THE EXTENDED ROLE OF THE MOLECULAR CHAPERONE CCT

ANDREAS SVANSTRÖM

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Faculty of Science

Department of Chemistry and Molecular Biology

The Extended Role of the Molecular Chaperone CCT
© Andreas Svanström

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Dedicated to my family

“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”

- Albert Einstein

ABSTRACT

The oligomeric chaperone CCT is a large ATP-dependent chaperonin that consists of two rings placed back-to-back with eight different paralogous subunits with a size of ~ 55 kDa that sit in each of the two rings. The function of CCT is mainly to fold the abundant proteins actin and tubulin, components of the cytoskeleton. However, several studies have shown that CCT has a wide diversity of low-abundant substrates. In addition, CCT and monomeric subunits of CCT have been shown to influence cytoskeletal organization and processes that the cytoskeleton mediates. The aim of this thesis was to study the role of CCT beyond the folding of proteins.

We have overexpressed the subunits of CCT as monomers and demonstrated that monomeric CCTδ has an unknown function at the plasma membrane. The overexpression of monomeric CCTδ mainly induced lamellipodia retraction fibres and the function of monomeric CCTδ at the plasma membrane was shown to be dependent on a wild-type ATP-binding site and a wild-type apical domain of CCTδ. By reducing the levels of individual subunits of CCT, we report in a second study a function of CCTε to regulate the activity of the transcription factor SRF, which controls the transcription of cytoskeletal genes such as actin, via the transcription activator MRTF-A. Cells depleted of CCTε have an increased SRF-mediated transcription in an SRF-luciferase gene reporter system. Monomeric CCTε was shown to interact directly with MRTF-A and the interaction site was identified as the apical domain of CCTε and the c-terminal half of MRTF-A. Consistent with an increased SRF-transcription upon the reduction of CCTε levels, the overexpression of monomeric CCTε delayed the translocation of MRTF-A to the nucleus in serum-stimulated cells. In our final study, we addressed the possibility of CCT to affect the number of actin filaments via the interaction between CCT and the actin filament severing protein gelsolin. We showed that CCT binds to the activated severing conformation of gelsolin and that CCT inhibits activated gelsolin to sever actin filaments.

Taken together, we present several studies that independently identify the CCT oligomer, or its individual subunits, to affect processes related to the cytoskeleton. Thus, there is a close interplay between CCT and the cytoskeleton that extends beyond the dependency of actin and tubulin to be folded by CCT.

Keywords: Actin, CCT oligomer, CCTδ, CCTε, Cell morphology, Gelsolin, MRTF-A.

ABBREVIATIONS

AMP-PNP	Adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate
ATP	**A**denosine **t**riphos**p**hate
BSA	**B**ovine **S**erum **A**lbumin
CAS	**C**haperone **A**ssisted **A**utophagy
CCT	**C**haperonin **C**ontaining **T**CP-1
Cdc20	**C**ell **D**ivision **C**ycle protein **20**
Cpn	**C**ha**p**eroni**n**
DAG	**Dia**cyl**g**lycerol
DNA	**D**eoxyribo**n**ucleic **a**cid
EGFP	**E**nhanced **G**reen **F**luorescence **P**rotein
EGTA	**E**thylene **G**lycol **T**etraacetic **A**cid
ER	**E**ndoplasmic **R**eticulum
ERM	**E**zerin/**R**adexin/**M**oesin
FRET	**F**luorescence **R**esonance **E**nergy **T**ransfer
FqRET	**F**luorescence **q**uenching by **R**esonance **E**nergy **T**ransfer
GR	**G**lucocorticoid **R**eceptor
HSP	**H**eat **S**hock **P**rotein
IP_3	**I**nositol tris**p**hosphate
MAP	**M**icrotubule **A**ssociated **P**rotein
MICAL-2	**MIC**rotubule **A**ssociated monooxygenase, calponin and **L**IM domain containing **2**
mRNA	**M**essenger **RNA**
MRTF-A	**M**yocardin **R**elated **T**ranscription **F**actor **A**
NAC	**N**ascent **A**ssociating **C**omplex
NHE1	**N**a-**H** **e**xchange isoform **1**
NPF	**N**ucleation **P**romoting **F**actor
PI3K	**P**hosphatidyl**i**nositide **3**-**K**inases
PIP_2	**P**hosphatidyl**i**nositol bi**p**hosphate
PLC	**P**hospho**l**ipase-**C**
RNA	**R**ibo**n**ucleic **a**cid
S6K	**S6**-**K**inase
sHSP	**S**mall **H**eat **S**hock **P**rotein
SRF	**S**erum **R**esponse **F**actor
SRP	**S**ignal **R**ecognition **P**article
SS	**S**ignalling **S**equences
STAT3	**S**ignal **T**ransducer and **A**ctivator of **T**ranscription **3**
TCAB1	**T**elomerase **Ca**jal **B**ody protein **1**
TCP-1	**T**ailless **C**omplex **P**olypeptide **1**
TM	**T**rans-**M**embrane
UPS	**U**biquitin **P**roteosome **S**ystem
VHL	**V**on **H**ippel–**L**indau tumor suppressor protein

PUBLICATIONS

I. Spiess, M., M. Echbarthi, **A. Svanström**, R. Karlsson and J. Grantham (2015). "Over-Expression Analysis of All Eight Subunits of the Molecular Chaperone CCT in Mammalian Cells Reveals a Novel Function for CCTdelta." J Mol Biol 427(17): 2757-2764.

II. Elliott, K. L., **A. Svanström**, M. Spiess, R. Karlsson and J. Grantham (2015). "A novel function of the monomeric CCTepsilon subunit connects the serum response factor pathway to chaperone-mediated actin folding." Mol Biol Cell **26**(15): 2801-2809.

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PREFACE

Life has evolved during billions of years in a process first described by Charles Darwin called natural selection, wherein an organism's survival depends on the beneficial traits arisen from changes in their genome. This evolutionary pressure has resulted in the life seen on earth today, including humans. Our genome contains several thousands of genes and depending on which of these genes are active, different cells at a specific place and with a certain function are produced to constitute our organs. Each cell possesses its own individual intracellular biological system where a key process is the transcription of DNA to RNA and the translation of RNA to protein, also referred to as the central dogma. The cell then utilizes the RNA and proteins to be able to fulfil necessary processes. Christian Anfinsen first described that a proteins's amino acid sequence determined the overall structure of the protein. Obtaining a correct structure, known as a native state, is essential for a protein to become biologically active and to fulfil its cellular responsibilities. A group of proteins called molecular chaperones are proteins that assist other proteins that require aid to obtain their native state. A lack of such assistance and consequently the failure of such a protein to reach its native state may lead to malfunctioning cellular processes due to a loss-of-function of the specific protein. Moreover, these non-native proteins can also form toxic protein aggregates. The focus of this thesis is the chaperonin containing TCP-1 (CCT), which is a molecular chaperone that is essential for the folding of two abundant proteins, actin and tubulin. These proteins are major components of the cytoskeleton, an intracellular skeleton that controls important processes including those that determine cell morphology, vesicle and protein transport as well as cell motility. Furthermore, CCT influences the cytoskeleton network beyond the folding of actin and tubulin, which is demonstrated by the requirement of CCT to fold proteins related to the actin cytoskeleton, such as myosin II, and by CCT to influence actin dynamics. The work presented in this thesis focuses specifically on the role of CCT beyond its interactions with folding substrates.

INTRODUCTION

PROTEOSTASIS

Protein homeostasis, known as proteostasis, comes from the Latin “Staying the same” and is defined as the balance of protein synthesis, protein folding and protein degradation (Figure 1), which is essential for maintaining cellular health. The molecular chaperones are a group of proteins named after the word chaperone, which is derived from the purpose of guarding a process to ensure that the process is accurately performed. The molecular chaperones are constitutively expressed, such as CCT, or expressed upon stress, such as the heat shock proteins (hsp), and function to assist in protein folding and protein assembly. Therefore, the molecular chaperones are essential for upholding the cellular health.

PROTEIN FOLDING

Proteins are constituted of a chain of amino acids, a polypeptide, which is made at the ribosome from the translation of a template messenger RNA (mRNA). A single mRNA chain interacts with several ribosomes, forming a complex known as the polysome (Kiseleva, 1989), so translation of several proteins can occur simultaneously. The proteins emerging from the ribosomes contain amino acids that can be hydrophilic or hydrophobic in their nature, which will influence the folding of each protein. As the cellular environment is hydrophilic, the hydrophobic amino acids are normally buried within the core of a correctly folded, native, protein (reviewed by Ellis and Minton, 2006).

Although proteins fold by the rules of thermodynamics to a native conformation that harbours the lowest level of free energy, a large pool of proteins need assistance to over-come energy barriers that hinder possible intermediate-folding and misfolded states to reach a native state (reviewed by Bartlett and Radford, 2009; Kim et al., 2013). A protein lacking this folding assistance may instead end up exposing the hydrophobic amino acids on its exterior and aggregate as a result of having the hydrophobic amino acids interact with hydrophobic residues of other proteins. The requirement of having a folding assistance is evident in a cellular environment where proteins endure a crowding effect caused by the high cellular macromolecular concentration (200-300 mg/ml) that promotes protein misfolding and protein aggregation (reviewed by Ellis and Minton, 2006; van den Berg et al., 1999).

