIPC synthase	195	ND
PIF1	33	97 762	DNA helicase mitochondrial	259	0.06
IRC10	32	68 217	DNA repair	ND	ND
COX15	32	54 794	Hydroxylation haeme O to haeme A	ND	ND
SPC34	32	34 171	Dam1/DASH complex	ND	0.10
SHS1	31	62 706	Septin	5620	0.23
RSR1	31	30 486	GTPase (CDC24 interaction)	ND	ND
DPH1	30	48 679	Diphthamide synthesis	2562	ND

NCBI Inr *Saccharomyces cerevisiae* database of 11 143 sequences was used for Mascot searches. CCT subunits (italics) and other proteins listed according to MudPIT score with a cutoff set at 30. Copy numbers are taken from the Swissprot database (<http://expasy.org/sprot/>), and estimated synthesis rate (in protein per second) are from Arava *et al* (2003). ND, not determined.

release of proteins from the chaperonin as the major determinant. We applied the multidimensional chromatography-mass spectrometry approach, MudPIT, to our CCT preparations (Graumann *et al*, 2004). Before subjecting CCT to liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS), we centrifuged the CaM resin eluates on 10–40% sucrose gradients to further purify the CCT complexes (Figure 1B) and reduce contamination by nonspecific proteins and by *bona fide* calmodulin-binding proteins. The CaM resin wash step was performed with or without ATP, and in the analysis similar numbers of tryptic peptides were obtained from digestion and LC-ESI-MS analysis of both treatments; CCT-ATP (Table I) and CCT+ATP (Table II)—9352 and 9221 peptides, respectively. Tables I and II show the top matches obtained for the two samples. As expected, the eight CCT subunits dominate both histograms (Figure 1C) and, gratifyingly, the next most abundant ATP-elutable matches include some proteins known to interact with CCT, namely the folding substrates actin and  $\beta$ -tubulin, and folding regulators Plp1p and Plp2p (Stirling *et al*, 2006, 2007). Immediately following these hits are a previously undiscovered set of CCT-interacting proteins, the septin GTPases (Figure 1C, blue text). We also identified the ATP-elutable,

actin-related protein Arp2p just above the arbitrary cutoff for these lists (Melki and Cowan, 1994; Gavin *et al*, 2002). Thus, it is likely that many of the novel CCT-interacting proteins represent *bona fide* partners of CCT. Of further note is that additional polypeptides with significant scores appear in the ATP-treated sample including the type V myosin motor, the dynein heavy chain, an mRNA decapping protein (EDC3), and SWI3, a subunit of the SWI1/SNF chromatin remodelling complex (Table II). Finally there are a few proteins that are only partially removed by ATP treatment such as the previously described CCT-binding protein Vid27p (Aloy *et al*, 2004) and Rpl23Ap, Rpb7p, and Rtt102p.

We performed controls for nonspecific binding of yeast proteins to the calmodulin column in the presence and absence of the ATP wash steps by analysing the final washes prior to specific elution of CCT from the CaM resin. We identified 307 hits with MudPIT score >30, our cutoff value (see Supplementary Table S2). Only signals from the most abundant substrates are present in the ATP wash because the spectra are dominated by the most abundant proteins in yeast cytosol (i.e. glycolytic enzymes). The Ssa/Ssb chaperones are highly represented and despite the high affinity that certain Ssa and Ssb family proteins have for calmodulin

**Table II** CCT–3CBP interacting proteins in the presence of ATP, identified by LC-ESI-MS

Gene name	Score	Mass (Da)	Polypeptide
<i>CCT2</i>	4560	57 510	<i>Chaperonin subunit</i>
<i>TCP1/CCT1</i>	4308	60 899	<i>Chaperonin subunit</i>
<i>CCT8</i>	3607	61 965	<i>Chaperonin subunit</i>
<i>CCT7</i>	3409	60 211	<i>Chaperonin subunit</i>
<i>CCT4</i>	2992	57 967	<i>Chaperonin subunit</i>
<i>CCT3</i>	2761	59 404	<i>Chaperonin subunit</i>
<i>CCT5</i>	2607	61 152	<i>Chaperonin subunit</i>
<i>CCT6</i>	2371	60 171	<i>Chaperonin subunit</i>
RPL23A	117	14 578	60S ribosome subunit
VID27	73	89 190	WD40 (yeast/plants)
RTT102	49	21 177	SWI/SNF and RSC complex
MYO4	48	170 092	Type V myosin motor
TID3	42	80 552	Kinetochore-associated Ndc80 complex
RPB7	42	24 330	RNA pol II subunit 16
SEY1	40	89 662	Membrane organisation—GTP binding
YKL088W	40	65 312	Phosphopantothenoylecysteine decarboxylase
EAP1	40	69 719	eIF4E-associated protein
YOR356W	39	70 103	Mitochondrion—flavoprotein-type oxidoreductase
CCC1	36	34 229	Vacuolar Ca <sup>2+</sup> /Mn <sup>2+</sup> transporter
HS1155	36	110 642	U2-snRNP-associated splicing factor
VAR1	36	47 149	Mitochondrial ribosomal protein small subunit
YRR1	36	93 321	Zn2-Cys6 zinc-finger transcription factor
RPG1	35	110 333	Translation initiation factor 3 complex (eIF3)
ENP2	35	81 983	Nucleolar protein
RPL36A	34	11 117	N-terminally acetylated protein 60S ribosome
MNN1	33	89 058	Golgi alpha-1,3 mannosyltransferase
DYN1	33	473 843	Microtubule motor
CPN60	33	60 999	Mitochondrial chaperonin
DOP1	32	195 648	Mitochondria/cell morphogenesis
EDC3	32	195 648	Enhancer of mRNA decapping protein 3
PSF3	32	21 952	DNA replication complex GINS protein PSF3
PEX25	31	45 111	Peripheral peroxisomal membrane peroxin
SLA2	31	109 442	Transmembrane actin-binding protein
BCH1	30	82 567	Chs5p–Arf1p-binding proteins (ChAPs)
PDX1	30	45 466	Dihydrolipoamide dehydrogenase (E3BP)
SWI3	30	92 984	SWI/SNF chromatin remodelling complex

NCBI *Saccharomyces cerevisiae* database of 11 143 sequences was used for Mascot Searches. CCT subunits (italics) and other proteins listed according to MudPIT score with a cutoff set at 30.

