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Review Article

The structure and evolution of eukaryotic chaperonin-containing TCP-1 and its mechanism that folds actin into a protein spring

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Actin is folded to its native state in eukaryotic cytosol by the sequential allosteric mechanism of the chaperonin-containing TCP-1 (CCT). The CCT machine is a double-ring ATPase built from eight related subunits, CCT1–CCT8. Non-native actin interacts with specific subunits and is annealed slowly through sequential binding and hydrolysis of ATP around and across the ring system. CCT releases a folded but soft ATP-G-actin monomer which is trapped 80 kJ/mol uphill on the folding energy surface by its ATP-Mg^{2+}/Ca^{2+} clasp. The energy landscape can be re-explored in the actin filament, F-actin, because ATP hydrolysis produces dehydrated and more compact ADP-actin monomers which, upon application of force and strain, are opened and closed like the elements of a spring. Actin-based myosin motor systems underpin a multitude of force generation processes in cells and muscles. We propose that the water surface of F-actin acts as a low-binding energy, directional waveguide which is recognized specifically by the myosin lever-arm domain before the system engages to form the tight-binding actomyosin complex. Such a water-mediated recognition process between actin and myosin would enable symmetry breaking through fast, low energy initial binding events. The origin of chaperonins and the subsequent emergence of the CCT–actin system in LECA (last eukaryotic common ancestor) point to the critical role of CCT in facilitating phagocytosis during early eukaryotic evolution and the transition from the bacterial world. The coupling of CCT-folding fluxes to the cell cycle, cell size control networks and cancer are discussed together with directions for further research.

Introduction

Chaperonin proteins are ATPases which assemble into single- and double-ring protein machines in whose central cavities non-native proteins can be bound and sequestered and helped to fold to their native states driven by ring cycle(s) of ATP binding and hydrolysis, which may be concerted or sequential [1]. A popular general mechanism of chaperonin action is the Anfinsen cage model, so named in order to convey two central ideas first, that the enclosed substrate protein folds autonomously along its normal pathway or pathways and secondly that the chaperonin cavity isolates the folding protein from unwanted interactions with other proteins [2]. Anfinsen's pioneering studies on RNase folding and correct disulfide bond formation led to the now long accepted dogma of autonomous protein folding; the tertiary structure of every protein is encoded in its own unique polypeptide sequence [3]. Consequently, it is also now generally accepted that chaperonins, and indeed most molecular chaperone systems, must always make use of relatively non-specific and energetically weak mutual molecular interactions with substrate proteins to nudge them along the mildly rugged landscapes towards their thermodynamic minima — the native folded state. Ergo, the route to the bottom of the folding funnel, is the same whether or not a protein diffused there of its own accord or with a

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little resetting from the action of a molecular chaperone. An excellent review of the history of the experiments and the emergence of the ideas underpinning the development of a general theory of molecular chaperones can be found in Finka et al. [4]. It can be dangerous to develop general theories or laws concerning a biological process or a set of processes because, as stated in the title of the famous essay by Theodosius Dobzhansky [5], *nothing in biology makes sense except in the light of evolution*. Not to undermine Anfinsen's ideas, in his Nobel Prize essay when discussing the thermodynamic model for protein folding, he does cite possible exceptions to his general model such as kinetic accessibility due to complex reconfigurations of some polypeptide chains.

This review describes how the eukaryotic cytosolic chaperonin-mediated folding system acts upon the ubiquitous cytoskeletal protein actin which is an exception to the Anfinsen dogma because actin is unable to fold efficiently, or perhaps at all, without catalytic assistance [6] from the chaperonin-containing TCP-1 (CCT and also named TCP-1 ring complex — TRiC). The goal is to persuade the reader, by focusing on the origin and mechanism of the CCT–actin system, that CCT is a highly specific protein machine whose core activity is to capture and manipulate the non-abundant folding intermediates of its core obligate substrates: the actins, tubulins and WD40 propeller repeat proteins [7]. The CCT system has been incorporated into the standard model of chaperonin behaviour despite its obviously exceptional structural properties, restricted substrate spectrum and evolutionary history. A psychological driver for the widespread misunderstanding that CCT is a general chaperonin is because it is the only chaperonin found in the cytosol of all eukaryotes and thus, if it is concerned with folding only a few substrates, what is doing the general protein quality control? It is likely that the majority of this general and bulk quality control function in eukaryotic cytoplasm is largely performed by other systems such as the Hsp90 proteins which interact with ∼10% of the cellular proteome [8].[1] Similar to the CCT network of ∼300 genes and proteins [9], most of the Hsp90 interaction network of more than 1100 proteins has little connection to classical protein folding and cell stress functions as normally understood. CCT and Hsp90 are both evolutionary ancient systems which have accreted large numbers of physical and genetic interaction partners over the past 1–2 Gyr [7].

Understanding the incremental novelties that led to the emergence of the eukaryotes is one of the great problem areas in biology. What were the new molecular processes that permitted cells to become large enough to engulf other cells? How do large cells control their increased volume, develop complex internal membrane systems and generate enough energy to maintain their metabolism? We think that the emergence of the symmetry breaking CCT–actin system was an important driver of the transition from the bacterial world to the eukaryotic one. Actin proteins are packaged up with folding energy through the action of CCT and this stored energy manifests itself as unusual water-binding entropy which allows energy to be recoverable through perturbation of sub-diffusive water shells in the F-actin filament. Also discussed is how the symmetry breaking allows the development of the actomyosin system of muscle function in multicellular organisms 1.5 Gyr after the emergence of the first eukaryotic cells.

We have developed a model for the CCT system in which it acts as a cellular counting device to integrate cell cycle and cell size behaviours [7], and we highlight the interplay between CCT and complex signalling systems in different cell types in many organisms. CCT gene copy number disruption in cancer correlates with poor prognosis in many cancer types and CCT2 is recognized as an Epi-driver gene conferring selective growth advantage in tumours [10]. Further studies are urgently required to reveal the specific molecular roles of CCT in these fundamental cellular processes and their disruption in cancer.

Evolution of chaperonins

Group I versus Group II chaperonins

The chaperonins were categorized as a specific group of chaperones that displayed striking similarity in protein sequence and biochemical properties: GroEL from prokaryotes; rubisco-binding protein from plant chloroplasts (cpn 60) and Hsp60 from mitochondria [4]. The discovery of GroEL was made through genetic analysis where it was found to be essential for bacteriophage λ growth and protein assembly in *Escherichia coli* [11]. These Group I chaperonins are characterized by an arrangement of two rings, each composed of seven protein subunits [12]. The rings stack back-to-back, creating a central cavity, open at either end. Each subunit has a three-domain topology. The equatorial domain includes the inter-ring contact residues and an ATP nucleotide-binding site. The intermediate domain communicates allosterically between the equatorial and apical domains.

[1]Website Hsp90Int.DB

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The apical domain includes the binding sites for substrate recognition and also the GroES co-chaperonin lid which caps the cavity. The binding and hydrolysis of ATP in the equatorial domains orchestrates all the concerted domain motions that propagate throughout the multimer to drive the folding cycle of the chaperonin [1].

Group II chaperonins possess a high degree of structural heterogeneity, being composed of one, two, three or eight distinct subunit proteins, making up an eight-subunit ring or nine-subunit ring in the case of the three subunit complexes [13]. The archaebacterial thermosome [14] and CCT chaperonins constitute a separate Group II sub-group because they show strong sequence homology and evolutionary relatedness [15]. The two rings are set flush in the closed forms of both the eight-subunit thermosome and CCT rings such that each subunit associates with just one subunit from the opposite ring [14,16]. In contrast with the GroEL–GroES chaperonins, no co-chaperonin lid is associated with any of the Group II chaperonins and instead the apical domains possess an extended apical protrusion that closes the cavity like a camera iris.

The Group I and Group II chaperonins last shared a common ancestor at least 3 Gyr ago and there is no reason to suppose that they still have identical common mechanisms and protein folding functions. The protein folding mechanism of GroE is still being argued over after 30 years of study [4], partly because it is such a promiscuous enzyme and model substrates such as green fluorescent proteins and evolutionary heterologous systems such as *E. coli* GroE folding of the plant enzyme rhodanese continue to be studied. Moreover, as discussed [7], GroE negative bacterial species exist and GroE's substrate spectrum is almost completely non-overlapping in different bacterial species, whereas CCT is essential in all eukaryotes and its core substrate spectrum is highly conserved. Additionally, substrate-specific GroEL complexes are required for the folding of NdhH, a subunit of the chloroplast NADH dehydrogenase-like complex which is not foldable by the cpn 60 constitutively expressed in *Arabidopsis thaliana* [17]. GroE-mediated folding assistance is important for protein quality control during rapid growth and under stress, and its presence also supports evolutionary flexibility [18]. CCT may be a supreme example of evolutionary capacitation, because it facilitated the emergence of the modern actins, tubulins and WD40 scaffolds whose dynamics underpin so much of eukaryotic biology [7].

Evolution of chaperonin rings in LUCA from a peroxiredoxin fold

We have traced the emergence of chaperonins back to the last universal common ancestor of all organisms (LUCA). While refining the yeast CCT crystal structure datasets [16], we searched for PLP2 density using fitting routines and consistently found matches between its thioredoxin domain and the apical domains of the CCT subunits. Detailed computational fold-analysis suggested that the most likely origin of the chaperonin fold was a peroxiredoxin-like fold [19]. The peroxiredoxins evolved from thioredoxins, which are one of the very oldest protein folds, and a putative trajectory for the evolution of the Ur-chaperonin assembly from a primitive TRX protein is shown in Figure 1. Peroxidases are antioxidant enzymes and were present in early life forms because they allowed cells to cope with oxidative stress arising from hydrogen peroxide damage caused by high fluxes of UV radiation hitting earth in the absence of ozone. Peroxiredoxins are not superefficient H_2O_2 catalysts like catalases, and, at high H_2O_2 concentrations, they probably became over-oxidized themselves. This would have limited their use as peroxidases but may have triggered their oligomerization into high molecular mass structures that subsequently acted as rudimentary chaperones by acting as holdases that bind unfolded protein. Since PRXs are highly abundant proteins, and assuming this was the case in LUCA, oligomerization of PRX may also have contributed to cell survival in other ways; the oligomerization of a highly abundant protein component effectively leads to a decrease in molecular crowding and this subsequently results in lowered thermodynamic activity coefficients for other soluble components. The ability of the cell to modulate its own degree of crowding in response to stress can be described as an entropy buffer, whereby the monomer–oligomer phase transition produces large changes in the activity of other molecules in the cellular environment.