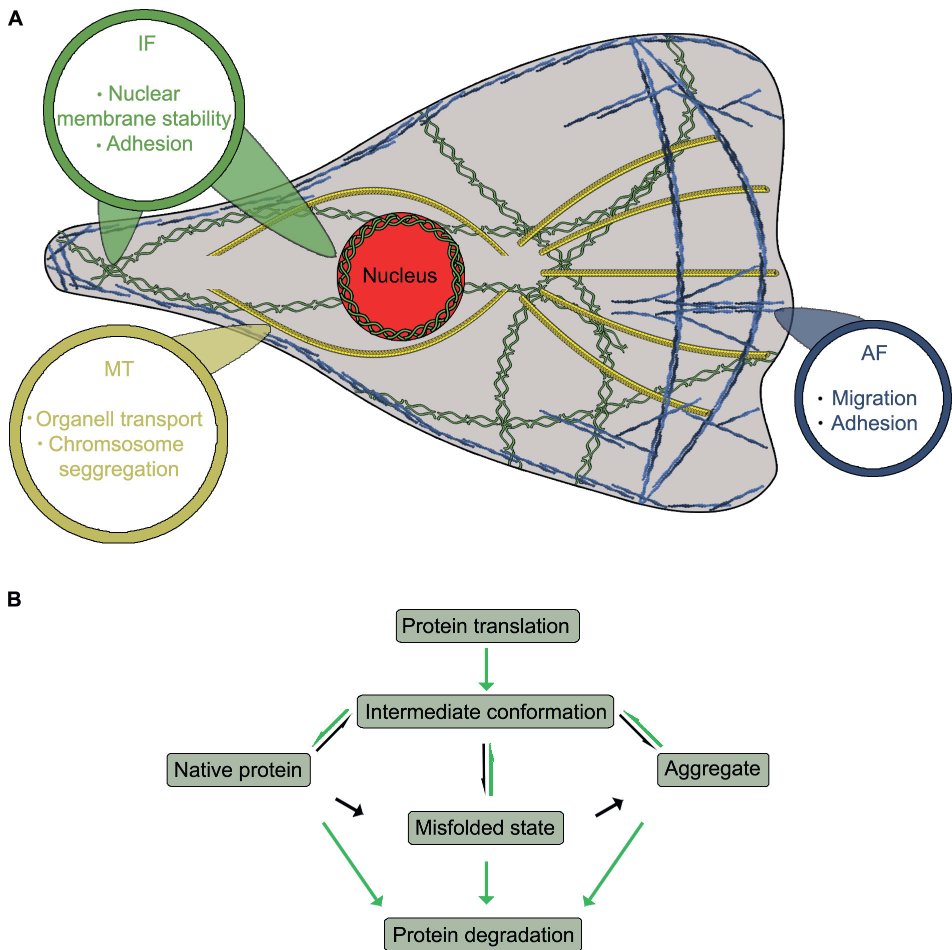

Figure 1 The mammalian cell. A The cell morphology and several subcellular processes are controlled by the cytoskeleton (MT: Microtubules, AF: Actin filaments, IF: Intermediate filaments)(reviewed by Pollard and Earnshaw, 2008) in a highly crowded environment. **B** Proteostasis is tightly regulated by molecular chaperones to promote the correct folding of proteins. The steps in proteostasis that are mediated by molecular chaperones are indicated by green arrows, whereas black arrows indicate steps that may result in endangering cellular health. Native and non-native proteins are degraded via Ubiquitin/Proteosome System (UPS) or via autophagy.

During protein translation, discontiguous amino acid interaction sites within a native protein will not be able to form as the protein sequence is not complete. Thus, prior to a complete protein translation, the polypeptide may attain non-native amino acid interactions that are energetically favourable in an incomplete protein sequence. Furthermore, although translated proteins have their complete set of amino acids that are required for a protein to reach a native state, proteins may still arrest in a non-native state. Thus, the molecular chaperones may, depending on the specific protein, be required both co- and post-translationally in order to aid proteins to become native. In addition to the requirement of chaperones during protein synthesis, chaperones are also required during cellular stress. In general, a protein that reached a native state is a vulnerable structure as a consequence of it being held together by weak internal non-covalent bonds. Thus, different stress factors such as heat may therefore unfold a native protein (reviewed by Ellis and Minton, 2006). In conclusion, molecular chaperones are required co- and post-translationally to ensure that proteins attain a native state and during various stress-conditions to re-fold denatured proteins to a native state.

The mammalian molecular chaperone network is diverse and includes many stress-induced hsp's, proteins originally shown to have an induced expression during heat-stress (Kelley and Schlesinger, 1978 and reviewed by; Kim et al., 2013), and their constitutively expressed homologues. Categorized by size, these are the small hsp's (shsp's), hsp40, hsp60, hsp70, hsp90 and hsp110. The ability of a cell to fold and assemble proteins also relies on several other chaperones such as HOP and Prefoldin as well as the molecular chaperone CCT (reviewed by Kim et al., 2013). The importance of an individual chaperone is reflected by the cellular consequences of having a chaperone deleted, such as increased protein aggregation or cell death. The genes encoding for the subunits of the molecular chaperone CCT have been shown to be essential for cell viability in yeast (reviewed by Stoldt et al., 1996).

THE INITIAL INTERACTION OF PROTEINS WITH CHAPERONES

In eukaryotic cells the emerging polypeptides at the ribosomes during mRNA translation encounter the ribosomal binding proteins nascent-chain-associating-complex (NAC) and signal recognition particle (SRP). While SRP mainly binds and targets peptides to the endoplasmic reticulum (ER) by recognizing signalling sequences (SS) and trans-membrane (TM) regions of the emerging peptides, NAC has been shown to interact with virtually all polypeptides (del Alamo et al., 2011). In yeast, deletion mutants of SRP (Nyathi and Pool,

2015) and NAC (Koplin et al., 2010) have shown that only a SRP deletion has an effect on protein folding, where cells display increased protein aggregates. Never-the-less, deleting NAC increased the expression of molecular chaperones such as hsp70 (del Alamo et al., 2011), whilst a double knockout of both NAC and hsp70 increased protein aggregation in comparison to hsp70 alone (Koplin et al., 2010). Additionally, overexpressing NAC in SRP deletion mutants results in a reduction of protein aggregates (Nyathi and Pool, 2015). These data suggest that both SRP and NAC function as molecular chaperones in a first line of defence toward protein misfolding (Figure 2).

Hsp40/hsp70

The nascent proteins emerging at the ribosomes have been shown to interact co-translationally with hsp40 and hsp70 (Beckmann et al., 1990; Frydman et al., 1994). Hsp40 is an ATP-independent chaperone that passively inhibits protein misfolding and protein aggregation by binding to target proteins (Langer et al., 1992) and functions to deliver the proteins to hsp70 (Szabo et al., 1994) (Figure 2). Hsp70 is in contrast to hsp40 an ATP-dependent chaperone that according to the conventional model passively reduces the risk of a protein to misfold and aggregate by kinetic partitioning. In this model, ATP-bound hsp70 transiently sequesters proteins exposing hydrophobic residues. Upon ATP-hydrolysis, a process strongly induced by hsp40 (McCarty et al., 1995), hsp70 undergoes conformational changes that trap the interacting protein, without effecting the folding conformation of the specific protein. As a result, the protein will have a decreased risk of misfolding and aggregating. Following the release of the substrate from hsp70, the substrate will have the chance to fold to a native state (reviewed by Goloubinoff and De Los Rios, 2007). Substrate binding and release is a combinatory result of the ATP-hydrolysis of hsp70 and of nucleotide exchange factors (NEF's) that exchange ADP to ATP to release the hsp70 bound substrate (Szabo et al., 1994).

Translated proteins with an intermediate or misfolded state may have non-native residues exposed on the exterior structure of the protein. The hydrophobic residues that are recognized by hsp70 will be used by hsp70 to unfold the proteins, where hsp70 may if required undergo several cycles of substrate binding and release in order to unfold the proteins (reviewed by Finka et al., 2015).