(Shevchenko *et al*, 2002) it is likely that some Ssa/Ssb chaperone signal is the result of the specific interactions known to occur with CCT (Lewis *et al*, 1992). Several mitochondrial protein interactions are also observed; however, all of these hits are encoded by nuclear genes, and are therefore potential clients of CCT, except Var1p, found in the ATP-washed complex (Table II).

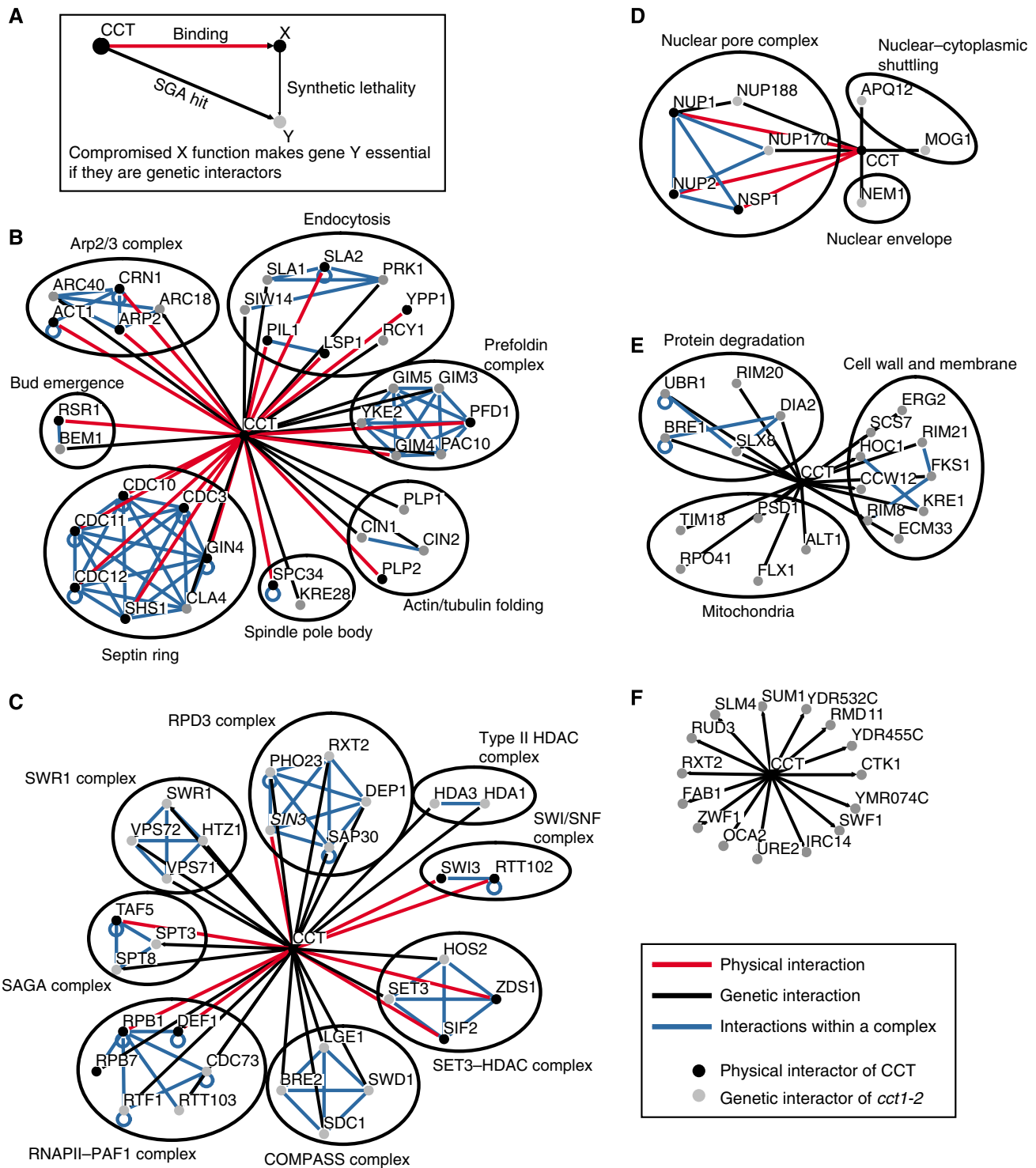
Overall, our proteomic analyses of the CCT–3/6CBP fusions revealed a total of 72 protein–protein interactions, many of which are novel (Table I and Supplementary Table S1). Moreover, our functional proteomics experiment (Figure 1C) revealed several ATP-elutable CCT-binding proteins, which may represent substrate proteins or cofactors and regulators of CCT activity. These data significantly expand the known CCT interactome and point to several possible novel cellular roles for CCT.

### Genetic interactors of CCT

Genetic interactions can imply that the interacting genes work in the same, or parallel, cellular pathways to execute a common function. This information can be extremely useful for placing a gene in a biochemical pathway or identifying redundancies *in vivo*. In recent years, SGA technology has allowed the creation of extensive genetic interaction networks based on large-scale synthetic lethal genetic

interaction screens (Tong *et al*, 2001, 2004) and for specific cellular processes (Zhao *et al*, 2005).

To explore the extent of CCT function in the cell, we used the *cct1-2* temperature-sensitive allele to perform an SGA screen with the ~5000 viable yeast deletion mutants. From all of the possible synthetic interactions identified, a total of 72 gene deletions were validated by random spore analysis as growing more slowly at 30°C (permissive temperature for *cct1-2*) when in combination with *cct1-2* (Supplementary Table S3). The genetic interactions probably result from an overlapping function between the SGA hit and some CCT-interacting protein whose folding is perturbed when CCT is disrupted (Figure 2A). In other words, the SGA hits would not typically interact with CCT physically but instead functionally overlap with a CCT substrate that misfolds in the *cct1-2* strain. The exception to this logic would be *PLP1*, which works in a complex with CCT to promote efficient protein biogenesis (Stirling *et al*, 2006). Consequently, genetic interactors of CCT point towards those cellular pathways that involve a CCT substrate (Figure 2). We placed SGA interactions into functional groupings along with relevant CCT physical interactions from our studies and the literature (Figure 2; Gavin *et al*, 2002, 2006; Ho *et al*, 2002). Our analysis, combined with gene ontology annotations of the interacting genes, reveals strong biases towards the cytoskeleton and chromatin