Subsequently, the merging of a PRX domain with the ancestral form of the equatorial domain may have contributed to the stabilization of the oligomeric species, thereby shifting the PRX protein from peroxidase activity to chaperone activity and giving rise to a very early Ur-Chaperonin (Figure 1). The insertion into the equatorial domain could also have triggered the proposed transition from a classic PRX fold, which became overloaded, to the new and stable apical domain fold. As a consequence of the functional recycling interaction between PRX and TRX, the emergence of the Ur-Chaperonin from PRX proteins may have resulted in co-evolution of chaperonins with TRX-like cofactors like the phosducins, which stabilize substrate interaction with the chaperonin, and substrates like the C-terminal domains of eukaryotic α- and β-tubulins which possess a TRX-like domain with a type II circular permutation.

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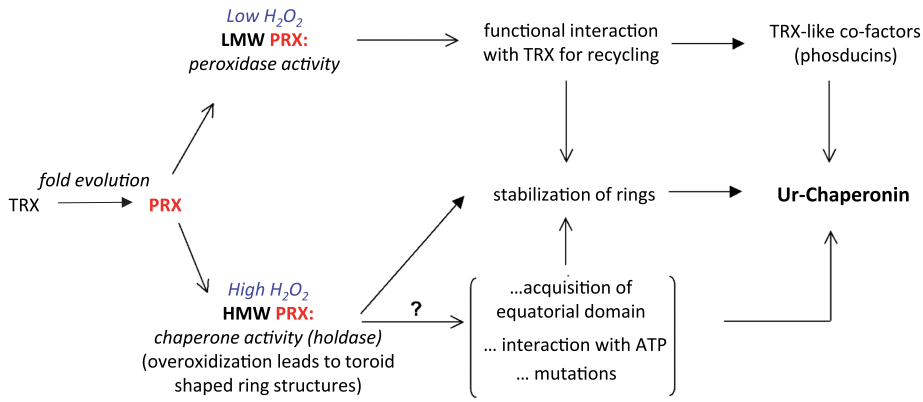

Figure 1. Evolution of chaperonins from a peroxiredoxin fold.

Evolution of the chaperonin fold. Schematic diagram of the evolutionary origin of the chaperonin fold. Under high oxidative stress, PRX oligomerizes and acts like a chaperone. The stabilization of this chaperone-active conformation — possibly due to events of random mutations, interaction with ATP or the merging with the equatorial domain, the order of which is unknown — would have led to the earliest chaperonin: the Ur-Chaperonin. Abbreviations: TRX, thioredoxin; PRX, peroxiredoxin; LMW, low molecular weight; HMW, high molecular weight.

CCT genetics

History and cloning of mouse testis CCT genes

The chaperonin of the eukaryotic cytosol is a Group II chaperonin and is referred to as CCT, which stands for chaperonin-containing tailless complex polypeptide 1 (TCP-1), or also as TRiC (TCP-1-ring complex). The mouse TCP1 gene was molecularly cloned over 30 years ago by Willison [20] because the protein was highly expressed during spermatogenesis and the *t*-complex locus had very interesting and unusual genetic properties and involvement in sterility phenotypes [21,22]. TCP1 was found to be a subunit of the cytosolic chaperonin in 1992 [6,23–25]. The CCT protein assembly was characterized as a chaperonin because of its essential role in folding the cytoskeletal proteins actin [6] and tubulin *in vitro* [25] and *in vivo* [26,27]. In contrast with Group I chaperonins, CCT does not behave as a stress protein, in the sense that it is not up-regulated under heat shock or other stress conditions [23]. CCT consists of eight different proteins, encoded by eight different genes: Tcp1, Cctb, Cctg, Cctd, Ccte, Cctz, Ccth and Cctq [28]. The sequence homology between the CCT subunits is low and typically in the order of 30%. Each chaperonin subunit consists of three domains, as first identified for GroEL: the equatorial domain harbouring the ATP-binding site, an intermediate domain and the apical domain involved in substrate binding [29]. The sequence differences among the eight CCT subunits are located mainly in their apical domains [16,30,31], indicating their individual specificities towards substrate binding which is confirmed by the fact that the two cytoskeletal proteins actin and tubulin, which are the predominant substrates of CCT with respect to mass occupancy [7,9], interact with specific subunits [16,32–34]. Mammalian testis CCT is biochemically heterogeneous because it contains CCTζ subunit isoform CCTζ2 [35], which probably relates to the increased requirement for tubulin folding during spermatogenesis. In humans, these CCTζ isoforms, CCT6A and CCT6B, are also present plus an additional CCT8 gene (CCT8-2L) not found in mouse. Coming full circle, a recent study of CCT expression and function in the testis of the planarian flatworm, *Schmidtea mediterranea*, discovered four testis expressed CCT paralogues, CCT1, CCT3, CCT4 and CCT8, that are all essential for spermatogenesis [36] and notably they comprise the substrate-binding arc of CCT.

Genetic analysis of CCT in *Saccharomyces cerevisiae*

The yeast genome harbours just the eight CCT subunit genes which are all essential for viability: Tcp1, Cct2, 3, 4, 5, 6, 7 and 8 encode proteins which are orthologous to the corresponding mouse TCP-1, CCTβ, γ, δ, ε, ζ, η and θ proteins [37]. Early genetic analysis of Cct in yeast showed involvement of Tcp1 in actin and tubulin biogenesis [38], Cct2 in tubulin biogenesis [39], Cct2 in actin and tubulin biogenesis [40] and Cct4 in actin function *in vivo* [41]. Subsequently, Cct6 was implicated in Tor2 regulation of actin function [42], and it also shows connectivity to ribosome biogenesis [43]. A genetic screen for gene mutants which exacerbated the already deleterious effects of mutations in the yeast cytoplasmic actin gene (ACT1) uncovered the anc2 gene [41]

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which became a vital tool in CCT research. Anc2 (actin non-complementing 2) is a mutation in a conserved glycine residue in the CCT4 subunit (G345D) [32]. CCT_{anc2} has lost its complex allosteric behaviour and shows simple Michaelis–Menten kinetics in its ATPase cycle *in vitro* [44]. CCT_{anc2} complex has an altered actin interactome. It is bound by three times more PLP2 cofactor than wild-type complex and by Hsp42 which is not detected on wild-type complexes [9], suggesting that a stress response is constitutively active in this mutant even though CCT_{anc2} actin folding activity is only reduced two-fold *in vitro* [44]. Much else in the anc2 strain appears to be wild type which points yet again to the restricted role for CCT in protein folding [7]. Stuart et al. [45] investigated the Arrhenius behaviour of CCT_{anc2} during a single actin folding cycle *in vitro* and showed that its deviation from linearity at temperatures above 30°C tracked the temperature-sensitive behaviour of the anc2 strain observed *in vivo*. Dekker et al. [16] used CCT_{anc2} to obtain the 3.8 Å crystallographic dataset of CCT–α-actin complex because co-crystals made with wild-type CCT always diffracted very poorly (>10 Å). The CCT_{anc2} machine is probably damped and less dynamic than wild-type CCT.

CCT structure

The atomic structure of the closed form of α/β thermosome was determined in 1998 [14], but it took much longer to obtain the 3.8 Å crystallographic dataset for the closed form of yeast CCT–actin complex [16] and a 5.5 Å dataset for an open form of bovine testis CCT [46]. Early single particle electron microscopy (cryo-EM) approaches obtained 3D reconstructions of apo-CCT, ATP-CCT, tubulin–apo-CCT complexes and α-actin–apo-CCT complexes [32,47] and, in combination with biochemical analysis of actin and tubulin CCT-binding motifs [48,49], this led to our sequential allosteric model for CCT-mediated folding of actins and tubulins [33,34]. Figure 2A shows the atomic model of skeletal muscle α-actin which we used as the substrate for the mouse testis CCT–α-actin cryo-EM structure (Figure 2B) and the yeast CCT–α-actin crystal structure

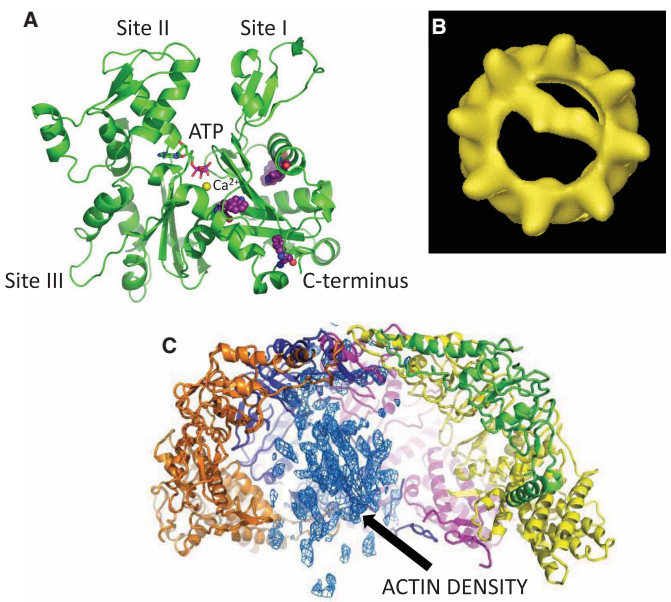

Figure 2. Structures of actin and CCT–actin complexes.

(**A**) Atomic structure of rabbit skeletal muscle G-actin (PDB code: 1ATN) in the standard front view with the subdomains 1–4. Ca^{2+} (yellow sphere) and nucleotide (ATP shown as ball and stick) are bound in the cleft between the subdomains 3 and 4 (the large domain), and 1 and 2 (the small domain). CCT-binding sites, as identified by a β-actin peptide array and site-directed mutagenesis [48,53], are indicated; site I in subdomain I, site II in subdomain 2 and site III in subdomain 3. In subdomain 4, the C-terminus is indicated and the four space filled residues are the only tryptophan residues found in actin — tryptophan 356 is blue-shifted, suggesting it is located in a strained environment [54]. (**B**) 3D electron microscopy reconstruction of complex between nucleotide-free mouse testis CCT–α-actin showing specific CCT subunits interact with the two domains of actin (taken from Llorca et al. [32]). (**C**) Cut-through of the 3.8 Å yeast CCT–ACT1 complex showing the electron density attributable to actin inside the cavity coloured in blue [16].

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(Figure 2C). Subsequently, several cryo-EM laboratories have increased the resolution for closed apo-CCT [50] and open-state CCT [51]. The most recent structures for the open-state forms reveal astonishing flexibility and distortion of the Z-shaped CCT2 subunit [51], which was apparent in the 5.5 Å open-state crystal structure of a bovine CCT ring [46]. The structural cycle of CCT subunit motions and rearrangements in conjunction with nucleotide states will require several more higher resolution reconstructions to obtain the complete 'movie' of sequential actin folding [52], but the trailer is already available to watch.