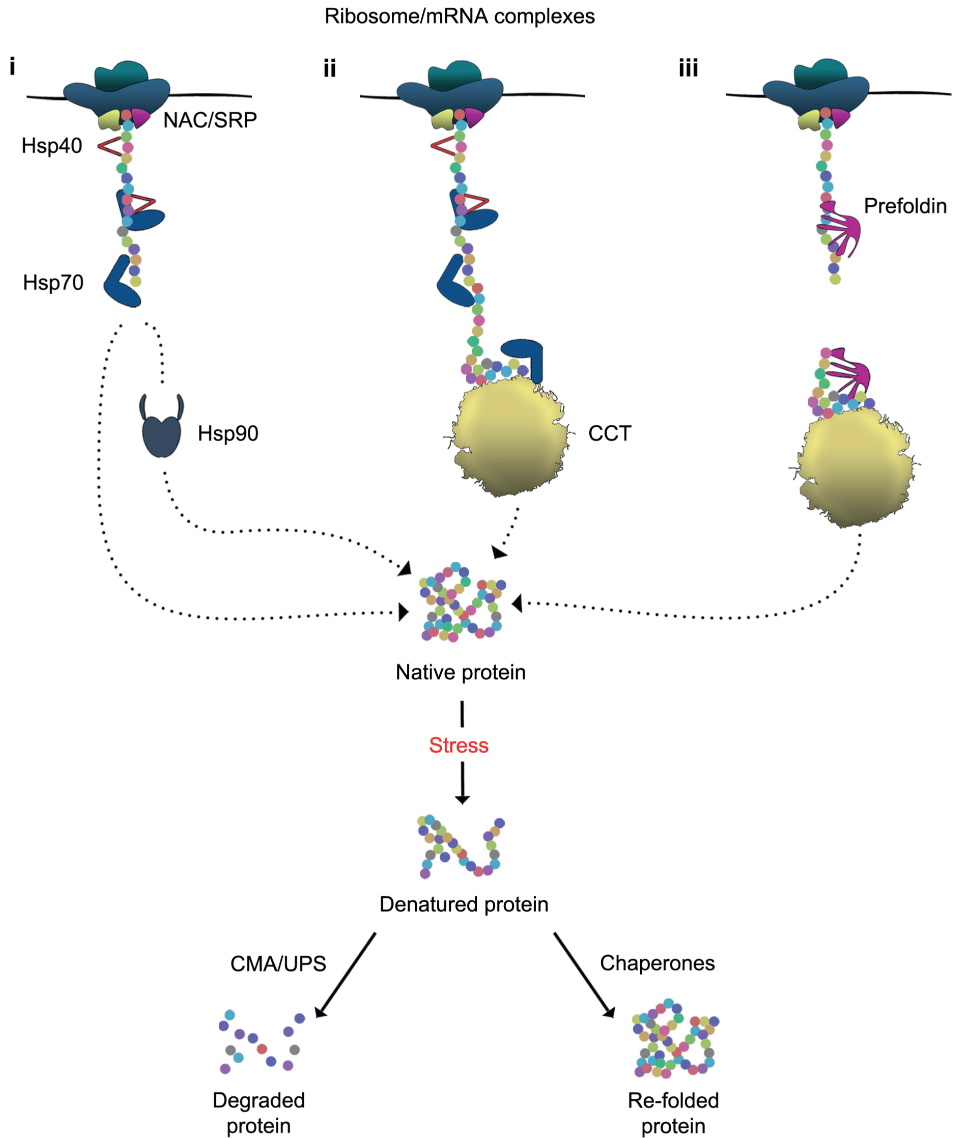

Figure 2 Folding and degradation pathways mediated by mammalian chaperones. Translating proteins from the ribosomes interact with numerous chaperones co- or post-translationally as indicated by different folding pathways **(i, ii, iii)** to reach a native state (Dotted arrows). Upon stress and protein denaturation (Black arrows), proteins that are assisted in their degradation by chaperones are degraded via chaperone-mediated autophagy (CMA) or via the ubiquitin proteasome system (UPS). Alternatively, denatured proteins are aided by chaperones to regain a native state.

It has been suggested that the unfolding of non-native proteins by hsp70 is mediated by a power-stroke from hsp70 that is caused by ATP-hydrolysis, which according to the model traps and exerts a mechanical force on the interacting substrate of hsp70. There is however a discrepancy whether or not the ATP-hydrolysis generates enough energy to unfold the interacting substrate. Therefore, an alternative re-folding model has been described, where the hsp70 chaperones bind a non-native protein and use "entropic pulling" to unfold the protein (reviewed by Finka et al., 2015).

Non-native translated proteins that require unfolding and re-folding to reach a native state have the risk of aggregating. Therefore, the hsp70 family has expanded to include a chaperone that increases the ability of a cell to disaggregate aggregated proteins. In humans, the hsp110 chaperone functions together with the hsp40/hsp70 system to actively reduce aggregates. It has been shown that hsp40 stimulates the folding activity of hsp110 similarly as for hsp70 (Mattoo et al., 2013). Moreover, the study showed that the system of hsp40/hsp70/hsp110 becomes more effective in protein disaggregation while also consuming less ATP in comparison to the hsp40/hsp70 system.

Non-native and aggregated proteins that are unable to obtain a native-state undergo proteolysis in the cytosol via the ubiquitin proteasome system (UPS) or in the lysosomes via autophagy, the latter being divided into macro-, micro- and chaperone mediated- autophagy (CMA). Both the UPS and macro-autophagy assisted protein degradation relies on the ubiquitination of a target protein, a process mediated by a combination of ubiquitin related enzymes, which is then recognized and degraded (reviewed by Korolchuk et al., 2010). CMA on the other hand mediates degradation via cognate hsp70 (hsc70) that recognizes proteins via a varying, charge dependent, pentapeptide that becomes accessible during protein denaturation, complex disassembly or possibly during protein translocation (Reviewed by Cuervo and Wong, 2014). Proteins recognized by hsc70 are delivered to the monomeric lysosome-located trans-membrane protein LAMP-2A, which oligomerizes and creates a barrel for protein translocation, leading to protein degradation within the lysosome. During the transition from a monomeric to oligomeric LAMP-2A receptor complex, hsc70 unfolds the bound protein and subsequently releases from the protein. It has also been shown that protein degradation via endosomal micro-autophagy shares the mechanism of using hsc70 mediated substrate recognition. However, the hsc70 substrates are instead delivered to late endosomes and the interaction between hsc70 and the endosomal membranes are mediated by

electrostatic charges and not by LAMP-2A (Sahu et al., 2011). As a result, proteins recognized for degradation by hsc70 are engulfed by the late endosomes, which will later fuse with the lysosomes.

Small heat shock chaperones

In case of increased cellular stress, many proteins may denature and consequently increase the workload on the hsp40/hsp70 chaperone network by their requirement to re-fold. However, the re-folding of proteins during stress will result in a wasteful consumption of ATP, as proteins that are re-folded will again be unfolded due to the stressful cellular environment. Therefore, the cells utilize passive small heat-shock proteins (shsp's) that bind multiple unfolded proteins during cellular stress to prevent their aggregation. When the stress is relieved, proteins are released from the shsp's and may again attain a native state via the hsp40/hsp70 system (Ehrnsperger et al., 1997; Lee and Vierling, 2000). In humans there are 10 genes encoding shsp's (Kappe et al., 2003), all suggested to function as ATP-independent chaperones (Jakob et al., 1993), which are activated by different factors such as phosphorylation, temperature and pressure that control their oligomer state (Reviewed by Haslbeck and Vierling, 2015). Importantly, the re-folding of denatured proteins by the hsp40/hsp70 system is shown to be dependent on an excess concentration of shsp's compared to hsp40/hsp70 (reviewed by Haslbeck and Vierling, 2015). Thus, the shsp's not only reduce the cellular ATP-consumption during stress but also function to ensure that the hsp40/hsp70 system is operational by relieving the immediate re-folding workload.

Hsp90

Proteins that require additional aid in attaining a native conformation may be assisted by hsp90, which has been shown to bind a wide range of substrates mainly categorized as kinases and nuclear receptors (reviewed by Li et al., 2012). Although some of these substrates are already in a native state prior to encountering hsp90, they require the assistance of hsp90 to become active. This is the case for the native glucocorticoid receptor (GR) that requires a transfer from hsp40/hsp70 system, via the co-chaperone HOP, to the ATP-dependent (Panaretou et al., 1998) hsp90 to become active (reviwed by Lorenz et al., 2014; Pratt and Toft, 2003). Hsp90 has also been shown to be non-essential for the folding of VHL but required for the UPS mediated degradation of VHL (McClellan et al., 2005). Thus, as in the case of hsp70, hsp90 is directly involved in mediating protein degradation.

CHAPERONINS

The chaperonins are multi-oligomeric chaperones that form a barrel-like structure of two back-to-back stacked rings that together create a central cavity (Figure 3). Categorized by their protein sequence, there are two groups of chaperonins that have apparent differences in their overall protein structure. The chaperonins belonging to group I have a co-enzyme that encloses the central cavity and are found in bacteria (GroEL/GroES), mitochondria (Hsp60/Hsp10) and chloroplasts (Cpn60/Cpn10). In contrast, the chaperonins belonging to group II have a build-in lid and are found in archea (Thermosome) and in the cytosol of eukaryotes (referred to as CCT and TCP-1 Ring Complex, TRiC) (reviewed by Horwich et al., 2007). The two groups are also different in the number of subunits located within each of the two rings as well as in their subunit heterogeneity. While group I are hepta-homooligomeric assemblies, group II are octa- (Phipps et al., 1993) to nona-oligomeric assemblies (Knapp et al., 1994) with one, two or three different subunits in archaea and nine different subunits in eukaryotes (reviewed by Horwich et al., 2007; Kubota et al., 1997; Yamamoto and Yohda, 2016). In the specific case of CCT, eukaryotes have been shown to have two rings that each consists of eight different subunits (α-θ in mammals, 1-8 in yeast) that sit in a defined ring-order (Kalisman et al., 2012; Leitner et al., 2012). In addition, mammals have been shown to have a ninth subunit (CCTζ-2) that is testis-specific and suggested to be interchangeable in the eight-symmetry ring of CCT (Kubota et al., 1997).