**Figure 2** Genetic and physical interactions of *cct1-2* reveal numerous protein complexes functionally connected to the CCT chaperonin. Network diagrams incorporating either physical and genetic interactions (B–D) or only genetic interactions (E, F) are shown. (A) Schematic method used to implicate CCT-binding proteins as putative substrates by integrating SGA and proteomic analyses. The presence of *cct1-2* leads to misfolding of a substrate ‘X’ that exhibits a known genetic interaction with gene ‘Y’. Therefore, the SGA hit connecting *cct1-2* and gene ‘Y’ actually reflects loss of function for a CCT substrate. Protein complexes or functional groupings are circled and labelled in each case and sorted into (B) cytoskeletal functions, (C) chromatin remodelling functions, (D) nuclear functions, and (E) other functional groups found within the SGA hits. (F) The remaining 15 SGA interactions with *cct1-2* such that all 72 SGA interactions are represented in this figure. Genetic interactions are shown in black, physical interactions are shown in red and interactions within the protein complex groupings are shown in blue. SGA hits are represented by grey nodes and physical interactors are represented by black nodes.

modification (Figure 2B and C, and Supplementary Tables S3 and S4). Additional connections were identified between CCT and the nuclear pore complex (NPC) (Figure 2D), and other

functional modules (Figure 2E). Figure 2F shows the remainder of the SGA interactions not grouped in other panels, such that all 72 genetic interactions are shown in Figure 2.

As expected, various components of the actin/tubulin folding machinery were identified, including the prefoldin complex, tubulin folding cofactors C and D (*CIN2* and *CIN1*, respectively), and the phosphocofactor-like protein *PLP1*. The cytoskeletal connections were expected because of the known participation of CCT on actin and tubulin folding, yet they reveal the depth of physical and genetic connections of CCT to the cytoskeleton and its various effectors (e.g. the endocytic machinery; Figure 2B).

The bias towards chromatin remodelling components may be a partially nonspecific phenomenon, as some of the genes identified have more than 100 synthetic genetic interactions (*LGE1*, *BRE1*, *CDC73*, *HTZ1*, *SWR1*; Tong *et al*, 2004; Collins *et al*, 2007). Nonetheless, CCT likely assists in the biogenesis of several histone deacetylase complexes (Pijnappel *et al*, 2001; Guenther *et al*, 2002). Also, the Set3p, SAGA, and RNAPII–Paf1p complexes that are implicated in chromatin modification contain both genetic and physical connections to the chaperonin, suggesting likely connections to CCT (Figure 2C).

In addition, several physical and genetic interactions suggest that CCT has a functional connection to the NPC and nuclear transport (Figure 2D). This was a totally unexpected result, and, although genetic interactions alone could be considered as secondary cytoskeletal effects, the fact that there are several genetic and physical interactions with CCT (Figure 2D and Supplementary Tables S1 and S3) supports the biological significance of this result.

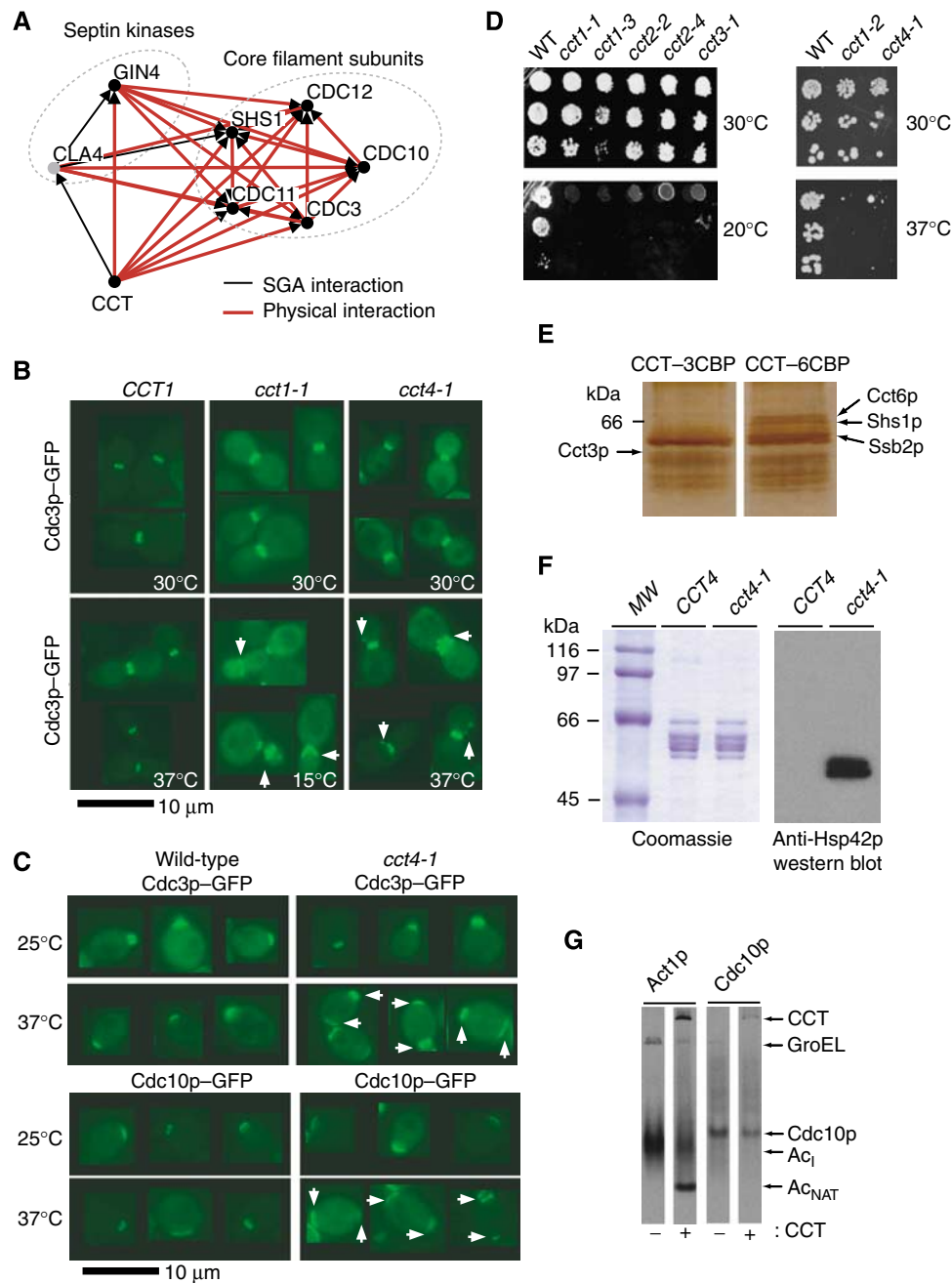
To expand upon our genomic analysis, we examined the cross-correlation coefficients (CC) of yeast gene expression for the cytoskeletal network (Figure 2B) as calculated by Sceptrans (Kudlicki *et al*, 2007; shown as a simple heatmap in Supplementary Table S5). The genes in the cytoskeletal network are tabulated as shown in Figure 2, where genes have been grouped in accordance with their functional subgroup, with the addition of the tubulin genes for comparison. The heatmap shows strong correlations between genes within a subgroup, as indicated by hot spots along the diagonal. There are strong correlations between CCT and *PLP2*, the bud-emergence subgroup, the prefoldin subgroup, and the kinase *CLA4* of the septin subgroup. Interestingly, the prefoldin subgroup also correlates strongly with *PLP2* consistent with the proposed role of Plp2p in actin folding (Vainberg *et al*, 1998; Stirling *et al*, 2007). The correlation between individual CCT subunits and substrates ACT1 and tubulins is variable, with values ranging from 0.51 to  $-0.36$ . Positive correlation coefficients are found between CCT subunits and septins, with one septin component, *SHS1*, having the highest CC. Interestingly, we were able to identify a direct interaction between Cct6p and Shs1p because Shs1p co-elutes specifically with Cct6p in sucrose gradient fractions containing dissociated subunits of the CCT complex (Figure 3E). As exemplified in Tu *et al* (2005), a positive correlation between CCT and other genes will not necessarily identify substrates *per se*, but may strongly suggest co-involvement of gene pairs in the same cellular process, akin to a genetic interaction. Hence this information reinforces the idea of CCT involvement in the septin network, possibly mediated through Cla4p. We note the strong correlation between CCT and prefoldin genes, providing another line of support for the transient character of CCT–prefoldin interaction (Hansen *et al*, 1999), which was not detected in our MS analysis.