CCT ring-order data

Llorca et al. [32] obtained a 3D EM reconstruction of mouse CCT–rabbit–α-actin complexes which showed the specific binding interaction between the small and large domains of actin in a 1 : 4 configuration on CCT (Figure 2B). Using 2D EM projections of antibody-decorated complexes, they proposed that there were two 1 : 4 binding modes, but the current revised ring-order data from mass spectrometry cross-linking analysis [55,56] and fitting to our 3.8 Å X-ray structure dataset of yeast CCT–rabbit–α-actin [16] allow for an alternative explanation for the two binding mode model (Supplementary Data, S1). The reinterpretation of the EM top view images (Supplementary Appendix 1 and Figure 1C,F) shows that actin-Sub 4 binds the CCT8 subunit which is consistent with all our subsequent biochemical analysis ([16,57] and see Figure 7 of this manuscript). Liou and Willison [58] observed several CCT microcomplexes and proposed a unique ring order from 5040 possibilities based on the assumption that the dimers and trimers of CCT subunits represented fragments of single rings. Voronoi area calculations of subunit–subunit interfaces around the CCT ring, with the subunit new order, are used to infer strengths of intersubunit interactions [59], but it is notable that, using CCT-3CBP affinity chromatography, a stable trimeric complex of the CCT3–CCT6–CCT2 subunits can be detached from CCT [16]. Instead of constituting an arc of the ring, this trimer consists of the axis of symmetry subunits across the rings, CCT6 and CCT2, and one of the two subunits adjacent to CCT6 within the ring, the CCT3 subunit. Recent cryo-EM structures [51] of the open state of CCT show CCT6 tilted towards CCT8, its other neighbour, with a large gap to CCT3, so there must be complex binding rearrangements of the open states within and across the rings. Perhaps, these states reflect aspects of CCT assembly and disassembly processes [60] in addition to allosteric transitions regulated by nucleotide occupancies and substrate binding.

CCT apical domains

CCT protein recognition sequences and structure

The X-ray structure of GroEL [29] allowed the building of homology models of CCT apical domains [30] using the first few CCT protein sequences [15]. Subsequently, the crystallographic models of the CCT3/γ apical domain at 2.8 and 2.2 Å gave multiple views of the CCT residues involved in substrate binding and their evolutionary conservation [31]. As well as their location and their flexibility, the physical properties of the substrate-binding sites in GroEL and CCTγ are fundamentally different, because most of the residues in the substrate-binding region of CCTγ are charged (Figure 3). The differences between the substrate-binding sites in GroEL and CCT in terms of their location, flexibility and physical properties indicate that they employ different molecular mechanisms to achieve their particular cellular roles. This is consistent with the fundamental functional difference between CCT and GroEL which is their substrate-specificity [7]. CCT has to recognize a specific partially folded protein rather than the non-specific property of a protein being unfolded. The specific substrate binding of CCT may thus involve polar interactions for interacting with Gln/Asn-rich sequences (Figure 3A) and electrostatic complementarity as binding mechanisms which are vital for high-affinity recognition of low abundance substrates like the cell cycle regulators Cdc20 and Cdh1 [7]. Pappenberger et al. [31] obtained 12 crystallographically independent structures of the CCTγ apical domain and several side-chains of the substrate-binding site show characteristic altered orientations (Figure 3B; e.g. R316, E358), but most backbone conformation is largely unaffected by the interactions. There is, however, a notable movement of the backbone around E246 by 1.5 Å. The side-chains of R313 and E246 are within 4 Å of each other in the absence of a crystal contact, but move apart as both find alternative interaction partners in the crystal contact. This backbone shift is propagated C-terminal of E246 until the gap in the model at the ill-defined N-terminal half of the helical protrusion after K248. Interactions at the substrate-binding site can thereby alter the conformation of the flexible helical protrusion region, which is involved in intersubunit contacts. This is the first and only high-resolution glimpse so far of an allosteric response that spreads the news of actin binding to the CCT3–CCT6–CCT8 arc of the ring [16]. Phylogenetic analysis has continued with the increase in CCT

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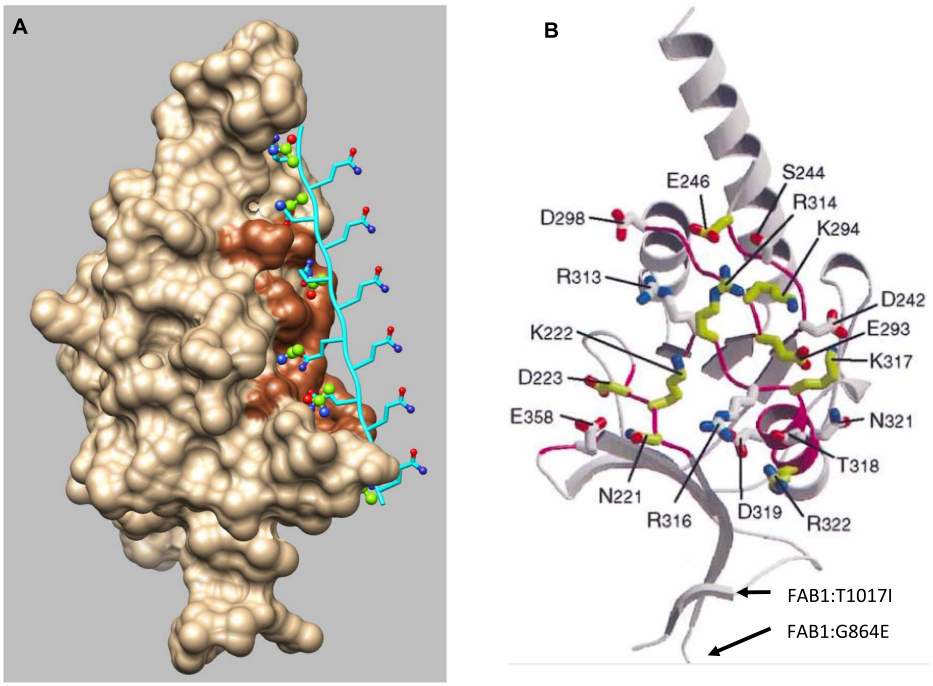

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CCTγ	N	K	D	D	S	E	E	K	D	R	R	R	K	T	D	N	R	E
FAB1	S	K	A	M	P	E	G	A	G	F	N	K	P	Q	V	I	R	K

Figure 3. CCT3/γ domain in FAB1p involved in autophagy.

(**A**) Multiple high-resolution X-ray structures of the mouse CCT3/γ apical domain (PDB: 1GML and 1GNI) revealed the chemical properties and dynamic flexibility of its substrate-binding surface [31]. CCT3/γ has a high affinity for Gln/Asn-rich segments in yeast proteins *in vivo* and the image shows a space-fill model of the CCT3/γ apical domain with its solvent-accessible-binding surface highlighted in brown and a stretch of bound poly-Gln β-strand occupying the long stretch of anchoring sites [61]. (**B**) Homology modelling of the apical domain region of the CCT3/γ gene insertion in FAB1. Yeast FAB1 is a 2278 amino acid residue protein (P34756). There are remnants of an ancient insertion of a complete CCT gene between residues 642–1250, which are clearly detected by alignment with any of the eight CCT polypeptide sequences. The strongest local match is with the mouse CCTγ (P80318) apical domain region E210–S380 displaying 28.1% identity over 158 aligned residues. We used the Phyre^2 web portal [62] to build a homology model of the FAB1 apical domain and the top scoring model was with the 2.2 Å resolution mouse CCTγ apical domain template, PDB: 1GML [31]. The image shows a front view (rotated 90° clockwise compared with the view in (**A**) of the apical domain with the 18 residues constituting the binding surface shown with ball-and-stick side-chains. (**C**) The equivalent residues in the FAB1 model are very divergent with only 3/18 residues being conserved and nine residues which are charged in CCTγ changing to polar or hydrophobic in FAB1. The two FAB1 mutants at the base of the domain are arrowed.

sequences available to confirm the evolutionarily conserved core of the apical domain which ranges from four residues in CCT8 to 17 in CCT4 [16] (Figure 7).

CCT3/γ domain in FAB1p involved in autophagy

Since an important neo-function in the emerging eukaryotic cell [LECA (last eukaryotic common ancestor)] was the development of the internal membrane system and phagocytosis, it is of some note that the FAB1/PIKfyve protein, which is conserved from yeasts (FAB1) to humans (PIKfyve), contains an ancient insertion of the CCT3/γ chaperonin. Here, I present a model of the FAB1 CCT apical domain (Figure 3B,C) based upon our X-ray structures of mouse CCT3/γ [31]. Fab1/PIKfyve is a lipid kinase which converts

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phosphatidylinositol-3-phosphate [PtdIns(3)P] to phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P_2]. Phosphoinositides (PtdInsPs) are key variables in eukaryotic membrane lipid compositions. An organelle identity code model, based on PtdInsPs, has been proposed for establishing and maintaining organelle-specific properties such as size, shape and function. PtdInsPs are rapidly interconverted by the action of specific PtdIns kinases and phosphatases, and each of the seven PtdInsP found in human cells has a stereotypical organelle distribution.

Yeast Fab1 regulates several vacuolar functions, including acidification, morphology and membrane traffic, and forms a complex with a 21 heat-repeat scaffold protein, Vac14p, and an antagonistic PtdIns (3,5)P_2-specific 5-phosphatase, Fig4p [63,64]. The conserved CCT3/γ apical domain of FAB1 binds Vac14 directly and a mutation at the base of the apical domain fold, *fab1*-T1017I, makes yeast defective for growth at 38°C [63]. A second mutant *fab1-2*-G864E, also located at the base and structurally adjacent to T1017 and one of a pair of highly conserved glycine residues in all CCT subunits, loses binding ability for Vac14p (60). From our own work on the whole genome SGA screen, we suggest a parsimonious model in which FAB1 evolved during or after the complete expansion of the CCT genes in LECA. Our SGA screen found FAB1 as one of the 72 interactors with the *cct1-2* mutant [9], suggesting that interactions between these two evolutionary related systems, CCT and FAB1, continued after the gene duplications which gave rise to the CCT3/γ subunit gene and a copy of which became inserted into FAB1. CCT itself is also closely involved in autophagy in higher eukaryotes where it appears to have an important role in the assembly processes of the organelle [65].