Although the chaperonins are different to each other in their subunit composition, all subunits have a common domain structure with an equatorial domain that binds and hydrolyses ATP, an intermediate domain that mediates the exerted force from the equatorial domain upon ATP-hydrolysis and an apical domain that binds folding substrates. By comparing the primary structure of GroEL and CCT, the chaperonins show most conserved homology in the equatorial and intermediate domains (Kim et al., 1994). In contrast, the apical domains have diverged throughout evolution, which reflects differences between the chaperonins in their selection of substrates. In the evolution of CCT, each subunit has been phylogenetically conserved between the eukaryotic species (Archibald et al., 2000), while the subunits has diverged between themselves. The apical domains of the CCT subunits display the highest amino acid sequence diversity between the subunits of CCT (Kim et al., 1994) and provides the folding substrates of CCT with several different interaction sites.

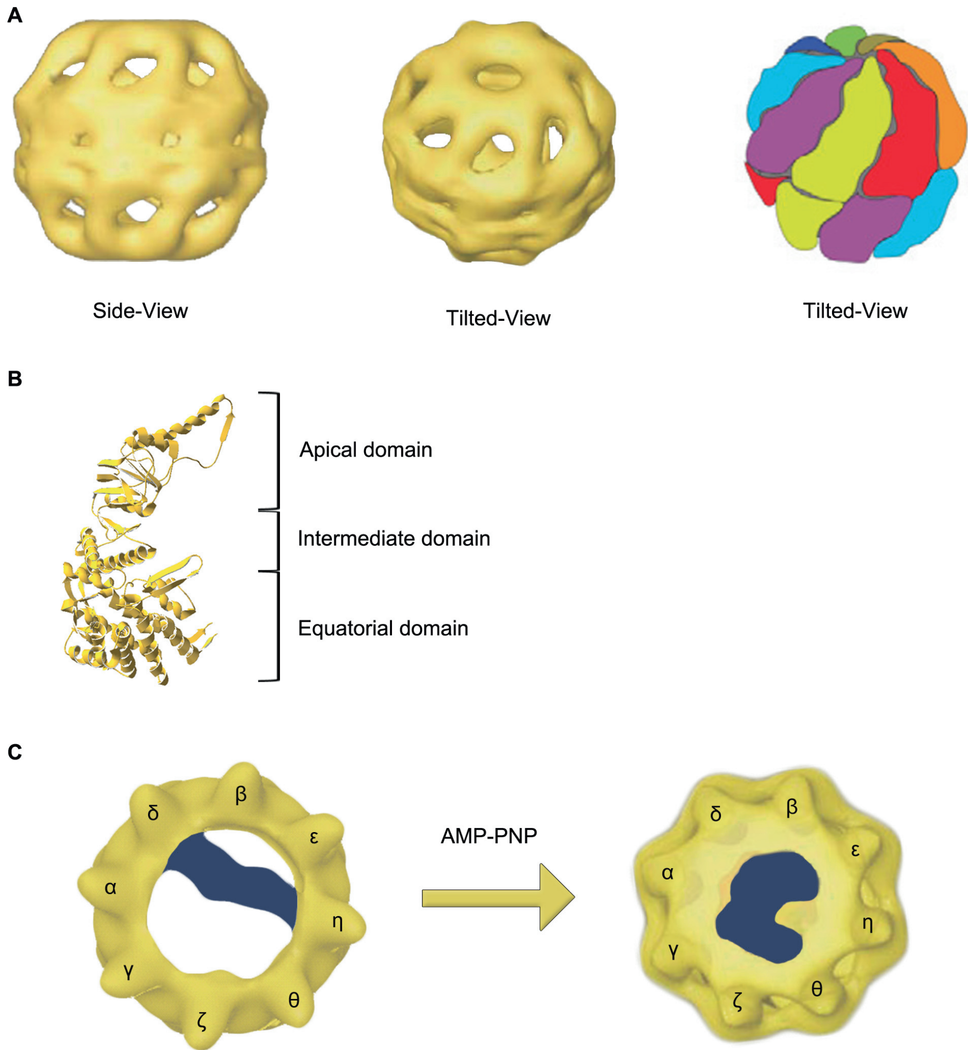

Figure 3 Architecture of CCT and folding mechanism of actin by CCT. A Side-view and tilted side-views of the CCT oligomer that discloses a barrel-like structure consisting of two-rings sitting back-to-back (Llorca et al., 2001) The tilted side side-view shows a fixed orientation of the eight subunits that faces each other, separated by the chaperonin cavity (Spiess et al., 2015). **B** Each subunit is divided into the apical, intermediate and equatorial domain (Ditzel et al., 1998). **C** Actin is positioned at its interaction site with CCT in a quasi-native conformation (Left) (Llorca et al., 1999) and is folded by CCT to a native state in the presence of the ATP analogue AMP-PNP (Right) (Llorca et al., 2001).

Subsequently, CCT is able to recognize different binding motifs of the same folding substrate, such as for the highly abundant proteins actin and tubulin that stretch over the central cavity of CCT and bind to subunits of CCT in a specific geometry (Llorca et al., 2000; Llorca et al., 1999) due to the defined subunit ring-order of CCT (Kalisman et al., 2012; Leitner et al., 2012).

The apical domains of CCT have been shown to have a combination of hydrophobic and polar residues to recognize substrates (Joachimiak et al., 2014). As actin has been shown to have its hydrophobic residues buried within its native structure and to bind CCT via charged and polar residues (Hynes and Willison, 2000), actin is suggested to bind CCT in a quasi-native state. This distinguishes CCT from general chaperones such as the bacterial homologue GroEL (reviewed by Horwich et al., 2009) and hsp70 that recognize folding substrates via hydrophobic residues (Rudiger et al., 1997). In addition, heat-stress that is known to denature proteins and expose their hydrophobic residues does not induce the expression of CCT, confirming that CCT is not a general chaperone and that CCT has an important role in upholding regular processes required on daily basis (Reviewed by Horwich et al., 2007). Studies made in yeast have demonstrated that CCT is an essential chaperone for viability (reviewed by Stoldt et al., 1996) and reduced levels of CCT in mammalian cells has been shown to result in growth arrest (Grantham et al., 2006). In contrast to the expression level of CCT during heat-stress, GroEL and thermosome have an induced expression level, supporting their role as general chaperones.

In conclusion, CCT is remarkably different in the group of chaperonins by binding to late protein folding intermediates and by binding proteins in a specific geometry due to its subunit heterogeneity and defined subunit ring-order.

Interactome of CCT

Studies have shown that the immunoprecipitation of CCT results in mainly the co-precipitation of actin and tubulin (Grantham et al., 2006; Thulasiraman et al., 1999), demonstrating that actin and tubulin are the major folding substrates of CCT. However, the interactome of CCT has been suggested to be considerably larger and estimated to be 7 % of the proteome (Yam et al., 2008). This wide-screen proteomic approach showed that CCT preferentially binds to proteins, not necessarily folding substrates, displaying a β-sheet enrichment and to proteins with a size range of 40-75 kDa. A second study employed both a

proteomic and genomic (synthetic genetic array analysis) approach to identify direct binding partners as well as proteins that are involved in processes indirectly dependent on the function of CCT (Dekker et al., 2008). By using this approach, CCT was shown to interact with 136 genes and proteins and concluded to have a total of 227 interactions, corresponding roughly to 3-4 % of the yeast proteome. These interactions included numerous physical and genetic interactions with cytoskeletal dependent processes, such as with the septin ring-complex proteins that are essential for cytokinesis.

The results from the wide-screen approaches are in concurrence with previous, as well as more recent, studies that have shown CCT to interact with a diverse pool of substrates. CCT has been recognized to be involved in the biogenesis of β-sheet rich proteins such as the cell division cycle protein cdc20p (Camasses et al., 2003) and the oncogenic signalling transduction protein STAT3 (Kasembeli et al., 2014). Both increased activity of STAT3 (reviewed by Yu et al., 2009) and increased expression of CCT (Yokota et al., 2001) has been linked to cancer and CCT may therefore be coupled to cancer progression via STAT3. Additional folding substrates include both the TCAB1 protein that assembles into cajal bodies responsible for telomerase function (Freund et al., 2014) and the fragment of myosin II heavy chain, heavy meromyosin subfragment (HMM), necessary for actin filament contraction (Srikakulam and Winkelmann, 1999). Although the HMM of 140 kDa exceeds the folding chamber size of CCT, studies have shown that CCT may fold individual domains of a polypeptide and thereby circumvent the size limitation of its chamber (Russmann et al., 2012).

The number of interacting folding substrates to CCT, which are dominated by low abundant proteins relatively to actin and tubulin, is conclusively many and there has also been studies showing that some of these substrates need the assistance of CCT to assemble into larger complexes. This is the case for Gα- (Farr et al., 1997; McLaughlin et al., 2002) and Gβ-proteins that require CCT for their folding and where CCT is necessary for the dimerization of Gβ and Gγ (Wells et al., 2006). Supporting the assembly function of CCT, evidence for the participation of CCT during VHL assembly has also been shown (Feldman et al., 1999).