### Analysis of septin function in yeast bearing mutant CCT subunits

The physical association of CCT with all five septin subunits (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p) and a septin kinase (Gin4p), as well as the genetic interaction between *cct1-2* and the *CLA4* septin kinase deletion strain (Figures 1C, 2B and 3A) strongly suggest a role for CCT in modulating septin function. Septins assemble into a defined core complex that polymerises in a GTP-dependent fashion into filamentous structures. During the G1 phase the septins form a patch at the incipient bud site before expanding into a disk during bud emergence and then they re-organise into an hourglass-shaped collar around the bud neck, which then splits during M phase to form a pair of rings before cytokinesis and septin disassembly. This entire process is regulated by the kinases Cla4p, Gin4p, and Swe1p, and the phosphatase Rts1p (Versele and Thorner, 2005).

To explore a role for CCT in septin function, we examined the organisation of two GFP-tagged septin subunits (Cdc3p and Cdc10p) in cells bearing cold- and heat-sensitive alleles of *CCT1*, *CCT2*, *CCT3*, and *CCT4*. The localisation of Cdc3p and Cdc10p is normally confined to the patch or collar structures described above. We found that the pattern of septin localisation was altered for both Cdc3p and Cdc10p in cold-sensitive *cct1-1* and in heat-sensitive *cct4-1* cells (Figure 3B and Table III-A). The morphology of the septin collar was disrupted in a significant percentage of *cct1-1* and *cct4-1* budded cells at the non-permissive temperatures (Figure 3B and Table III-A). Moreover, we observed aberrant cortical GFP-tagged septin patches in the mutant cells (Figure 3B and Table III-A). Interestingly, these phenotypes were observed in cold-sensitive *cct1-3* cells for the localisation of Cdc10p but not Cdc3p (Table III). Finally, we observed, in unbudded *cct4-1* cells, septin patches that seemed to be retained following cell division while a new septin structure was assembled on the opposite side of the cell (Figure 3C and Table III-B). Importantly, none of the above phenotypes were observed in *cct1-2*, *cct2-4* or *cct3-1* alleles or in deletions of the CCT cofactors *PLP1* or prefoldin (*pac10Δ*) (Table III-A). This suggests that the defects specifically arise because of disrupted CCT function, and not simply cytoskeletal protein dysfunction. Even cells lacking both *PLP1* and *PAC10*, which have a severely disrupted actin cytoskeleton (Stirling *et al*, 2006), had largely wild-type septin localisation, although a small but significant percentage of septin collars displayed anomalies in these cells, potentially through an effect on the chaperonin itself, given the functional interactions between CCT and Plp1 and prefoldin (Table III-A).

It is possible that septin defects are only seen in some CCT mutant strains because of the relative severity of the alleles. If the *cct1-1* and *cct4-1* alleles were much stronger than the other alleles tested they may be revealing a general septin defect in response to a dysfunctional CCT holo-complex. In support of the specific nature of the defects, we observed that the *ts* growth defects in *cct1-2* cells was at least as severe as those of *cct4-1* cells and that all of the *cs* alleles tested show strong growth defects when grown at 20°C (Figure 3D). Therefore, at the non-permissive temperatures used in our septin localisation experiments (15 and 37°C), all of the *ts* and *cs* alleles should have shown defects if the septin mislocalisation were a nonspecific result of abrogating CCT function. This observation of allele specificity suggests the