CCT ATPase

Chaperonins are machines driven by cycles of ATP binding and hydrolysis, and they all show strong inter-ring negative cooperativity, meaning that the rings are generally in alternative states [1]. Binding cooperativity is positive when the presence of an already bound ligand enhances the binding of another to the macromolecule. Within the CCT rings, there is weak positive cooperativity which may be concerted or sequential, and this aspect of the cooperativity effect is very difficult to distinguish kinetically using Hill coefficients of ATPase kinetics. Bovine CCT ATPase has a Hill coefficient of 2.0 [66,67], and the yeast CCT enzyme Hill coefficient is 1.9 [44]. Rivenzon-Segal et al. [68] used single particle EM and Fourier analysis to show that bovine CCT has a sequential cycle as a function of ATP concentration. Reissmann et al. [69] showed a gradient of ATP affinities with the CCT1–CCT4–CCT5–CCT2 arc having the highest affinity, but did not distinguish whether their power stroke model is concerted or sequential, but Gruber et al. [59] recently used Arrhenius analysis to show that the cycle is clearly sequential and potentially bidirectional starting from the CCT3/CCT1 arc of the ring. Computational phylogenetic analysis is another method for revealing conserved allosteric networks across the type II chaperonin family and this may help identify residues involved in these sequential cycles [70].

Evolution of the CCT ATPases

Figure 4 shows a scheme outlining an evolutionary trajectory from a two gene precursor (4/5 type and upper branch type, B) through mutations in CCT8 and then CCT6 and CCT3 to yield the intermediate actin holdase complexes while retaining all the strong ATPases on the opposite side of the ring. The extant, fully developed asymmetric CCT complex binds actin across the ring [32]. The CCT6/CCT3 gene duplication produced one subunit that must have had to displace a 4/5 type subunit, and it is likely that this step gave rise to the disturbed subunit interfaces in the CCT8–6–3 arc of the ring [46,51,55].

Yeast CCT–actin system

As soon as we had developed the sequential allosteric model for actin and tubulin folding by mammalian CCT in 2001, my laboratory decided to start to analyze CCT in the yeast *Saccharomyces cerevisiae*. Why? First, we wanted to apply the power of yeast genetics to the CCT system to generate mutations in CCT subunits and other components, substrates and cofactors and to perform more complex genetic screens and proteomic analyses. Secondly, we had found that testis CCT is very inactive *in vitro* in the folding of actin and tubulin

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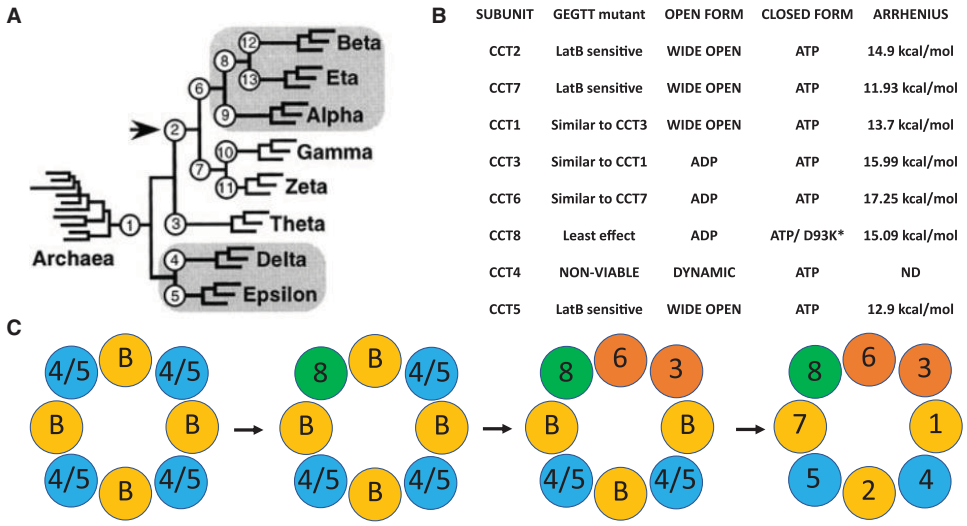

SUBUNIT	GEGTT mutant	OPEN FORM	CLOSED FORM	ARRHENIUS
CCT2	LatB sensitive	WIDE OPEN	ATP	14.9 kcal/mol
CCT7	LatB sensitive	WIDE OPEN	ATP	11.93 kcal/mol
CCT1	Similar to CCT3	WIDE OPEN	ATP	13.7 kcal/mol
CCT3	Similar to CCT1	ADP	ATP	15.99 kcal/mol
CCT6	Similar to CCT7	ADP	ATP	17.25 kcal/mol
CCT8	Least effect	ADP	ATP/ D93K*	15.09 kcal/mol
CCT4	NON-VIABLE	DYNAMIC	ATP	ND
CCT5	LatB sensitive	WIDE OPEN	ATP	12.9 kcal/mol

Figure 4. ATP cycle connected to the phylogeny of the eight CCT subunits.

(**A**) Phylogenetic analysis showing the ancient duplications and the most parsimonious branching model for the CCT genes as they evolved from an Archaeal thermosome-like precursor [13] ~2 Gyr ago [15]. (**B**) Table summarizes results of mutational, structural and kinetic analysis of yeast CCT ATPase. Column 1: effects of mutations in binding motif (GDGTT to GEGTT) [71]; CCT 4 mutant is lethal. CCT2 and CCT5 have similar gene expression phenotypes, CCT8 is essentially wild type. Column 2: single particle electron microscopy of yeast CCT complexes in different nucleotide occupancy states; the nucleotide partially preloaded state (NPP state) and ATP-bound state [51]. Column 3: all subunits are bound by ATP in the closed form crystal structure and CCT8 is probably catalytically inactive (Figure 7: [16]). Column 4: activation energies of steady-state ATPase activity (400 μm ATP) of the seven viable CCT mutants ([59]; wild-type value = 14.04 kcal mol^{-1}). CCT subunits are listed in the same order as the phylogenetic tree from top to bottom. (**C**) Scheme showing evolutionary trajectory from a two gene precursor (4/5 type and upper branch type), (**B**) through mutations in CCT8 and then CCT6 and CCT3 to yield the intermediate actin holdase complexes while retaining all the strong ATPase on the opposite side of the ring. The extant, fully developed asymmetric CCT complex binds actin across the ring [32]. The CCT6/CCT3 gene duplication produced one subunit that must have had to displace a 4/5 type subunit, and it is likely that this step gave rise to the disturbed subunit interfaces in the CCT8-6-3 arc of the ring [46,51,55,59].

chains, and we planned to develop more control over the biochemical purification of CCT from yeast and improve the folding activity of CCT *in vitro*.

The productive yeast CCT–ACT1–PLP2 complex

'Getting hold of CCT': a unique protein-tagging approach

Tagging of CCT for protein identification and purification purposes using N- or C-terminal tags has limitations, because both the N- and C-termini of CCT subunits are localized on the inside of the complex. Inserting C-terminal tags in yeast subunit genes, Cct1, Cct2 or Cct4, allowed the CCT complex to be pulled down, albeit with low yields, and C-terminal tagging of Cct3 or Cct7 did not result in viable strains [72]. However, based on the crystal structure of the apical domain of mouse Cctγ [31], a solvent-accessible loop was identified in the homologous location in the yeast Cct3 subunit that was located on the outside of the yeast CCT holocomplex, and a 56 amino acid sequence with three tags (Strep-tag, CBP-tag and His_8-tag) was introduced into this loop. Viable yeast strains were obtained which permitted efficient purification of the CCT holocomplex using calmodulin affinity chromatography despite CCT not being an abundant protein in yeast; ~3000 complexes per haploid cell [7]. This tagging strategy was performed for both the Cct3 and Cct6 subunits, yielding tagged holocomplexes named CCT-3CBP or CCT-6CBP. Yeast strains containing CCT-3CBP or CCT-6CBP are viable and grow with phenotypes indistinguishable from wild-type strains and *in vitro* folding assays show that the purified CCT-3CBP and CCT-6CBP are fully functional. This approach has been extended to inserting eGFP domains into the same location in all eight CCT subunits and visualizing them using cryo-EM [73].

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Actin folding and unfolding behaviour *in vitro* and thermodynamics

Actin is totally dependent on CCT for acquiring its correctly folded native state *in vivo* and *in vitro*. The complete dependence of actin folding on CCT prohibits the use of standard analysis of forward folding pathways from denatured states. Instead, the kinetics and energetics of actin folding were first characterized based on the unfolding pathway of actin, using thermal denaturation in conjunction with a DNase I-binding assay to measure the degree of native actin conformation [74]. It is important to understand that the native state of G-actin is stabilized by two cofactors: a tightly bound nucleotide (ATP/ADP) and a divalent metal ion (Mg^{2+} or Ca^{2+}), called "the clasp". The removal of these cofactors, through cation chelation by EDTA or EGTA, breaks the clasp and initiates denaturation of G-actin into an inactive conformation, referred to as I_3, which is unable to polymerize and is aggregation prone. We resolved three spectrally independent species, relating to native actin (N), the denatured state I_3 and the reversible, equilibrium state of cation-free nucleotide-bound actin (I_1) and cation-free nucleotide-free actin (I_2) [75]. EDTA-induced actin unfolding can complete within 10 min, depending on temperature and osmolarity, after which the actin I_2 state can be refolded into native actin by adding CCT-containing rabbit reticulocyte lysate [75,76]. EDTA denaturation of actin is the standard method for producing the CCT-binding ensemble of actin unfolding intermediates in our laboratory [45,57,72]. Figure 5 shows the proposed free-energy landscape for actin folding, unfolding and CCT interactions and is further discussed in the section 'CCT folds actin into a spring'.

Phosducin-like cofactors in yeast — tubulin/PLP1 and actin/PLP2

The phosducin-like proteins (PLPs) are cofactors which enhance the binding of the actin, tubulin and Gβ protein substrates to CCT [77,80]. In most eukaryotes, two different PLPs mediate actin and tubulin binding, essentially acting as specificity factors which enhance the respective substrate complexation

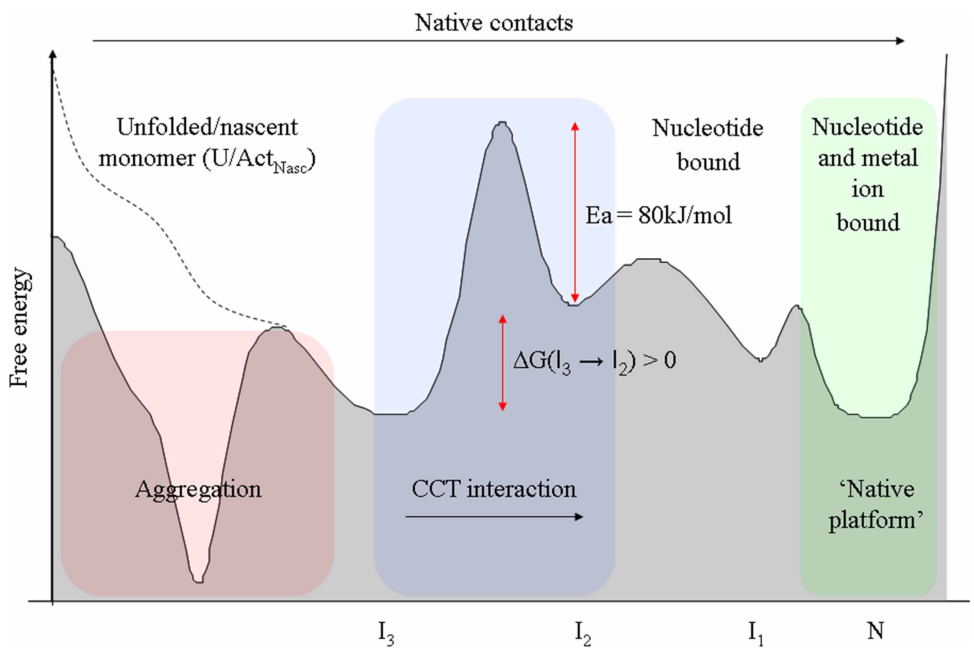

Figure 5. Energy landscape: proposed one-dimensional free-energy landscape for actin folding.