The Gβ-protein and several other β-sheet rich folding substrates of CCT such as cdc20p belongs to a family of WD-repeats that contain a repetitive stretch of β-sheets that often ends with the amino acid tryptophan (W) followed by an asparagine (D) (Neer et al., 1994). The WD-repeat proteins resemble a propeller-like structure and 17% of the WD-repeat proteins in

yeast have been shown to bind CCT (reviewed by Valpuesta et al., 2002). As the stability of the propeller-like structure depends on the closure of the ring of WD-repeats (reviewed by Smith et al., 1999), the cdc20p was suggested to use the cavity of CCT to arrange its WD-repeats (Camasses et al., 2003). However, as the size of the WD-repeat proteins that interact with CCT ranges between 55 kDa to 100 kDa, several of the WD-proteins may exceed the size of the folding chamber of CCT (reviewed by Valpuesta et al., 2002). Instead, CCT was suggested to assist a pool of interacting WD-repeat proteins in regulating their activity or assembly with other proteins.

CCT and Huntingtons disease

The neurodegenerative disease Huntington (HD) is a disease caused by genetic mutations in the huntingtin gene that give the huntingtin protein a repetitive stretch of polar glutamine residues. As a result, the huntingtin protein aggregates and form highly ordered amyloid structures (reviewed by Bates et al., 2015). A recent study showed that an exogenous addition of the apical domain of CCT1 ($CCT1^{AD}$) to neuronal cells results in a cytosolic and nuclear localization of $CCT1^{AD}$ and that $CCT1^{AD}$ delayed the onset of huntingtin aggregation and reduced the huntingtin mediated neurotoxicity (Sontag et al., 2013). $CCT1^{AD}$ was therefore suggested to function as a therapeutic drug to inhibit the progression of HD. This is consistent with a previous study, where the over-expression of CCT1 and CCT4 in yeast changed the morphology of huntingtin aggregates and where the CCT oligomer was shown to prevent huntingtin aggregation (Tam et al., 2006). Thus, the CCT oligomer may provide a platform that via the apical domains of the subunits stabilizes the huntingtin protein and prevents huntingtin from aggregating.

Regulation of CCT activity

Besides the role of CCT to fold and assemble interacting proteins there are co-factors such as the mammalian phosducin-like proteins 1 (McLaughlin et al., 2002), 2 (Stirling et al., 2007) and 3 (Stirling et al., 2006) that in their native states have shown to alter Gα, actin and tubulin folding by CCT. In addition, the phosphorylated phosducin-like protein 1 has been shown to enhance the release of the Gβ-protein from CCT (Lukov et al., 2006; Plimpton et al., 2015). Another study has demonstrated that PI3K and S6K, downstream targets of extracellular signalling, phosphorylate CCTβ of the CCT oligomer (Abe et al., 2009). As wild-type CCTβ but not the non-phosphorylated mutant rescued cells displaying impaired cell proliferation as

a result of reduced levels of CCTβ by siRNA, it was concluded that the phosphorylation of CCT effects cell proliferation, although the mechanism of this is yet to be determined.

CCT DURING PROTEIN FOLDING AND CONFORMATIONAL CHANGES OF CCT

CCT has been shown to be essential for the folding of actin (Gao et al., 1992) and tubulin (Gao et al., 1993). In addition, the CCT co-chaperone prefoldin has been shown to bind actin and tubulin co-translationally and to deliver them to CCT post-translationally (Vainberg et al., 1998) (Hansen et al., 1999). The biological function of this delivery system has in the case of actin been shown to increase the rate of the production of native actin (Siegers et al., 1999). There is however a discrepancy whether CCT interacts co- or post-translationally with actin (Frydman and Hartl, 1996) and CCT has been shown by cross-linking to be interacting with actin peptides co-translationally (McCallum et al., 2000), although to a minor extent. Another study showed that CCT and the hsp40/hsp70 folding system interact co-translationally with luciferase (Frydman et al., 1994). In contrast to the cytosolic chaperone CCT, luciferase is a peroxisomal protein (Keller et al., 1987). A study that heat-shocked and denatured luciferase in HEK cells following the targeting of luciferase to different cellular compartments (Nucleus, cytoplasm, ER, peroxisomes) showed that the kinetics of luciferase re-activation was the same irrespectively to the location of luciferase (Hageman et al., 2007). Thus, luciferase is not a *bone fide* substrate of CCT. Therefore, additional studies are required before determining the co-translational role of CCT. In the context of chaperone co-operativity, hsp70 has been shown in complex with CCT (Cuellar et al., 2008) and the folding of VHL requires both hsp70 and CCT (Melville et al., 2003).

Protein folding by CCT requires CCT to undergo major conformational changes. All of the subunits of CCT have an ATP-binding site located in the equatorial domain that hydrolyses ATP to induce the conformational change that travels via the intermediate domain to the substrate binding apical domain. The ATP-hydrolysis has been shown to be executed in a positive intra- and negative inter-ring manner (Kafri et al., 2001). As subunits in the ring of CCT hydrolyse ATP, the apical domains move toward the centre of CCT and seal the cavity from the cytoplasm (Meyer et al., 2003). A study suggested that the ATP-binding and hydrolysis occurred in a hierarchy among the subunits of CCT, where the ATP bound to CCTα was first hydrolysed, followed by the specific order of ATP-hydrolysis on CCTγ, β and ζ (Lin and Sherman, 1997). These findings supported a sequential model of ATP-hydrolysis within the ring of subunits of CCT, which was consistent with the subunit order of CCT that

was determined the same year (Liou and Willison, 1997) as the specific order of CCTα, γ, β and ζ more or less covered a movement throughout the whole ring of subunits of CCT. The sequential folding mechanism of CCT was further supported by a study made using electron microscopy (EM), which showed that the conformation heterogeneity of the CCT subunits decreased with increased concentrations of ATP (Rivenzon-Segal et al., 2005). By using the same subunit-ring order of CCT as before (Liou and Willison, 1997), the ATP-hydrolysis of CCT was suggested to be sequential and not concerted, as the placement within the ring of the subunits that displayed the highest conformational changes at low ATP-concentrations (α, δ, η, θ) was consistent with the sequential model (Lin and Sherman, 1997). However, recent studies revealed a different subunit ring-order of CCT (Kalisman et al., 2012; Leitner et al., 2012) that is considered to be correct, as the studies used a superior cross-linking method compared to the earlier biochemical assays (Liou and Willison, 1997) and to the alignment of the amino acids of the CCT subunits to the crystal structure of CCT (Dekker et al., 2011). Studies using the new ring-order the have shown that the subunits of CCT bind ATP with different affinities, where the subunits showing highest affinity for ATP (α, β, δ, ε) (Reissmann et al., 2012) are located to one side of the ring. By applying the correct subunit ring-order of CCT to the previous EM study (Rivenzon-Segal et al., 2005), the subunits displaying the highest conformational changes in the presence of ATP are CCTα, β, γ and δ. Although the subunits α, β, δ, ε from the ATP-affinity study are not identical with the α, β, γ and δ subunits from the EM study, the quadruplets are only one step away from each other in the correct subunit ring-order of CCT. A new power-stroke model was therefore proposed, where the four subunits displaying the highest ATP-affinity were suggested to transfer an ATP-driven power-stroke to the other side of the ring. However, it was discussed that the power-stroke model will be consistent with both a concerted and a sequential folding mechanism of ATP-hydrolysis (Reissmann et al., 2012).

By using the subunit ring-order of CCT (Kalisman et al., 2012; Leitner et al., 2012), the N-terminal domain of actin and tubulin locates at the CCT subunits (Llorca et al., 2000; Llorca et al., 1999) displaying the highest ATP-affinity (Reissmann et al., 2012). Additionally, actin and tubulin that appear to be in a native state, but not yet released from CCT, have their N-terminals moved toward their C-terminal side and form a compact density on the apical domains of CCT (Llorca et al., 2001). Later studies also demonstrated that the packing of the actin C-terminus, aided by the ATP-hydrolysis of CCT, is the final stage in actin folding on

CCT (Neirynck et al., 2006; Stuart et al., 2011). Following C-terminal packing, actin is suggested to be released from CCT and to have an ATP molecule incorporated into its nucleotide pocket (Neirynck et al., 2006).

Together, these observations are consistent with the initially described folding theory (Llorca et al., 2001) known as the sequential mechanical folding of actin and tubulin, wherein actin and tubulin are held at two places on CCT while a stepwise ATP-mediated movement drives actin and tubulin to a compact native conformation. Although this sequential model relied on the first described subunit ring-order of CCT (Liou and Willison, 1997), the currently suggested power-stroke model also fits with the folding mechanism of actin and tubulin by CCT (Llorca et al., 2001).