**Figure 3** Genomic and proteomic analyses identify a role for CCT in septin cytoskeleton function. (A) Physical and genetic interactions of yeast CCT with septin core components and septin kinases. (B) Localisation of GFP-Cdc3p in budded wild-type *CCT1*, *cct1-1* cold-sensitive and *cct4-1* heat-sensitive yeast cells. White arrows point to mis-shapen septin rings or additional patches of septin signal in *cct* mutant cells. *CCT1* wild-type strain at 15°C which had normal septin localisation is not shown (see Table IIIA). (C) Localisation of GFP-Cdc3p and GFP-Cdc10p in wild-type and *cct4-1* cells at permissive (25°C) and non-permissive temperatures (37°C). White arrows point to aberrant sites of septin localisation. (D) Allelic series of *CCT* *cs* and *ts* allele bearing strains to determine relative sensitivities of different alleles. CCT mutant cells were serially diluted, spotted onto rich growth media and grown at the temperatures indicated for 48 or 72 h. (E) Identification of Shs1p-Cct6p interaction. CCT-3CBP and CCT-6CBP were purified by CaM resin and sucrose gradient velocity sedimentation, after which the fraction of 18% sucrose normally containing individual CCT subunits was subjected to another CaM resin purification step. The eluates were run on an SDS gel and silver-stained and key bands were identified by PMF-MS as indicated. (F) Western blot: CaM resin-purified CCT from wild-type cells and mutant *cct4-1* cells, blotted with anti-Hsp42p antibody, shows co-migration of Hsp42p with mutant CCT but not with wild-type CCT. (G) *In vitro* translation of Act1p and Cdc10p in *E. coli*-derived extract with (+) or without (-) added yeast CCT. Ac<sub>I</sub> and Ac<sub>NAT</sub> indicate the actin folding intermediate and native actin, respectively, according to Pappenberger *et al* (2006).

existence of a physical interaction between the elements under study, a fact we had already observed with purification and mass spectrometry of CCT and septins (Figure 1), as well as with the observation of a direct interaction between Cct6p and the Shs1p septin subunit (Figure 3E). In light of

these findings, we note that certain very general downstream effects of septin disruption have been observed previously in CCT mutant yeast, including multi-budded cells and abnormal chitin deposition (Vinh and Drubin, 1994; Stirling *et al*, 2007).

**Table III** *In vivo* septin defects in *cct* mutant strains

Strain	Genotype	Temperature	Normal rings (%)		Abnormal rings (%)		Additional patches (%)		Total no. of cells analysed	
			CDC3	CDC10	CDC3	CDC10	CDC3	CDC10	CDC3	CDC10
<i>A. Septin localization in budded cells</i>										
MLY100	WT	15	98.2	100.0	1.8	0.0	0.0	1.0	110	100
		30	100.0	97.8	0.0	1.1	0.0	1.1	98	91
MLY110	<i>plp1</i> Δ	30	100.0	99.0	0.0	1.0	0.0	0.0	87	100
MLY111	<i>pac10</i> Δ <i>plp1</i> Δ	30	94.6	96.1	5.4	3.9	1.1	0.0	93	102
MLY118	<i>pac10</i> Δ	30	92.5	93.5	7.7	1.9	0.0	4.7	104	107
DUY558	<i>CCT1</i>	15	100.0	100.0	0.0	0.0	2.9	1.9	103	107
		25	94.5	99.1	5.5	0.9	1.8	0.9	109	113
		30	98.0	98.1	2.0	1.9	3.0	2.8	100	106
		37	98.2	98.2	1.8	0.0	1.8	1.8	112	111
DUY559	<i>cct1-1</i>	30	100.0	100.0	0.0	0.0	1.0	3.6	98	110
		15	<b>55.6</b>	<b>62.0</b>	<b>44.4</b>	<b>38.0</b>	<b>18.8</b>	<b>12.0</b>	117	108
DUY560	<i>cct1-2</i>	25	100.0	100.0	0.0	0.0	0.9	0.0	110	104
		37	96.2	100.0	3.8	0.0	1.9	0.0	106	101
DUY561	<i>cct1-3</i>	30	100.0	100.0	0.0	1.8	2.7	5.5	111	110
		15	96.0	<b>76.6</b>	4.0	<b>23.4</b>	1.0	<b>29.0</b>	101	107
CUY466	<i>cct2-4</i>	30	99.1	100.0	0.9	0.0	0.9	0.0	112	110
		15	94.1	98.1	5.9	1.9	4.0	3.8	101	106
CUY492	<i>cct2-2</i>	30	ND	ND	ND	ND	ND	ND	ND	ND
		15	ND	ND	ND	ND	ND	ND	ND	ND
CUY462	<i>cct3-1</i>	30	96.3	100.0	3.7	0.0	4.7	0.0	107	104
		15	92.3	94.6	7.7	5.4	3.3	2.2	91	93
DDY299	<i>cct4-1</i>	25	98.8	99.1	1.2	0.9	3.6	5.6	83	108
		37	<b>84.6</b>	<b>82.4</b>	<b>15.4</b>	<b>17.6</b>	<b>14.1</b>	<b>11.0</b>	78	91
<i>B. Septin localization in unbudded cells</i>										
			Normal single patch (%)		Opposing extra patch (%)		Total no. of cells analysed			
			CDC3	CDC10	CDC3	CDC10	CDC3	CDC10		
MLY100	WT	30	100.0	100.0	0.0	0.0	77	57		
DUY558	<i>CCT1</i>	25	91.1	93.8	8.9	6.2	79	81		
		37	95.4	94.9	4.6	5.1	65	59		
DDY299	<i>cct4-1</i>	25	100.0	100.0	0.0	0.0	54	95		
		37	<b>80.7</b>	<b>79.7</b>	<b>19.3</b>	<b>20.3</b>	57	59		

(A) Aberrant septin ring morphology or additional patches of GFP-septin signal were scored for the strains indicated at the noted temperatures. Scores differing significantly from wild type ( $P < 0.01$ ) are reported in bold. (B) The presence of additional septin patches in unbudded *cct4-1* cells was scored and reported as in A. ND, not determined.

To assess directly the impact of CCT disruption on the interaction with septins, we analysed the proteins interacting with mutant CCT complexes isolated from *cct4-1* cells grown at permissive temperature by LC-ESI-MS in a similar manner as performed for CCT-3CBP (gene lists not shown). Compared to wild-type CCT, two main differences were observed. First, we noted a high level of Hsp42p on the mutant CCT complex, an interaction that was independently confirmed by western blotting with anti-Hsp42p antibodies (Figure 3F and Haslbeck *et al*, 2004). This interaction is consistent with *cct4-1* cells harbouring a functionally abrogated CCT complex, as yeast Hsp42p is a promiscuous chaperone that may be recruited to stabilise or prevent the aggregation of the chaperonin. The second difference observed is that none of the five septins were detected on the mutant CCT complex. Taken together, these data strongly support the idea that loss of CCT interaction underlies the formation of the aberrant septin structures observed in the *cct4-1* mutant (Figure 3B and Table III).