This representation is a one-dimensional simplification of the rough multi-dimensional free-energy landscape for actin. States in rapid equilibrium are grouped and labelled according to the specific folding intermediates that have been experimentally identified: I_1, I_2 and I_3 [75,76]. Monomeric, chemically unfolded or nascent actin, U/Act$_{Nasc}$, spontaneously folds to form the intermediate I_3. At moderate to high protein concentration, this species irreversibly forms aggregates, which may represent the lowest free-energy actin species. At low protein concentration, I_3 is kinetically and thermodynamically isolated with respect to ligand-free folded actin I_2, and further folding of actin independent of CCT is not observed on any physiologically significant time scale. The forward folding reaction (I_3/I_2) is associated with ATP-dependent CCT interaction and activity (arrow in blue-shaded area). I_2 is thermodynamically unstable with respect to the unfolded, CCT-binding, intermediate I_3. Unfolding from I_2 to I_3 is very slow at room temperature > tens of minutes depending upon species and organ source [57,74].

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Table 1 Nomenclature and properties of phosducin-like genes

Group	Phosducin I	Phosducin II	Phosducin III
Human Ph-like genes (UniProtKB primary accession number)	**PDCL (Q13371)**	**PDCL2 (Q8N4E4) PDCL3 (Q9H2J4)**	**TXND9 (O14530)**
Yeast Ph-like genes (UniProtKB primary accession number)	Not present in genome	***PLP2* (Q12017)**	***PLP1* (Q04004)**
Gene deletion *Saccharomyces cerevisiae*	N/A	Lethal	G-protein and β-tubulin inhibition
Gene deletion *Dictyostelium discoideum*	Inhibition of G-protein signalling and $G_{\beta\gamma}$ dimer formation	Lethality/cell division collapse after 5 days	No phenotype
Gene deletion *Caenorhabditis elegans*	Not done	Not present in worm genome	Embryo division arrest with astral microtubule defects
Biochemical functions: (i) Substrate interaction	Competes for CCT binding to $G_t\alpha$ and luciferase (*in vitro*) and actin (*in vivo*) K_i for CCT ~190 nM	Excess GST-PDCL3 inhibits actin folding by CCT *in vitro* (reticulocyte lysate)	Excess TXND9 (2 μM) inhibits actin and tubulin folding by CCT *in vitro* (reticulocyte lysate)
(ii) CCT ATPase	Not tested	Not tested	CCT–actin, CCT–tubulin ATPase inhibited 10-fold
EM structure	1–5 across CCT ring	Not done	1–5 across CCT ring (α/β-tubulin binds in *cis* with PLP1)
CCT-binding sites	H-2, P190, S2-H5 loop, C-ter 24aa	NH_2-ter and C-ter	NH_2-ter and C-ter 8aa

The three monophyletic groups of the phosducin gene family are defined by Blaauw et al. [83]. The Phosducin I group includes the retinal/pineal gland phosducin of vertebrates which does not interact with CCT [81]. In the Phosducin II group, mammals contain an additional, meiotic germ cell-specific gene, PDCL2, and the mouse copy can rescue *S. cerevisiae PLP2* deletion lethality [84]. Biochemical and EM analysis of bovine testis CCT/rat PDCL interactions (PDCL = PhLP1 in refs [81,85]). Biochemical and EM analysis of bovine testis CCT/human TXND9 interactions [80]; TXND9 = PhLP3 in [80,82]. Genetic analysis of PhLP genes: *Dictyostelium discoideum* [83,86], *Caenorhabditis elegans* [87], *S. cerevisiae* [80,88,89]. Yeast PLP2 stimulates yeast CCT–actin folding 30-fold *in vitro* [53].

with CCT. The phosducin-like family of proteins are thioredoxin-fold proteins related to the canonical phosducin proteins which sequester the Gβ/γ subunits of trimeric G-proteins and inhibit interaction with Gα subunits [77,81]. Three subgroups of phosducin-like proteins are present in eukaryotes [82,83] and a summary of their properties is shown in Table 1. Figure 6 shows, in cartoon form, the cellular and biochemical connections between phosducins and trimeric G-proteins, phosducin-like proteins and CCT.

In vivo in *S. cerevisiae*, Plp1p and Plp2p are involved in tubulin and actin biogenesis, respectively [88,89]. Additionally, genetic screens in yeast with temperature-sensitive mutants of Plp2p revealed several genes involved in G1/S cell cycle progression [89] and the TOR pathway component, SIT4, is linked genetically to Plp2, as they share numerous suppressors [89].

Our laboratory showed that Plp2p, but not Plp1p, stimulates actin folding *in vitro* [57], and we developed the following scheme for Plp2p action.

We also found that the dissociation rate of CCT–Ac_I complexes to be faster than the folding rate so that, even in large excess of CCT, only 15% of the actin folding intermediate, Ac_I, is CCT-bound [72]. The utility for an activity such as Plp2p can be easily understood because it shifts the equilibrium to the right by stabilizing the Ac_I–CCT interaction complex. Plp2p increases the yield of folded actin by 30-fold, and we estimated

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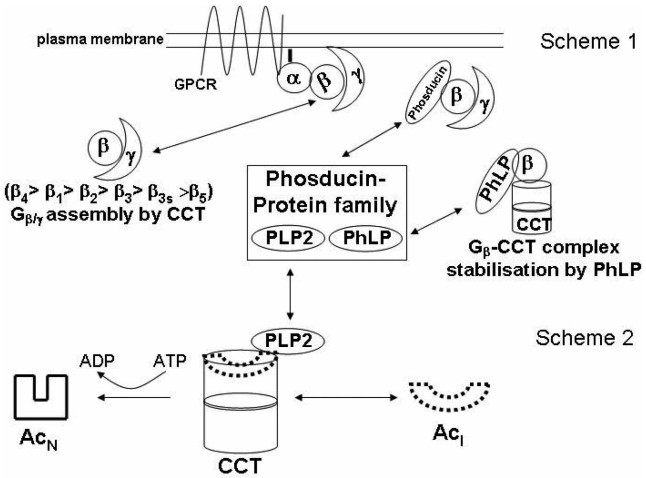

Figure 6. Phosducin-like proteins: connections with trimeric G-proteins, phosducin-like proteins and CCT.
Scheme 1 shows a cartoon of a G-protein coupled receptor (GPCR) interacting with a trimeric G-protein complex $G_{\alpha\beta\gamma}$. For the light-activated, rhodopsin GPCR, the phosducin cofactor acts as a chaperone for sequestration and transport of $G_{\beta\gamma}$ dimers [77]. Several $G_{\beta\gamma}$ dimer pairs in other human GPCR systems are assembled by CCT but to different extents [78]. The human phosducin-like protein, PhLP, stabilizes certain G_{β}–CCT complexes [79]. Scheme 2 shows our model for the role of Plp2p in stimulating actin folding by CCT through stabilization of the CCT–Ac_I encounter complex [57].

that Plp2p has low nanomolar affinity for the CCT–Ac_I complex [57]. Plp2 mRNA synthesis is cell cycle-regulated and this property allows for cell cycle-dependent actin folding [7,90].

CCT–actin system — summary mechanism

Figure 7 shows a schematic map of the interactions between CCT, actin and Plp2p in yeast [16,45,57,72]. The actin map shows the CCT-binding sites, I, II and III [48] and hinges [91], and the essential actin-binding D244 residue located in actin subdomain 4 [53,57], which biochemically cross-links to the CCT8 subunit [16]. Yeast actin (ACT1) binding to yeast CCT induces protease resistance in Cct4p and Cct8p [57]. The PLP2 component of the map in Figure 7 shows its interactions with CCT subunits, CCT1, CCT4 and CCT8 [16].

Stuart et al. [45] established a spectroscopic assay by selectively labelling the C-terminus of yeast actin with acrylodan and observed significant changes in the acrylodan fluorescence emission spectrum as actin is EDTA-unfolded and then refolded by CCT [75]. The variation in the polarity of the environment surrounding the fluorescent probe during the unfolding/folding processes allowed actin to be monitored as it folds on CCT. The rate of actin folding at a range of temperatures and ATP concentrations was determined for wild-type CCT and the $CCT4_{anc2}$. The maximum folding rate is observed at physiological ATP concentrations (2–4 mM), and we note that CCT-folding activity stalls at low ATP concentrations [57]. Binding of the non-hydrolysable ATP analog adenosine 5′-(β,γ-imino)-triphosphate to the ternary complex leads to 3-fold faster release of actin from CCT following the addition of ATP, suggesting a two-step folding process with a conformational change occurring upon closure of the cavity and a subsequent near-final folding step involving packing of the C-terminus to the native-like state.

Finally, we reiterate the importance of using rabbit α-actin to produce the yeast CCT_{anc2}–ACTIN–PLP2 complexes which produced the crystallographic breakthrough [16]. Yeast CCT and Plp2p form an unproductive complex with rabbit α-actin, due to sequence differences in rabbit α-actin compared with yeast Act1p [92], and presumably, the complex stalls during a late, release-like phase of the cycle because there was no Plp2p density observable in the electron density map.