In contrast to the conformational heterogeneity of the subunits of CCT that was shown at increasing ATP-concentrations (Rivenzon-Segal et al., 2005), the homo-oligomeric bacteria homologue GroEL displayed no such conformational heterogeneity (Danziger et al., 2003), which is consistent with a study that supported a concerted ATP-hydrolysis for GroEL (Yifrach and Horovitz, 1998). GroEL has been shown to undergo large ATP-dependent movements that occlude the substrate binding sites (Roseman et al., 1996), which releases the folding substrate into the charged and polar cavity of GroEL where it may fold to a native state (reviewed by Horwich et al., 2009). In the archaea CCT homologue, Mm-Cpn, the ATP-hydrolysis required for substrate folding also results in substrate release into the central cavity (Douglas et al., 2011). The Mm-Cpn is a homo-oligomeric chaperone (Kusmierczyk and Martin, 2003) that is predicted to have a concerted ATP-hydrolysis within a ring of subunits as in the case of GroEL. In contrast to the substrate that is released into the cavity of the GroEL and Mm-Cpn during protein folding, folding substrates of CCT such as actin stay associated with the apical domains during their folding (Llorca et al., 2001). This is consistent with the non-concerted movement of the subunits of CCT upon ATP-hydrolysis, which mediates a sequential or power-stroke driven substrate folding by CCT.

ACTIN PROTEOSTASIS

Actin is strictly dependent on the assistance of the chaperonin CCT to become native. Although the bacteria chaperonin ortholog GroEL can bind both denatured actin (Tian et al., 1995) as well as *de novo* synthesised actin (Pappenberger et al., 2006; Stemp et al., 2005), GroEL can not fold actin. CCT is thus an essential and non-interchangeable chaperone for

actin folding. In addition, CCT has been shown to affect the initial rate of actin filament polymerisation (Grantham et al., 2002) and can together with other actin and actin filament binding proteins influence actin filament polymerization. In early studies, actin filaments were concluded to be polarized structures as the decoration of the actin filaments by the actin filament binding protein HMM is unidirectional (Huxley, 1963). By the appearance of the complex, the two ends of the actin filaments became known as the barbed and pointed end, where the actin polymerization at the barbed end is faster than at the pointed end (Figure 4).

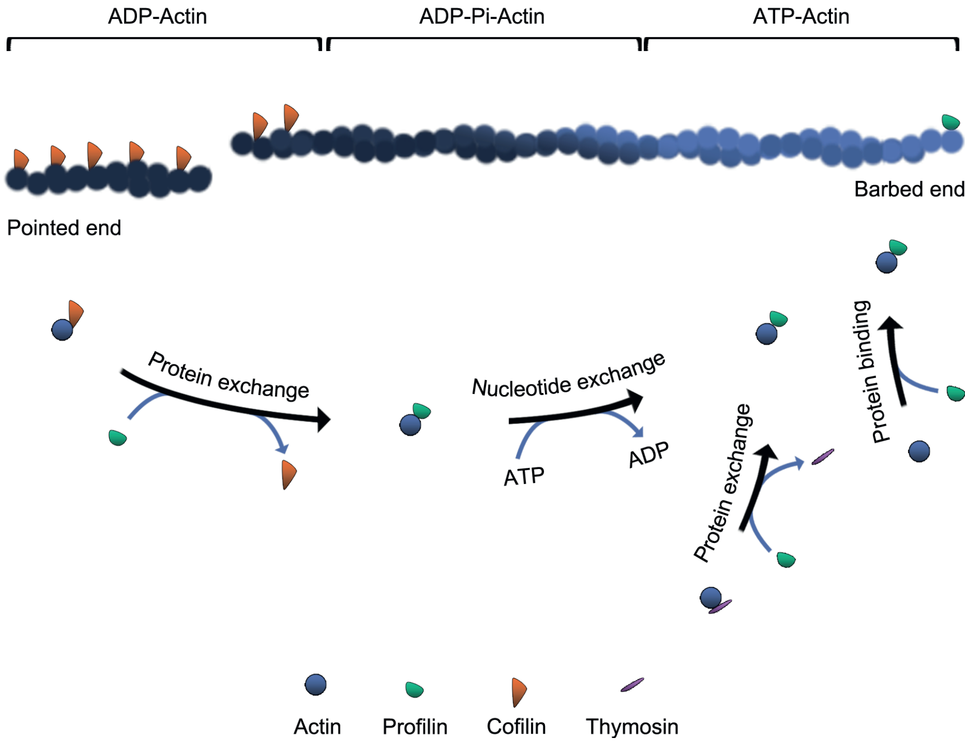

Figure 4 Actin dynamics. Actin monomers transferred directly or via thymosin to profilin are incorporated into the actin filament. Upon incorporation, the ATP on actin is hydrolysed to ADP-Pi and later to ADP, where ADP-actin is recognized by cofilin. Dense cofilin patches break the actin filaments and cofilin bound actin is re-cycled back to profilin where ADP is exchanged to ATP.

There are several factors that influence actin dynamics, including the cytosolic monomeric concentration of actin. Actin transcription is under the control of the serum response factor (SRF) and the transcription co-activator myocardin related transcription factor A (MRTF-A) (Vartiainen et al., 2007). Following actin transcription, newly synthesized and native actin may bind to a protein called profilin that incorporates actin into the barbed end of an actin filament in an ATP-bound state. Alternatively, actin may bind to a group of proteins called thymosins that function as an actin buffering system. By binding to thymosin, spontaneous actin polymerisation and nucleotide-exchange of actin is inhibited. The incorporation of monomeric ATP-actin into the actin filaments induces the hydrolysis of ATP to ADP, where the dissociation of the γ-phosphate of the ATP functions as a timer that once released makes the actin filament de-polymerize as a result of an increased instability of the actin filament. A protein called cofilin is responsible for the actin filament de-polymerisation by recognizing ADP-actin and by severing the actin filament at sites containing ADP-actin, resulting in actin monomer removal. ADP-actin is then transferred from cofilin to profilin that exchanges the actin-bound nucleotide from ADP to ATP, where upon actin is again in a state to be incorporated into the actin filaments. At a certain concentration of actin monomers, the association of actin at the barbed end is equal to the dissociation of actin at the pointed end, a process called treadmilling. In a pure system of native actin, the actin monomer concentration required for upholding the treadmilling is 0.1 μM, thus clearly different *in vivo* where the actin monomer concentration is 150 μM and where actin dynamics are changed by actin binding proteins such as thymosin, cofilin and profilin.

In addition, several other proteins form a complex network that together affect actin filament dynamics, including the actin nucleation promoting factors (NPF's). The NPF's include the seven protein complex arp2/3 that is known to nucleate branched actin filaments, the group of formins that enhance the incorporation of profilin-actin complexes to the actin filaments and the membrane anchored Ena/VASP proteins that direct bundled actin filaments to cell membranes. Actin filament dynamics are also affected by the actin filament cross-linking proteins that form stable actin structures (e.g. actin bundles/stress fibres), the actin filament membrane-anchoring proteins Ezerin/Radexin/Moesin (ERM) and the talin/vinculin/tensin proteins that link actin filaments to focal adhesions. Additionally, a protein called gelsolin increases actin filament dynamics by severing actin filaments and by increasing the number actin filament ends that are available for actin polymerization.

Upon cell growth stimulation, different proteins belonging to the Rho family will be activated and will influence actin dynamics via the complex network of actin binding proteins (reviewed by Jaffe and Hall, 2005). The Rho family proteins are known to induce stress fibres via rhoA, to induce branched actin networks known as lamellipodia at the leading edge of the cell via rac and to induce protrusions of bundled actin filaments called filopodia via cdc42. Together, the activation of the Rho family proteins leads to the activation of cellular processes such as cell migration and cytokinesis (reviewed by Blanchoin et al., 2014; Grantham et al., 2012; Pollard and Earnshaw, 2008).

GELSOLIN AND ACTIN FILAMENT DYNAMICS

A previous study identified that CCT binds to the actin filament severing protein gelsolin, although the biological significance of this interaction remained elusive as gelsolin did not behave as a folding substrate of CCT (Brackley and Grantham, 2011). Gelsolin was first discovered by its ability to solubilize actin gels and was by this ability referred to as gel-sol-in (Yin and Stossel, 1979). Of the two isoforms of gelsolin (Kwiatkowski et al., 1986; Yin et al., 1984), isoform-1 is secreted and functions in an actin scavenger system in the blood to avoid an increased viscosity caused by actin polymerization (Lind et al., 1986), whereas isoform-2 is cytoplasmic and severs actin filaments to increase actin dynamics (reviewed by Nag et al., 2013).

Gelsolin consists of three N-terminal domains that are linked to three C-terminal domains and gelsolin has been shown to sequentially induce an open conformation at increasing calcium concentrations by initially releasing the N-terminal domain from the C-terminal domain. The open conformation of gelsolin severs and subsequently caps barbed ends of actin filaments (Reviewed by Nag et al., 2013), where the capped filaments are uncapped from gelsolin by the binding of gelsolin to phosphatidylinositol biphosphate (PIP_2) (Janmey et al., 1987). Thus, the number of actin filaments available for actin polymerization increases by the calcium-induced severing activity of gelsolin in combination with the PIP_2 mediated release of gelsolin (Figure 5). Consistent with increased actin dynamics as a result of increased gelsolin activity, overexpression of gelsolin was shown to increase cell motility (Cunningham et al., 1991) via the Rho family protein Rac (De Corte et al., 2002). This is in agreement with a study showing that primary cells from gelsolin knock-out mice display increased actin bundles as well as a reduced motility in wound healing assays (Witke et al., 1995).