We propose that CCT is not involved in the biogenesis and folding of septin polypeptides, but rather performs a regulatory role that is clearly compromised in *cct* mutant cells. It is unlikely that CCT is required to fold septins *de novo* because they fold properly and polymerise when co-expressed in

*Escherichia coli*, which lacks CCT (Versele and Thorner, 2005). We translated the septin Cdc10p in an *E. coli*-derived extract supplemented with yeast CCT, a system we have developed for folding actin polypeptide chains (Pappenberger *et al*, 2006). Figure 3G shows the analysis for Cdc10p; the presence of CCT during translation and folding has no effect on the yield of the folded septin protein in contrast to Act1p, which is completely dependent upon CCT for folding to the native state. Interestingly, some radiolabelled Cdc10p appears to associate with CCT, consistent with our proteomic and *in vivo* results (Figures 1 and 3).

## Discussion

Efforts to understand the eukaryotic chaperonin CCT have been hampered because its essential and subtle mutant phenotypes are often masked by the pervasive actin and tubulin defects associated with its loss of function. Here, we present the first genome-wide analyses of CCT function using a combination of novel proteomic approaches and a synthetic interaction screen with a *ts* allele. Our findings provide a rich data set of 136 proteins/genes that interact with CCT, more than doubling the total number of known and putative CCT interactions (now at 227, from 91 previously

identified in the literature). Importantly, our study shows for the first time both known and many unexpected links between CCT and specific protein complexes and cellular processes (Figure 2), defining its position in numerous biological networks.

A contentious issue in the CCT field is the number and nature of the substrate proteins actively folded by CCT (Sternlicht *et al*, 1993; Thulasiraman *et al*, 1999; Grantham *et al*, 2006). How does one discriminate experimentally a substrate protein that is completely dependent upon CCT for its folding, such as actin, from one which is partially dependent through kinetic partitioning. This analysis is confounded by the existence of proteins that require CCT for higher order assembly processes, cofactor proteins and other binding activities. Our CCT interactome, combined with functional assays, should assist in the identification and verification of novel substrates and cofactors of the chaperonin.

### Cytoskeletal networks

Aside from the expected connections to actin- and tubulin-related processes, our analyses reveal unexpected physical and genetic connections to a third cytoskeletal system—the septin ring. The identification of strong, ATP-elutable interactions between CCT and all of the core septin components in *S. cerevisiae* suggested a physiologically relevant relationship between CCT and the septin ring (Figure 1). This proposition was also supported by the SGA analysis in which the septin kinase gene *CLA4* was identified as an interactor of *cct1-2*. Indeed, direct examination of GFP-Cdc3p and GFP-Cdc10p in various CCT mutant strains revealed clear septin localisation/assembly defects (Figure 3 and Table III). Septins probably do not need CCT for biogenesis or folding, but CCT may participate in an ATP-dependent septin assembly/disassembly process. Septin organisation is disrupted when CCT function is compromised in mutant cells *cct4-1*. The ATP-dependent allosteric behaviour of CCT isolated from *cct4-1* mutant cells was recently investigated (Shimon *et al*, 2008), showing that the intra- and inter-ring allosteric cooperativity was abolished in the CCT carrying the mutant subunit. The spectrum of CCT-interacting proteins is also perturbed in the mutant CCT complex from *cct4-1* cells (data not shown). The sudden appearance of a small heat-shock protein and the absence of septins in the mutant proteomics data may be a direct consequence of the perturbation of allostery.

### Chromatin modification and NPC networks

CCT was previously shown to assist the biogenesis of the SET3 histone deacetylase complex, defining a role for CCT in chromatin modification (Pijnappel *et al*, 2001; Guenther *et al*, 2002). We found that a large group of protein complexes seemingly impacted by CCT are involved in modifying or remodelling histones (Figure 2C and Supplementary Table S4). This suggests that the chaperonin impacts chromatin biology and epigenetics to an extent that was not previously appreciated, similar to that recently found for Hsp90 (Zhao *et al*, 2005). CCT displays interactions with complexes involved in histone methylation (i.e. COMPASS complex; Paf1-RNAPII complex), histone acetylation (i.e. SAGA complex), histone deacetylation (i.e. Rpd3 complex; Set3 complex; type II HDAC complex), chromatin remodelling (i.e. Swr1 complex; SWI/SNF complex), and proteins involved

in telomere maintenance (e.g. Yku80p and Pif1p; Supplementary Table S4).

Proteins involved in chromatin modification are usually localised to the nucleus, implying either that CCT performs a protein complex assembly function in the nucleus or that it assists the folding or assembly of nuclear proteins in the cytosol prior to their transit through the nuclear pore. Surprisingly, our data reveal a network of physical and genetic interactions between CCT, core NPC proteins and other factors associated with nuclear transport (Figure 2D). Although the genetic interactions of *cct1-2* with *NUP170* and *NUP188* could be indirect effects of cytoskeletal defects, the association of CCT with Nup1p, Nup2p, and Nsp1p suggests a direct relationship. This raises several possibilities: (1) CCT could be involved in the folding/assembly of the nuclear pore components themselves, (2) CCT could usher nuclear proteins from the ribosome to the NPC, releasing them directly to the pore in a state suitable for nuclear transport, or (3) CCT could transit the NPC to perform protein folding/assembly functions inside the nucleus, consistent with reports of nuclear localisation for CCT subunits (Souès *et al*, 2003). Further studies should help elucidate the precise role of CCT in nuclear protein folding/transport and/or nuclear pore function.

### TOR and cell cycle networks

A role for CCT in cell cycle progression, particularly at the G1/S phase, has been suggested (Yokota *et al*, 1999; Camasses *et al*, 2003; Grantham *et al*, 2006; Stirling *et al*, 2007). Several CCT substrates are cell cycle proteins (e.g. Cdc20p, Cdc55p, and Cdh1p), and the interaction between CCT and Cdc20p and Cdh1p has been well studied (Camasses *et al*, 2003). Notably, neither Cdc20p nor Cdh1p were identified in our MS analysis and their absence is most likely due to their low synthesis rate, being respectively  $100 \times$  and  $366 \times$  lower than Act1p (Table I and Arava *et al*, 2003). On the basis of Arava *et al* (2003), we estimate our present detection limit at  $\sim 50 \times$  lower synthesis rate than Act1p. Hence, detecting low abundance CCT substrates such as Cdc20p and Cdh1p could well be outside the current range of state-of-the-art MS technologies.