Bacterial actins and eukaryotic actins

Comparisons between bacterial actins and modern eukaryotic G-actin

It is abundantly clear that bacteria contain actin filament systems involved in cell shape control, cell division and other partitioning processes but in a comprehensive review of prokaryotic actin structure and filament

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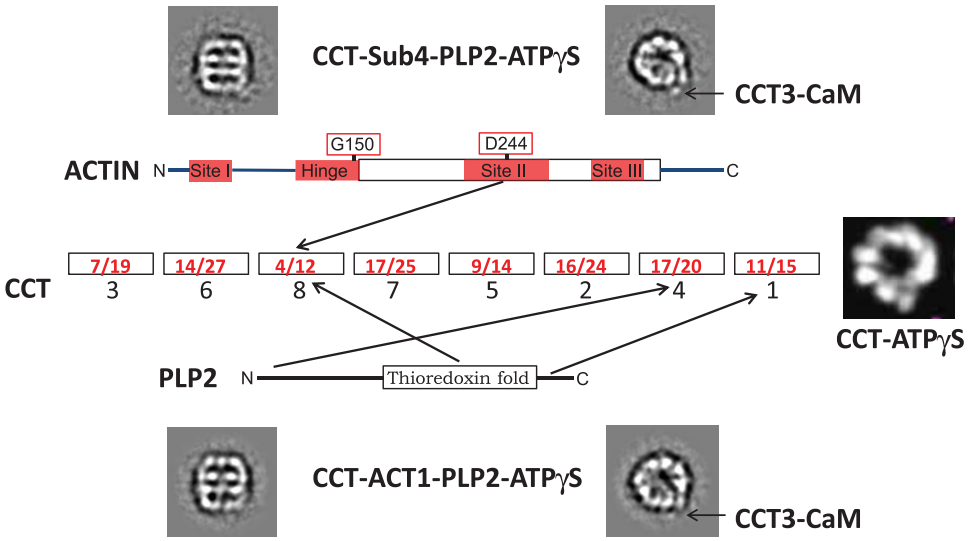

Figure 7. Yeast actin and PLP2-binding map.

The central CCT map shows the subunits numbered according to the order around the ring; the red numbers indicate the evolutionary conservation of the substrate-binding apical domain residues (identical/conserved) according to Dekker et al. [16]. The actin map shows the CCT-binding sites, I, II and III [48] and hinges [91], and the essential actin-binding D244 residue located in actin subdomain 4 [53,57], which biochemically cross-links to the CCT8 subunit [16]. Yeast actin (ACT1) binding to yeast CCT induces protease resistance in Cct4p and Cct8p [57]. The PLP2 map shows its interaction sites with CCT subunits, CCT1, CCT4 and CCT8 [16]. The thioredoxin domain of PLP2 binds ACT1 subdomain 4 and is abrogated by mutation D244S [57]. The four two-dimensional projections of yeast CCT complexes assembled in the presence of non-hydrolysable ATPγS according to refs [16,92] are placed above (CCT-Sub4-PLP2- ATPγS) and below (CCT–ACT1–PLP2–ATPγS) the central map (K. R. Willison, unpublished data). The top views on the right-hand side show the location of calmodulin bound to the CBP-tag in the CCT3 subunit. The handedness of the views is from the grid not from the actual ring order which is opposite. The actin and PLP2 proteins are intricately interacting with CCT subunits on the CCT4,1,3,6,8 arc of the asymmetric assembly. Empty CCT–ATPγS complex top view is on the far right-hand side.

dynamics, and their comparison with modern actins not a single mention of the CCT-mediated folding pathway of eukaryotic actins can be found [93].

The X-ray structures of MreB and ParM, FtsA and MamK show strong structural homology between themselves and with eukaryotic actin. MreB from mesophilic and thermophilic organisms shows very high structural similarity too; the actin/hexokinase/dnaK ATPase fold (AHK-fold) is clearly ancient [94]. There is a large body of structural work on the filaments that these bacterial actins form and much comparison with eukaryotic F-actin structure and behaviour to rationalize the properties of the bacterial cytoskeleton and understand how it evolved into the modern eukaryotic system [95]. But since the two-domain tree of life posits that eukaryotic actin evolved in the Archaeal lineage [96], it is in these bacterial species, presently unknown and possibly even extinct, that we should study the precursor actin systems and perhaps then understand what processes and changes produced the novelty. It is striking that the bacterial actin protein sequences are very divergent from one another but that eukaryotic actin became one of the most evolutionarily conserved proteins after it evolved. Doolittle [97] calculated that the change in the rate of sequence evolution of actin during the transition must have been 10–100-fold higher than the rate of change over the next 2 Gyr. We have argued that the evolution of CCT-dependent folding was the driving force behind the development and fixation of the 375 amino acid residue sequence of the eukaryotic actin ATPase, because it altered the biophysics of the molecule advantageously [76,94]. It is necessary to follow the energy in this story, as physicists are wont to say, but this is a hard task and we cannot observe the energetics by inspection of protein structures or images of filaments in cells.

In considering the differences between bacterial actins and modern actins, a very important element is the asymmetric nature of the filaments formed by each system. F-actin is an asymmetric, right-handed helix

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formed from two parallel filaments and therefore its two ends are always different, regardless of length, and this permits different rates of subunit addition and removal from each end and the existence of different proteins to cap them too. Furthermore, the symmetry breaking is assumed vital for the directional movement of molecular motors on F-actin. Like F-actin, ParM and MamK form pairs of protofilaments with subunits facing the same direction and they grow directionally although they can become dynamically unstable and break catastrophically. ParM polymerization kinetics indicates that conformational changes associated with polymerization does not occur which means they cannot break symmetry [98]. Surprisingly, MreB filaments are constructed from protofilaments paired in an antiparallel arrangement making them unlikely precursors for modern actin because, in an antiparallel, two-filament arrangement, there are always both copies of the subunit orientation at each end and, in the absence of a conformational change of the subunit, the rates of addition and loss at the two ends of a polar polymer must be identical, since every change and displacement at one end must happen at the other too [98]. This has far-reaching consequences for the molecular mechanism of MreB's function in the bacterial kingdom, given the surprisingly high degree of similarity between the structures of MreB from mesophilic and thermophilic organisms, and consequently for the search for the precursor of the modern eukaryotic actins.

Co-evolution and emergence of CCT and actin in LECA

The LECA (last eukaryotic common ancestor) is the precursor organism which gave rise to the entire eukaryotic lineage. Phylogenetic analysis of the CCT genes showed that they emerged as a cluster ∼2 Gyr ago [15], and we suggested that CCT and the ancient actins and tubulins co-evolved to yield the modern eukaryotic actin and tubulin filament systems which, compared with their precursors, had novel biophysical properties and dynamics gained through their elaborate folding pathways [33,94]. Our early model for the evolution of the CCT-cytoskeletal system postulated that the actin/hexokinase/Hsp70 ATP-binding fold (AHK-fold) [99] came to interact with an Ur-CCT through horizontal gene transfer during the mitochondrial symbiosis because early Archaeal genome sequences showed the presence of the thermosome-type II chaperonins, related to CCT, but no AHK folds [94]. But recent phylogenetic studies on the origin and gene contents of primitive cells are challenging the view that there are three domains on the universal tree of life, because new methods place eukaryotic core genes within the Archaea; thus, there are only two primary domains of life, Archaea and Bacteria, and eukaryotes arose through partnership between them [96,100]. The two-domain tree of life model is supported by exciting new data from metagenome sequencing of TACK archaea species which reveals a host of 'eukaryotic' protein systems [101,102]. These organisms contain actin-like genes and actin-binding folds like gelsolins. Interestingly, they also contain a host of WD40 repeat elements [7]. The actin-like proteins are more like ARPs than G-actin and are embellished with many insertions when aligned with the modern actins, which is also the case with eubacterial actins like MreB [93]. Our co-evolution model though still stands because these organisms have the classic Archaeal thermosomes and no-CCT like chaperonin genes. We propose that a Lokiarchaeota-like organism containing CCT and a modern actin cytoskeleton had developed the machinery required to be able to phagocytose the α-proteobacterium in the single unique symbiosis which produced LECA (Figure 8). Koonin [96] identifies phagocytic processes as a vital neo-function for the LECA organism. A phagocytosing cell would have gained huge selective advantage in being able to engulf particles and other cells and over time a variant developed which engulfed an entire bacterium, yielding the mitochondrial symbiosis and LECA.

CCT folds actin into a spring

There is no doubt that all actin-based motility systems, constructed from combinations of particular myosin molecular motors, cross-linking proteins and other protein components, behave macroscopically like springs to generate force and movements at length scales from nanometres to metres. We have long argued that special biophysical properties must have accreted to actin through the CCT-mediated folding process [15,34,94] and have developed a model that actin is a protein spring because G-actin contains stored potential energy (Figure 5), donated by the CCT ATPase cycle, which is recoverable upon ATP turnover in F-actin [76]. The maximum height of the energy surface that α-actin can re-explore in the context of the filament is 80 kJ/mol [75] before the back-transition state is reached and irreversible unfolding occurs and this quantity equates to ∼3–4 molecule's worth of ATP-binding energy. A spectroscopic manifestation of the strain caused by this energy storage is the observation that tryptophan 356 located in subdomain I of yeast actin accounts 51% of the intrinsic fluorescence which is highly blue-shifted [54]. This model gives rise to a deep question though; how, from a microscopic perspective, can a spring be built from protein? Although to some extent all folded

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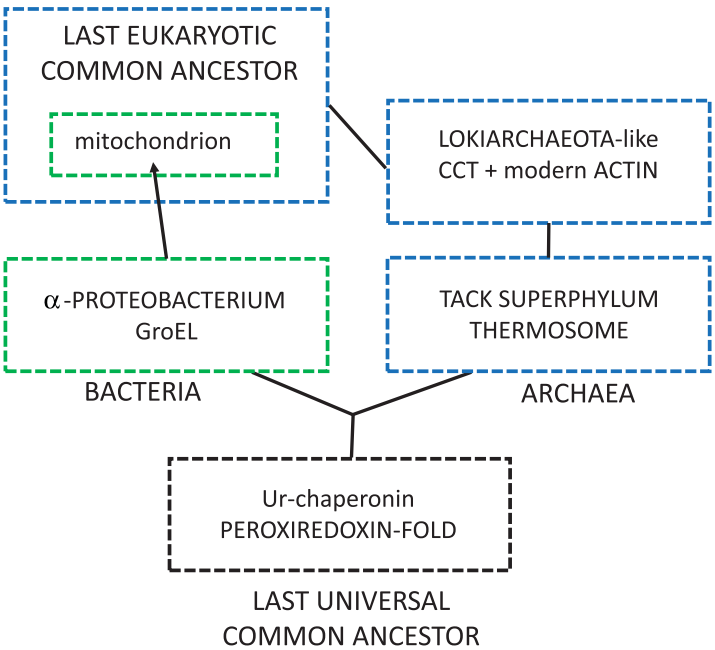

Figure 8. LECA and co-evolution of CCT and the eukaryotic cytoskeleton.