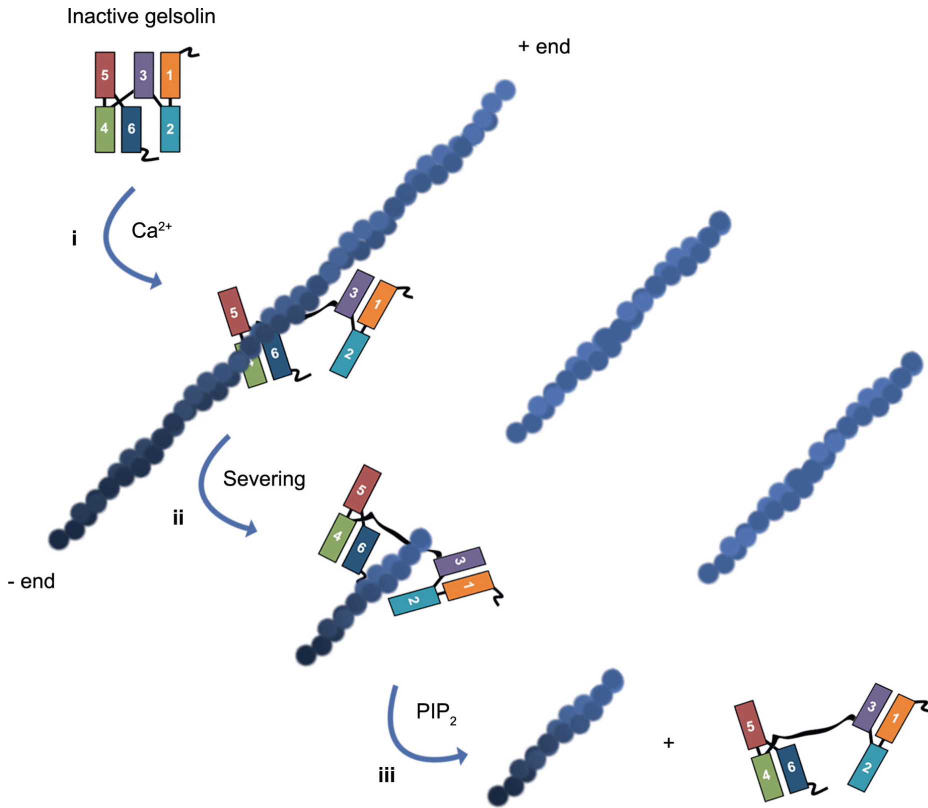

Figure 5 Actin filament severing by gelsolin. i) Gelsolin is activated by elevated calcium concentrations and forms an open conformation that recognizes actin filaments. **ii)** Gelsolin severs and caps the actin filaments by weakening the non-covalent bonds within the filament. **iii)** PIP_2 releases gelsolin from the actin filaments, leading to increased barbed ends (+) available for actin filament polymerization.

Subunits of CCT as monomers

Although CCT is mainly considered as an oligomer, defined by its barrel-like structure and apical protrusions that form a lid to exclude the outer cytosol from the secluded folding environment, there is increasing evidence that the subunits of CCT have functions when they are monomeric. The CCT oligomer is dynamic and dissociates to smaller assemblies and monomers at physiological potassium- and ATP-concentrations (Roobol et al., 1999a). Additionally, the expression levels of the individual CCT subunits in yeast are shown to be different to each other, where CCTα and CCTδ are shown to be the lowest and most abundant

subunits respectively (Matalon et al., 2014). In an oligomeric protein complex that requires all subunits in order to be functional, the individual expression of each subunit would be expected to be coherent. In contrast, different expression levels between the individual subunits may be a result of an individual function of a particular subunit, known as a moonlighting function. However, it may also be that the proteins in an oligomeric protein assembly interact with each other with different kinetics and some subunits are therefore required to express at different levels in order for the oligomeric complex to assemble.

A study that searched for suppressors to the inhibited growth displayed by the conditional yeast mutants tor2-21, last8-2, rsp5-9 and by the concomitant overexpression of SIT4p and SAP155p discovered that the overexpression of CCT6 rescued growth in all mutants (Kabir et al., 2005). The overexpression of CCT6 resulted in high levels of monomeric CCT6 and the growth rescue was dependent on the wild-type ATP-binding site of CCT6. In contrast to CCT6, CCT1, 4 and 8 only rescued growth in some of the mutants whereas CCT2, 3 and 7 did not rescue any mutant. The growth rescue of the mutants by CCT6 was therefore concluded to be a result of a monomeric function of CCT6. A second study in yeast showed that different yeast strains (*MA1-MA8*), each with an impaired ATP-binding site of a specific subunit of CCT (1-8), have different viability at a temperature of 15 °C and 37 °C (Amit et al., 2010). The CCT subunits have, in the context of the CCT oligomer, different binding affinities for ATP and the viability of the *MA1-MA8* strains can be correlated to the ATP-binding affinity of the CCT subunit that is being impaired (Reissmann et al., 2012). The effect of impairing the high ATP-binding affinity subunits of CCT has the most severe result on cell viability. The defined location of the subunits of CCT within the ring of the CCT oligomer is important for protein folding by CCT, where the initial conformational changes of CCT during the folding cycle of actin and tubulin are mediated by the apical domains of the subunits that have a high affinity for ATP. Thus, the viability of the *MA1-MA8* strains may reflect the total protein folding capacity of the CCT oligomer. However, in the specific case of actin, differences in abnormal actin patches and in sensitivity to the actin sequestering drug latrunculin A (Lat-A) in the *MA1-MA8* strains do not unambiguously correlate with the high ATP-affinity subunits of CCT or to the subunits of CCT that interact with actin during actin folding. Thus, the actin phenotypes may also be a consequence of a loss-of-function of a monomeric subunit. The study also showed that the *MA1-MA8* strains have different transcriptional responses compared to each other, in particular the *MA2* strain. As the

transcriptional responses do not correlate with the ATP-binding affinity of the subunits of CCT, which would indicate an impaired protein folding capacity of the CCT oligomer, it suggests that the transcription profile of the *MA2* strain is a result of an impaired monomeric function of CCT2. In addition, a general folding substrate of CCT can be expected to interact with several subunits of CCT, as in the case of actin and tubulin. Thus, this supports that the particular transcription profile of CCT2 is not a result of an impaired protein folding by the CCT oligomer, indicating a loss-of-function of monomeric CCT2.

In agreement with individual subunits of CCT having monomeric functions, a study showed that CCTα, γ, ζ and θ subunits co-purify with polymerizing microtubules in an ATP-dependent manner (Roobol et al., 1999b), which indicates that some CCT subunits function as microtubule-associating proteins. Another study demonstrated that reduced levels of individual subunits of CCT by siRNA result in subunit-specific cell-shapes in a mammalian cell-line (Brackley and Grantham, 2010). Cells depleted of CCTε adopted a narrow long elongated phenotype and as CCTε was shown to co-localize with actin filaments, it was suggested that monomeric CCTε functions to stabilize the actin filaments. Together, these studies provide convincing evidence that some monomeric subunits of CCT have individual functions. A summarized view of the functions that are mediated via CCT is presented in figure 6.

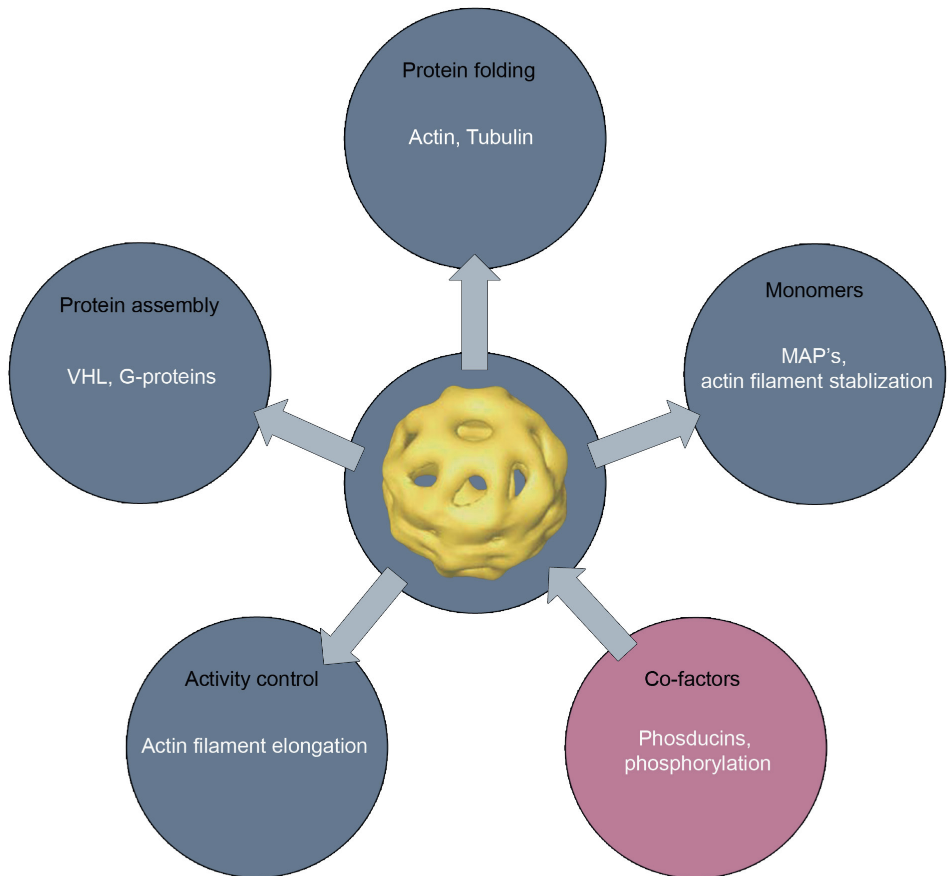

Figure 6 Functions regulated by CCT and the regulation of CCT. CCT oligomer assists in protein folding, protein assembly and in regulating the activity of specific cellular processes. In addition, the CCT subunits have been shown to have individual roles as monomers, such as microtubule associating proteins (MAP's). Functions mediated by CCT or by monomeric subunits of CCT are indicated in blue. The CCT oligomer is also itself a target for regulation by the phosducin-like proteins and by the phosphorylation of CCTβ, indicated in red.