The connection between CCT function and the G1 phase is not only through CCT-interacting cell cycle proteins. Signalling through the TOR kinase also regulates G1 progression and TOR has been functionally connected to CCT (Kabir *et al*, 2005). We identified interactions between CCT and proteins related to both TOR and the cell cycle (Supplementary Table S4). Particularly enriched are type IIA protein phosphatase components, including the catalytic subunits Pph3p and Sit4p, the regulatory subunits Cdc55p, Sap155p, and Tap42p, as well as the scaffolding subunit Tpd3p (Ho *et al*, 2002; Kabir *et al*, 2005; Gavin *et al*, 2006; Supplementary Table S4). Cdc55p is a known CCT substrate and the other interactions suggest that CCT may regulate phosphatase trimer assembly or the folding of multiple subunits.

Furthermore, *SIT4* has been linked genetically to the CCT cofactor *PLP2*, as they share numerous suppressors (Stirling *et al*, 2007). The precise role of CCT function in the G1/S phase of the cell cycle progression remains to be elucidated. Nonetheless, a picture emerges in which CCT is tightly coupled to cell cycle control not only through the biogenesis

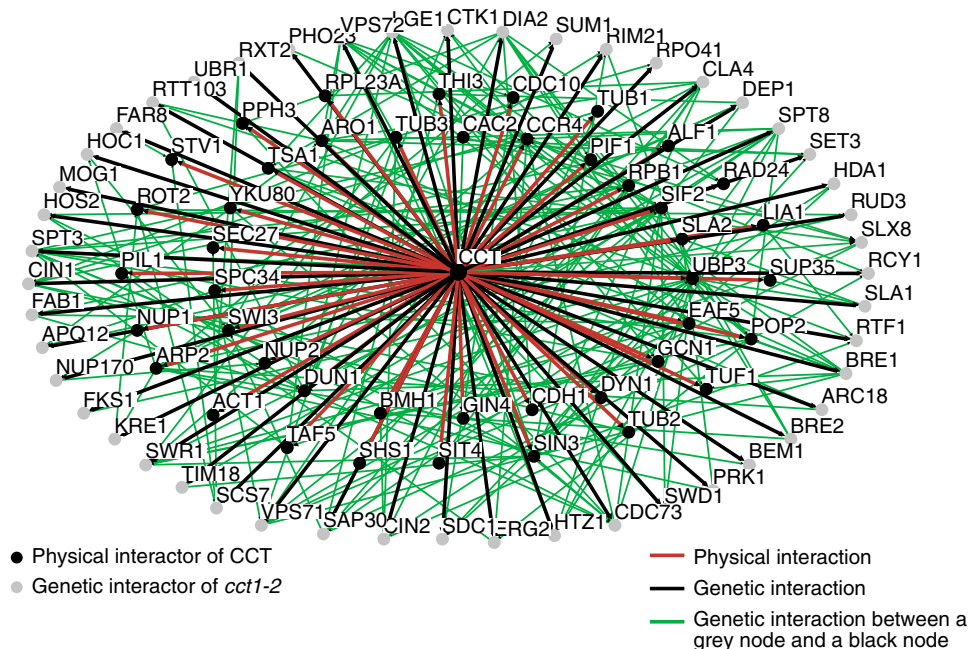
of actin, tubulin, and the APC activators Cdc20p/Cdh1p but also through the septins and probably several other targets (e.g. type II protein phosphatases).

To investigate a possible evolutionary coupling of CCT function to cell cycle control, we compared our data to the minimal eukaryotic genome of the intracellular parasite *Encephalitozoon cuniculi*. The tiny, compacted genome of *E. cuniculi* encodes only 1997 proteins (Katinka *et al*, 2001). Strikingly, we found that, of the ~20 conserved CCT-interacting proteins, several cell cycle proteins were present (Supplementary Table S6). This supports an evolutionarily ancient coupling of CCT to cell cycle control, with important implications for human cancer studies. It is difficult unravelling the role of CCT in G1 phase in mammalian cells (Grantham *et al*, 2006) and yeast (Stirling *et al*, 2007) because of the ‘nonspecific’ actin and tubulin phenotypes caused by disrupting CCT function but with new CCT targets in hand, more directed genetic approaches will be possible.

### Mining the CCT interaction network reveals putative CCT substrates

By integrating the CCT physical and genetic interaction networks with one another, we can begin to offer potential explanations for many synthetic sickness interactions we observed in our SGA screen. Figure 2A illustrates how physical interactors of CCT that share a genetic interaction with *cct1-2* are more likely to represent substrate proteins than those with no connection to the genetic interaction map. This is based on the assumption that if a CCT-binding protein X is a substrate, it may lose function, by misfolding, when CCT function is compromised. As a result, CCT loss of function phenocopies the loss of individual substrate proteins as evidenced by the actin and tubulin mutant phenotypes in

*cct*-defective cells (Ursic *et al*, 1994; Grantham *et al*, 2006). The analysis in Figure 4 (Supplementary Table S7A) expands the functional groupings in Figure 2 by adding another dimension, namely, published genetic interactions between our physical and genetic CCT interaction maps. Assuming that nearly all of the SGA hits arise due to a CCT substrate that misfolds in *cct1-2* cells, integrating the SGA data, CCT physical interactions and genetic interactions from the literature identifies CCT-binding proteins that have common genetic interactors with *cct1-2* and thus are more likely to be true substrate proteins. A simple example of this type of relationship exists for the known CCT substrate Tub2p—both *TUB2* and *cct1-2* genetically interact with the tubulin folding cofactor *CIN1*. Another example of this relationship exists for the CCT-binding protein Nup1p—both *NUP1* and *cct1-2* genetically interact with *NUP170*. Figure 4 shows that 44 CCT-binding proteins have reported genetic interactions with at least one of 48 genetic interactors of *cct1-2* (see Supplementary Table S7A for a complete list). This group of 44 proteins points to diverse interacting proteins, including known substrates (e.g. actin, tubulins, and Cdh1p), potential substrates, as well as some of the most interesting groups of interactors from our screens and the literature (e.g. septins: Shs1p, Cdc10p, and Gin4p; NPC: Nup1p and Nup2p; TOR signalling: Pph3p, Rot2p, and Sit4p). Also, analogous to Hsp90 (Zhao *et al*, 2005), the interaction of a protein with CCT and a cochaperone makes it more likely to be a genuine CCT substrate: 36 CCT-interacting proteins interacting with at least one of its putative cochaperones are listed in Supplementary Table S7B. Integrating our data sets in this way is a powerful tool for extracting information about the possible functions of CCT in the cell and will provide a basis for distinguishing CCT-binding proteins from *bona fide* substrates.