Proposed scheme for the evolution of the CCT-emerging actin system in the Lokiarchaeota-like archaeal organism [101]. The precursor organism (top right panel) probably evolved the ability to phagocytose bacterial particles and existed in a symbiotic niche with other species of bacteria. Eventually, an entire α-proteobacterium became engulfed and LUCA emerged with its internal energy source, the mitochondrion, which allowed cells to become larger. The modern actin cytoskeleton system then developed further complexities and novelties leading to the complex modern eukaryotic cell.

proteins are springs, in general the free-energy changes between the total energies of unfolded and folded states are rather small, $\Delta\Delta G \sim 5$–20 kJ/mol. Furthermore, within a protein structure, the coupling forces between open and closed states or other conformational equilibria are close to k_BT and proteins are buffeted by thermal noise and diffusion and macromolecular crowding inside cells. How has the CCT-folding pathway acted upon actin polypeptide chains such that they can store large amounts of folding energy and recycle it through the filament reproducibly?

All springs are made of compressible materials and are held in their open or closed energy-storing states by clamps or clasps. When a spring exerts force, in its reproducible, Hookian regime, it reduces from an expanded macro-state to a smaller one in accordance with Liouville's theorem [103] (and see Supplementary Data, S2 for a more detailed discussion on reproducibility in molecular machines). Suppose that ATP-G-actin has a larger phase volume than ADP-G-actin; then, upon ATP hydrolysis and phosphate release in F-actin, the filament would be able to contract in some or other dimension(s) and exert force. Relevantly, the partial specific volumes and adiabatic compressibility of ATP-G-actin and ADP-G-actin, in the presence of Ca^{2+}, have been determined by sound velocity measurements in solution [104]. ATP-G-actin has an extraordinarily soft global conformation compared not only to ADP-G-actin but also to all other globular proteins. The difference in partial specific volumes equates to the volume of ATP-G-actin being ∼2% greater than ADP-G-actin.[2] Since the amino acid composition does not vary between the two states, it can only be deposition of water molecules that distinguishes them and 2% of the G-actin monomer volume is equivalent to ∼50 water molecules. Frederik et al. [105] measured the effects of several crowding agents on the relative stability of ATP-F-actin versus ADP-F-actin and constructed a thermodynamic cycle in which ADP-F-actin is less hydrated than ATP-F-actin by 50 water molecules per monomer. They proposed that water is an allosteric regulator of G-actin activity and also that hydration of the nucleotide-binding cleft and other regions of the protein contribute to the molecular basis of actin filament stability. F-actin can be assembled from completely nucleotide-free G-actin monomers

[2]ATP-G-actin: partial specific volume = 0.744 cm^3/g; adiabatic compressibility 8.8×10^{-12} cm^2/dyne. ADP-G-actin: partial specific volume = 0.727 cm^3/g; adiabatic compressibility 5.8×10^{-12} cm^2/dyne.

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as long as they are stabilized in high sucrose solutions to prevent unfolding [106], and our group showed that nucleotide-free G-actin populates the CCT-binding conformation upon dilution from crowding agents [45,57], which experimentally connects unfolding states with F-actin states. Thus, the action of the CCT system is to reconfigure actin polypeptide chains into springs with unusual water-binding behaviour and preservation of folding energy through entropic mechanisms relayed by water molecules. Kabir et al. [107] used microwave dielectric spectroscopy to investigate the hydration shell of F-actin. Apart from the water molecules with lowered rotational mobility that make up a typical hydration shell, there are other water molecules around the F-actin which have a much higher mobility than that of bulk water. This layer of hypermobile water generates a solvent space of axially skewed viscosity along and around the actin cylinder, and they suggested that, upon interaction with myosin, an asymmetric space for diffusion is dynamically generated and myosin moves towards the direction of lower viscosity thus breaking the symmetry of the system [107]. We propose that the asymmetric water surface pre-exists in F-actin and that myosins can recognize it specifically but weakly. Cryo-EM reconstructions of F-actin filaments show that ADP-actin subunits are rotated by ∼12° with respect to the filament axis [108], and this motion is the observable macro-conformational change of the actin spring.

Implications for actomyosin

The formation of the actomyosin bond is exothermic and is driven by an increase in entropy resulting from the formation of a large hydrophobic interface between the headpiece of myosin and the underlying actin monomer target in the filament. When this binding energy is converted into mechanical work, the second law states that the work obtained must be paid for by a decrease in enthalpy or an increase in entropy. Since the first law requires conservation of enthalpy during a reaction, it seems therefore that the entropic penalty can only be paid for by absorption of Brownian heat from the medium. The problem with this type of Brownian ratchet idea is that the rate at which work can be performed by trapping thermal fluctuations depends strongly on the magnitude of the fluctuation trapped, as recognized originally by Huxley & Simmons [109]. Howard [110] has considered the time required for a protein the size of the myosin head to diffuse against an elastic load over a barrier of energy E:

$$t_k = g\left[\frac{k\mathrm{RT}}{E}\right]^{-1/2}\exp\left(\frac{E}{\mathrm{RT}}\right)$$

where k is the spring constant of the load and g is a constant. This equation estimates t_k = 1–2 ms for E = 25 kJ/mol but 15–30 s for 50 kJ/mol. Estimating 50–60 kJ/mol of ATP turnover and a muscle engine working at 60% efficiency, then the energy cannot be absorbed sufficiently rapidly by capturing a single fluctuation [103].

Transient time-resolved FRET has been used to determine the structural kinetic mechanism of the myosin power stroke, and it shows that actin induces rotation of the myosin light chain domain (LCD) to initiate the power stroke before phosphate dissociates from ATP-myosin and not afterwards as many models have proposed [111]. Muretta et al. [111] argue that power is not generated by the free energy of the LCD rotation itself but also by the concomitant formation of the actin–LCD-binding interface and the release of Pi which inhibits reversal of the binding transition. This mechanism is analogous to a 'thermally' activated ratchet and consistent with the original proposal [109] that there are two thermodynamic components to the myosin power stroke, first a fast elastic transition (>500 s^{-1}) followed by a second slower viscoelastic one. The cost of displacing a tightly bound water molecule during a protein interface collision is ∼8 kJ mol^{-1} at 310 K. This is an upper limit because loosely bound water molecules cost less to displace and hypermobile molecules in a second shell would incur even less penalty. Muretta et al. [111] suggest that the free-energy change for the reversible fast (f = 2 ms) elastic transition is ∼4.5 kJ mol^{-1}, only 2kT. If the actin–myosin interaction is comprised from entropic changes in groups of loosely interacting water molecules, it would permit an intricate, multivalent and accurate recognition process to happen between myosin and actin cheaply and without commitment to the irreversible fully engaged interaction of the power stroke. The viscoelastic, less reversible, second transition is the power stroke which converts/amplifies the structural change into directional force. This postulated water surface in conjunction with irreversible phosphate release is the symmetry breaking spring and pawl of the Brownian ratchet mechanism proposed 60 years ago by Hugh Huxley. The myosin 'power stroke' is actually a timed commitment stroke. Our new perspective helps in understanding the high efficiency of the muscle machine; unproductive myosin-ATP engagement with actin costs very little and does not waste a hydrolysis

cycle but just leaves the system to wait stochastically until the next myosin-binding site appears on the underlying actin filament water surface. The timing of the engagement between actin and myosin must be critical for the efficiency of the engine because late release will cause wasteful energy dissipation; the reproducibility argument of Jaynes [103]. It is logical to assume that the most rapid irreversible step in the system, which is the tight-binding step, is controlled by irreversible phosphate release from myosin-ATP.

A helpful analogy to consider is the one of pushing a child on a swing. The most effective point at which to push in time and space is just as the down stroke begins at the end of top of the rise. The binding site, the child on the seat (the myosin-binding site on actin) arrives, your hands (myosin) engage (actomyosin bond formation) at the stationary point to avoid energy loss (phase-dependent for actomyosin) and commit (ADP-Pi) and you let go (Pi release/ADP/ATP exchange on myosin) after some time period which is dependent on how much force you want to impart to the swinging child during that particular push. There is some similarity between our actin water shell recognition step and the first step of pushing the child; it is the phosducins and actin-regulated receptors which provide the visual information for the initial recognition process which allows the gentle interaction with the child's back!

In summary, our view is that this mutual recognition behaviour of actomyosin is the result of the elastic water surface produced ultimately by the CCT folding of actin. Over a long period of ∼2 Gyr, evolution and selection have designed a complex water surface which has enough unique structural properties to be weakly but specifically recognized by myosin and permit rapid and reversible engagement of a pair of large protein surfaces in the energetically cheapest fashion possible. New theoretical and computational approaches find that the water in the protein surface environment can be treated as two populations: one population of immobile molecules stuck in deep traps and another population of mobile molecules jumping between shallow traps [112,113]. Further investigation of G-actin and F-actin surface water structure and the role of the folding landscape in producing scale-free waiting times and actin polymer ageing is required to test these ideas concerning the actomyosin recognition mechanism [113].

Human CCT and disease

Inherited CCT syndromes

Hereditary sensory neuropathies show degeneration of the fibres in the sensory periphery neurons. Two mutations have been identified in CCT subunits (Table 2). CCT4 (C450Y) was identified in a stock of Sprague–Dawley rats and CCT5 (H147R) was found in a Moroccan family [114,115]. Interestingly, both CCT4 and CCT5 can be assembled into homomeric hexadecamers which allow them to be studied individually in the context of an assembled ring system [115]. Recently, Adams' laboratory has obtained the X-ray structure of closed forms of human CCT5 homo-oligomeric complex and the CCT5 mutant associated with the sensory neuropathy at 3.5 and 3.6 Å [116]. It may be possible to build the other seven homomeric CCT complexes *in vitro* and also use them as scaffolds to investigate CCT mechanism and post-translational modifications and develop drug and small molecule screening platforms for individual CCT subunits.

CCT-related genes

CCT has been found to have a role *in vivo* in ciliary function through interaction with Bardet–Biedl syndrome proteins some of which are homologous to CCT [157]. Bardet–Biedl syndrome is a polygenic disorder causing ciliary dysfunction in humans. Knockdown of cct1 and cct2 in zebrafish leads to BBS-like phenotypes [158]. Three BBS proteins which have homology to chaperonins, BBS6, BBS10 and BBS12, and a sub-complex of CCT proteins (CCT1, 2, 3, 4, 5 and 8) mediate the association of two β-propeller domain-containing proteins, BBS7 and BBS2, during the assembly process of the BBSome [158,159]. BBS6, -10 and -12 are vertebrate-specific proteins and it may be an evolutionary connection that one of the two absent CCT subunits in the BBS–CCT complex, CCT6, has a vertebrate-specific isoform, CCT6B, which is abundant in testis CCT. CCT6 self-interacts across the CCT rings which probably permits isoform interchange [16,35], and therefore, it is possible that one of the BBS subunits has hijacked this mechanism and is able to slot into the CCT6 position in the CCT ring system.