**Figure 4** Integration of the CCT interactome with known interactions reveals putative substrate proteins. Network diagram showing integrated physical and genetic interactors of CCT. Those physical interactors of CCT that exhibit a genetic interaction with at least one genetic interactor of CCT (green lines) are shown. The outer ring (grey nodes) represents SGA hits and the two inner rings (black nodes) represent physical interactors of CCT (see text for description and Supplementary Table S7A).

## Conclusion

The chaperonin CCT is essential for the folding of actin and tubulins and has relevance to developmental disease (Bouhouche *et al*, 2006) and cancer pathways (Camasses *et al*, 2003; Coghlin *et al*, 2006). In spite of its importance, most other major molecular chaperone families have been better studied than CCT. Our innovative method for isolating CCT-interacting proteins, together with our integrative, genome-wide analysis of chaperonin function substantially expands the CCT 'interactome' and points to several previously unknown cellular roles for the chaperonin. In addition to the two classical CCT-interacting cytoskeletal proteins, actin and tubulin, we identified a novel physical connection of CCT to a third cytoskeletal system, the septin ring, which requires CCT activity for its proper assembly and function *in vivo*. On the whole, our data will help to initiate a multitude of studies aimed at unravelling the role of CCT not only in the cell cycle but also in septin function, nuclear pore function and other cellular processes.

## Materials and methods

### Purification of CCT

CCT-3/6CBP complexes were purified according to Pappenberger *et al* (2006) using calmodulin affinity chromatography. CCT was analysed for its binding partner proteins immediately after calcium chelation elution or after further size fractionation on 10–40% sucrose and 15% glycerol gradients. The ATP incubation step was performed on CCT bound to the resin in the presence of nucleotide (5 mM ATP/0.5 mM ADP/15 mM MgCl<sub>2</sub>) followed by two incubations of five column volumes of wash buffer (20 mM Hepes pH 8.0, 150 mM KCl, 0.2 mM CaCl<sub>2</sub>, 2 mM DTT, 0.01% LDAO, 20% glycerol) supplemented with 5 mM ATP/0.5 mM ADP/15 mM MgCl<sub>2</sub> at 20°C for 20 min each.

### Construction of CCT-6CBP strain

The CCT-6CBP strain was constructed using an analogous scheme to the CCT-3CBP tagging method in which tags were inserted into loops predicted to be located on the outside surface of CCT based on crystallographic models (Pappenberger *et al*, 2006). A 55 amino-acid residue triple tag, containing the Strep-, His<sub>8</sub>- and calmodulin-binding peptide (CBP) sequences, was inserted into a loop located within residues <sub>368</sub>TENTDPKSCTILIK<sub>381</sub> at the base of the apical domain of CCT6p. This insertion site in CCT6p at P373|K374, is exactly equivalent to CCT3-P374|K375 (Pappenberger *et al*, 2006).

### Mass spectrometry

For peptide mass fingerprinting (PMF)-MS, CCT-6CBP yeast lysate was spun down for 1 h in the presence of protease inhibitors and 1 mM EDTA, then 1.0 ml was added to 100 µl of Calmodulin resin (Stratagene) in the presence of 5 mM CaCl<sub>2</sub> and incubated at 4°C overnight. The resin was washed three times with equilibration buffer (2 mM CaCl<sub>2</sub>) and wash buffer (0.2 mM CaCl<sub>2</sub>). The protein was eluted in 2 × 100 µl elution buffer containing 10 mM EDTA, and

24 µl was loaded onto a 10% SDS gel, stained with SimplyBlue and bands were cut-out for PMF-MS analysis. In-house analysis was performed on an ABI Voyager STR MALDI-MS as previously described (Passmore *et al*, 2003). In-house peptide sequencing was performed using a Waters PMF-electrospray MS. Purified CCT samples of wild-type cells and *cct4-1* mutant cells were analysed by LC-ESI-MS performed by Dr Thomas Halder, Toplab, Munich, Germany. Peptide data are available upon request.

### Yeast strains and manipulations

Yeast strains used are listed in Supplementary Table S8. Yeast plasmid transformations were performed as described (Amberg *et al*, 2005). Yeast were grown on rich YPD media or on synthetic complete media lacking uracil to select for plasmids (Adams *et al*, 1997). For the CCT allelic series, yeast were grown to late log phase before serial dilution and pinning onto YPD plates. The plates were grown at the permissive or non-permissive temperatures for the alleles for 24–72 h.

The *cct1-2* strain used for SGA (Supplementary Table S8) was derived from the heat-sensitive *tcp1-2* strain described by Ursic *et al* (1994). The SGA screens were performed in triplicate as described (Tong *et al*, 2001, 2004). All SGA replica pinning steps were conducted at 22°C with the exception of the final double mutant selection step, which was conducted at 30°C. Genes with the highest statistical probability of representing a true interaction were confirmed by random spore analysis at the *cct1-2* permissive temperature of 30°C. Anti-Hsp42p antibodies were a kind gift of Prof J Buchner.

### Microscopy

N-Terminal GFP fusion plasmids containing *CDC3* and *CDC10* were a kind gift of Dr Christopher Beh. Cells bearing one of these plasmids were grown to log phase before shifting to the non-permissive temperature, 15°C for cold-sensitive cells or 37°C for heat-sensitive cells, and grown for 16 or 4 h, respectively, before live cell imaging on a Leica DM 6000 epifluorescence microscope with the appropriate fluorescence filter. Images were analysed in Openlab 5.0.2 (Improvision). Cellular defects were scored manually and the  $\chi^2$  analysis was used to determine the significance of any differences from wild-type cells under the same conditions.

### Interaction analyses

Network diagrams in Figures 2–4 were generated using Osprey 1.2.0 (Breitkreutz *et al*, 2003a). Interactions between CCT-interacting nodes were overlaid automatically by Osprey according to the GRID database (Breitkreutz *et al*, 2003b).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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