Cancer and signalling

CCT perturbation indicates roles for the holocomplex and individual subunits in biological homeostasis and signalling processes. Early studies in yeast connected CCT6 activity to the TORC pathway [42], and the entire

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Table 2 Involvement of CCT in biological processes and disease states

Reference	Gene or process	Organism	CCT subunit	Biological system/process
[71,117,118]	VHL	Human	CCT7	Tumour suppressor
[119–121]	p53	Human	CCT2	Tumour suppressor
[122]	STAT3	Human	ND	Breast cancer signalling
[123]	PDC5	Human	CCT2	Apoptosis/tubulin
[124]	BAG3	Human	Entire CCT	Apoptosis
[125,126]	AML-ETO	Human	CCT8	Leukaemia
[127]	LOX1	Human	Entire CCT	Artherosclerosis
[128]	PHD3	Human	Entire CCT	Prolyl-4-hydroxylase
[129]	SMRT-HDAC3	Human	Entire CCT	Chromatin
[130]	TCAB1	Human	Entire CCT	Telomerase
[131]	α-synuclein	Human	Entire CCT	amyloid fibrils
[121,132]	Breast cancer	Human	TCP-1, CCT2	Drives survival
[133]	Glioma	Human	CCT8	mRNA overexpression
[134–136]	Colorectal cancer	Human	CCT2	mRNA overexpression
[137]	Head and Neck	Human	CCT8	mRNA overexpression
[138]	Lymphoma	Human	CCT8	mRNA overexpression
[139]	Small cell lung cancer	Human	CCT2	mRNA overexpression
[140]	Uterine cancer	Human	CCT2	Chemotherapeutic target
[141–144]	Hepatocellular cancer	Human	CCT3, CCT8	mRNA overexpression
[114,116,145]	Sensory neuropathy	Human	CCT5 (H147R)	Inherited syndrome
[114,116,145]	Sensory neuropathy	Rat	CCT5 (C450Y)	Mutant strain
[146]	Fibroblast motility	Human	CCT7	Motility
[65]	Autophagy	Human	CCT2,5,7	Required for function
[147]	Mitotic checkpoint	Human	Entire CCT	Disassembly of MCC
[148]	Sarcomere Z-disk	*Danio rerio*	CCT5 (G422V)	Thin filament assembly
[36]	Spermatogenesis	Flatworm	CCT1,3,4,8	Spermatogenesis
[149]	Cilia assembly	*Tetrahymena*	CCT3, CCT7	Microtubules
[150]	Invasion	*C. elegans*	CCT5	Basement membrane
[151]	Microvillus	*C. elegans*	Entire CCT	Intestinal epithelium
[152]	Lifespan extension	*C. elegans*	CCT8	Increased CCT
[153]	Fragile X-linked	*Drosophila*	CCT1	Cell identity
[65]	Autophagy	*Drosophila*	CCT5	Required for function
[154]	Protein translocation	*Arabidopsis*	CCT8	Stem cell identity
[155]	Morphogenesis	Yeast	CCT8	Ras signalling
[156]	Cell polarity	Yeast	CCT	Polarisome

TOR–CCT–phosphatase axis is highly conserved through to humans [160], although there are many more components of the network in humans (Supplementary Data S3). Chemical genetic screens discovered impairment of yeast fitness to rapamycin in diploids hemi-zygous for CCT subunit genes. CCT acetylation and lifespan extension are perturbed in yeast SIR2 mutants and TOR is clearly involved in lifespan regulation in worms and flies [156]. TOR complexes (TORC1 and TORC2) drive temporal control of cell growth through regulation of translation, ribosome biogenesis, nutrient import and autophagy. Table 2 shows a list of substantiated connections between CCT activity and biological processes in cancer and signalling processes, including invasion phenotypes in worms, stem cell behaviour in plants, biogenesis of cilia and fragile X syndrome.

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Table 3 The human protein atlas database and CCT expression

CCT1/TCP1 overexpression liver and breast unfavourable. Cytoplasm only.
CCT2 overexpression is unfavourable for liver, prostate, head and neck. Cytoplasm only.
CCT3 liver and renal overexpression. Cytoplasm and plasma membrane.
CCT4 liver and breast prognostic, seminoma highly expressed. Cytoplasm mainly but nucleoplasm also.
CCT5 overexpression is a prognostic marker for liver and renal cancer (unfavourable outcome). Cytoplasm and heavily expressed in nucleoli.
CCT6A liver, renal, breast, head and neck. Cytoplasm. Single cell intensity variation in U2 OS cells.
CCT6B not prognostic.
CCT7 liver, endometrial prognostic. Cytoplasm only.
CCT8 liver prognostic. Cytoplasm and nucleus and intermediate filaments.

CCT subunit mRNA levels are often unregulated in cancer (Table 2), and CCT protein overexpression is highly correlated with poor prognostic outcome in the human protein atlas database (Table 3).[3] This database also provides hugely useful information about CCT subunit protein expression and subcellular localization in cells and tissues and their prognostic utility in a large set of human cancers (Table 3).

The CCT2/β gene is proposed to have a functional role in mediating the p53 response because its down-regulation in human TERT fibroblasts confers resistance to both p53-dependent and p19ARF-dependent proliferation arrest, and abolishes DNA damage-induced G1 cell cycle arrest [119]. TORC1 regulates ribosome biogenesis via S6K and 4E-BP1, and the CCT2 subunit is a direct phosphorylation target of S6K [161,162] as is PDCL3, the human orthologue of yeast Plp2p (K.R. Willison, unpublished data).

CCT2 is an Epi-driver gene conferring selective growth advantage in tumours [10] and is one of a set of 67 cancer genes tested for epistatic interactions with each other to find subsets of correlated genes [163]. CCT2 is a member of subcluster II (MEN1, CCT2, FBN1 and TSC1) with roles in the regulation of focal adhesion and cell migration [163]. Willison [7] analyzed CCT gene epistasis in yeast and found that CCT genes are highly positively epistatic as predicted by his CCT-folding flux sensor model. Perhaps, the reason that CCT genes are epi-drivers in human cancer is due to the perturbation of the CCT flux sensor by polyploidization [164]. Amplification of one or two CCT genes will upset the replication and/or activities of CCT and perturb the very tight copy number distribution of CCT proteins resulting in aberrant calculation of actin and tubulin folding flux by the CCT interactome [7].

Malaria parasites and CCT

Parasites of the *Plasmodium* genus cause malaria and they move using actomyosin motors and associated proteins assembled in a complex called the glideosome. The *Plasmodium falciparum* actin proteins are more divergent compared with other eukaryotic actins, ∼80% homologous, and so are their eight CCT complex and three phosducin-like cofactor proteins [165]. PfAct1 is the actin component of the glideosome and its crystal structure displays no electron density for the last 10 residues at the C-terminus [166]. This has never been observed before in actin structures and points to some unusual stability features which may be related to its folding pathway and the CCT step involving the packing of the C-terminus in subdomain 1 [45]. Olshina et al. [165] found that PfAct1 could not be folded by rabbit or yeast CCT to yield native actin able to bind DNase I or gelsolin. Spillman et al. [167] showed CCT/TRiC to be an oligomeric complex in *Plasmodium* cytoplasm and that knockdown of the CCT8/θ subunit led to a severe growth defect in asexual development but did not alter protein trafficking in the RBC compartment. The PfCCT genes have been identified as some of the most up-regulated genes in artemesinin (ART)-resistant parasites [168] and when Ismail et al. [169] designed click chemistry-compatible activity-based probes incorporating the endoperoxide scaffold of ART, they identified CCT subunits and actin and tubulin as ART molecular target(s) in the asexual stages of the malaria parasite. Mutations in the propeller domain of the K13 propeller gene are commonly found in ART-resistant *Plasmodium falciparum* isolates in SE Asia and K13 propeller polymorphism seems to be a useful molecular marker of clinical ART resistance [170]. Kelch 13 is a WD40 β-propeller protein which is a common CCT

[3]Human Protein Atlas database: https://www.proteinatlas.org/ENSG00000150753-CCT5/pathology.

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substrate and CCT-binding protein [7] and, together, all this data suggest a central role of the CCT axis in malaria biology and possibly ART resistance.

Outlook

Intervention and chemical genomics

In addition to the long established tubulin inhibitor, benomyl, and actin inhibitor, lantrunculin, chemical biology approaches in *S. cerevisiae* have identified anti-fungal azoles coupled to CCT activity [171]. In the yeast *Candida albicans*, CCT is a key modulator of echinocandin susceptibility [172]. Echinocandin is the first-line therapy for candidiasis. Progress is being made in developing cell-based CCT inhibitors using peptide reagents [131,140,173] and HSF1A, a benzyl-pyrazole-based small molecule [174]. The development of chemical probes and inhibitors of CCT, which are selective for different eukaryotic organisms, will be an important area for future work.

Mechanism and cell biology of CCT

Further high-resolution structural analysis of CCT–actin complexes in the open and closed states are required to define further the atomic interactions between specific CCT subunits and their binding sites on actin folding intermediates [16] and the sequential nature of the annealing pathway which completes with the packing of the C-terminus of actin in subdomain 1 [45]. Higher resolution electron microscopy studies will highlight the role of nucleotide binding and hydrolysis in the extraordinary subunit motions in the open forms of mammalian and yeast CCT [51] and relate these to the kinetics of sequential ATP hydrolysis [59]. We need to understand what mismatches in binding sequences or timing prevent yeast CCT from productively folding mammalian α-actin [92] and stop mammalian CCT from folding native malarial actin with an incorrectly packed C-terminus [165]. The mechanism of CCT assembly in cells is little investigated so far [60,175], and further investigation should illuminate the roles of monomeric subunits, CCTδ and CCTε [176] in assembly processes and cellular regulation [177,178]. CCT is involved in interactions with Q/N-rich proteins [61] and amyloid [131] through subunit specific interactions and further understanding of these processes may help understand the *in vivo* roles of CCT in protein aggregation disease states.

Genetic approaches, especially in *S. cerevisiae*, have shown that individual CCT subunits have different 'functions' in cells [71] and much further work is required to unpick the interactions between the core CCT substrates [7] and CCT. We also need to relate the binding, folding and assembly processes that CCT mediates to our model in which CCT interactions allow networks to register the flux of newly folded cytoskeletal proteins which couple to cell growth control and time-dependent cell cycle mechanisms [7].

Abbreviations

Anc2, actin non-complementing 2; CCT, chaperonin-containing TCP-1; LCD, light chain domain; LECA, last eukaryotic common ancestor; LUCA, last universal common ancestor of all organisms; PLPs, phosducin-like proteins; TCP-1, Tailless complex Polypeptide 1; TRiC, TCP-1 ring complex.

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Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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