AIM, RESULTS AND DISCUSSION

PAPER I – OVER-EXPRESSION ANALYSIS OF ALL EIGHT SUBUNITS OF THE MOLECULAR CHAPERONE CCT IN MAMMALIAN CELLS REVEALS A NOVEL FUNCTION FOR CCT DELTA

In order to study monomeric functions of the CCT subunits we have employed a novel strategy that produces the subunits of CCT as monomers and that prevents the subunits assembling into an oligomeric complex by fusing each of the eight subunits to an N-terminal EGFP. According to a previous study, the intra- and inter-ring interactions between the subunits of CCT are placed in the equatorial domains of the subunits where the N- and C-terminus are positioned (Dekker et al., 2011). Therefore, the placement of EGFP at the N-terminus of each subunit of CCT may spatially disrupt the interaction between the subunits and render the subunits as monomers. To validate our approach, cell lysates of mammalian BALB 3T3 cells transiently transfected with a single EGFP-CCT subunit (α-θ) were loaded onto continuous sucrose gradients and separated by ultracentrifugation. By analysing the sucrose gradient fractions by SDS-PAGE and western blotting, we found that all of the eight EGFP-CCT subunits were predominantly monomeric, unlike the endogenous subunits that were found both monomeric and in oligomeric protein assemblies. This demonstrates that the EGFP-CCT subunits are unable to enter an oligomeric assembly in a mammalian cell line.

Strikingly, both the mammalian BALB-3T3 and B16F1 cell lines showed a pronounced, subunit-specific, plasma membrane protrusion formation when overexpressing EGFP-CCTδ. The expression levels of EGFP-CCTδ, which was 1.7 times higher than the endogenous levels of CCTδ, did not change cell proliferation and the cells were still viable at day 4 post-transfection. Thus, the CCTδ phenotype is not an artefact caused by endangering cellular health. As the expression levels of the different EGFP-CCT subunits were also shown to be relatively even, with the exception of EFGP-γ that was overexpressed to a lesser extent, the plasma membrane protrusion phenotype was concluded to be specific for monomeric CCTδ.

We then continued by studying the monomeric CCTδ induced protrusion phenotype by using time-lapse microscopy. The protrusion phenotype was shown to be mainly a result of retraction fibers from retracting lamellipodia, although newly formed filopodia were also observed to a lesser degree. Cells treated with Lat-A, a drug that sequesters actin monomers

and consequently de-polymerizes the actin cytoskeleton, abolished the phenotype, which reformed after the removal of Lat-A. Thus, the monomeric CCTδ induced protrusions can re-form upon re-building the actin cytoskeleton. In addition, cells co-transfected with the dominant negative cdc42 (cdc42T17N)(Coso et al., 1995), which remains inactive by preferentially binding GDP over GTP and thereby functioning as an antagonist for endogenous cdc42 by binding regulatory proteins such as nucleotide exchange factors (Feig and Cooper, 1988), abolished the phenotype. As active cdc42 triggers actin filament polymerization and filopodia formation (Reviewed by Jaffe and Hall, 2005), these results indicate a function of the actin cytoskeleton in mediating the formation of plasma membrane protrusions when overexpressing monomeric CCTδ.

Taking advantage of the EGFP fused to CCTδ, we were able to study the subcellular localization of EGFP-CCTδ by using structured illuminated microscopy (SIM). EGFP-CCTδ was shown enriched in the vicinity of plasma membranes and actin bundles visualized by phalloidin staining were localized within the EGFP-CCTδ induced protrusions. Consistent with the subcellular localization of CCTδ, a biochemical analysis showed that EGFP-CCTδ was located in the membrane fraction of cell extracts. Together, these data are compelling evidence that the plasma membrane protrusions are caused by monomeric CCTδ that is located at the cell periphery. To provide information on how CCTδ is inducing the plasma membrane protrusions, we sought to identify key residues of CCTδ that are necessary in the formation of these protrusions by introducing point mutations that will abolish the protrusion phenotype.

Previous studies showed that the subunits of CCT have an evolutionary conserved ATP-binding site of GDGTT (Kim et al., 1994) and that impairing the equivalent ATP-binding site of the CCT homologue GroEL ($GroEL^{D87E}$) reduces the overall ATP-hydrolysis by 50 % (Weiss and Goloubinoff, 1995). We sought to impair the ATP-binding site of monomeric CCTδ and study the effect on the protrusion phenotype.

A second mutation of interest was a G to D mutation at position 345 of CCT4 ($CCT4^{G345D}$), located at the outer surface of the apical domain, that was identified in a yeast strain (*CCT4-1*/*Anc2-1*) to cause abnormal de-localized actin structures and to result in the failure of the yeast strain to bud at a non-permissive temperature (Llorca et al., 1999; Vinh and Drubin, 1994). Using an *in vitro* assay, purified CCT oligomer from the *CCT4-1* yeast strain was

shown to have impaired ATP-induced allosteric transitions and to have a mild reduced folding of approximately 30% of native actin compared to wild-type CCT at the non-permissive temperature (Shimon et al., 2008). The reduced production of native actin by mutated CCT ($CCT4^{G345D}$) compared to wild-type CCT was also shown to be similar at the permissive temperature as at the non-permissive temperature. Thus, the data indicate that the reduced ability of mutated CCT ($CCT4^{G345D}$) to fold actin may not fully account for the yeast phenotype. Therefore, we sought to study if the G345D substitution of CCT4 impairs a monomeric function of CCT4.

We separately introduced the yeast D91E and G345D substitutions into the equivalent positions in mammalian CCTδ (EGFP-$CCT\delta^{D104E}$ and EGFP-$CCT\delta^{G357D}$) and showed that neither mutated subunit was able to induce the protrusion phenotype. Furthermore, by using SIM we showed that only EGFP-CCTδ located at the plasma membrane, which was in contrast to $CCT\delta^{D104E}$ and $CCT\delta^{G357D}$ that had a diffuse cytosolic staining. Thus, the protrusion phenotype is dependent on the plasma membrane localization of CCTδ. Consistent with the SIM data, biochemical analysis showed that EGFP-$CCT\delta^{G357D}$ and EGFP-$CCT\delta^{D104E}$ were not observed in the membrane fractions from the cell extracts. In conclusion, a wild-type ATP-binding site and a wild-type apical domain of CCTδ are both essential for the formation of the membrane protrusions and for the cell membrane localization of monomeric CCTδ.

We also showed that the apical domain of CCTδ (EGFP-$CCT\delta^{AD}$) alone was able to induce the phenotype, despite lacking an ATP-binding site. As the wild-type ATP-binding site of full-length CCTδ was shown to be essential for the formation of membrane protrusions, it may be that the EGFP-$CCT\delta^{AD}$ attains a conformation similar to the conformation induced by ATP-hydrolysis in the wild-type full-length CCTδ subunit.

Consistent with the subcellular localization in mammalian cells, yeast wild-type EGFP-CCT4 displayed enrichment at the cell periphery whereas EGFP-$CCT4^{D91E}$ and EGFP-$CCT4^{G345D}$ had a pan-cell staining. However, the sucrose gradient profile of EGFP-CCT4 from yeast cell lysates only showed, in contrast to mammalian EGFP-CCTδ, a peak of oligomeric protein assemblies. By using GFP-nanobody beads to precipitate the EGFP-CCT4 fusion protein, we showed that the oligomeric protein assemblies resemble endogenous CCT by the number co-precipitating subunits. The incorporation of EGFP-CCT4 into the CCT oligomer may be explained by the fact that the CCT oligomer in yeast is highly dynamic. Several studies have

reported the use of a high concentration of glycerol to stabilize the CCT oligomer (E.g. Dekker et al., 2008; Pappenberger et al., 2006). In addition, the membranes from the yeast lysates were cleared prior to the centrifugation of the sucrose gradients and the separation of the monomeric subunits and oligomeric protein assemblies. Thus, as we have already observed monomeric CCTδ at the membranes of mammalian cells, the lack of a monomeric peak of CCT4 from sucrose fractionated yeast lysates may be due to the pre-clearance of the membranes from the yeast lysates. Although no monomeric peak of EGFP-CCT4 was observed from the pre-cleared yeast lysates, a large quantity of EGFP-CCT4 was shown by biochemical analysis to be present in the membrane fraction from yeast lysates. Thus, it may be that a small pool of EGFP-CCT4 oligomerizes, whereas the monomeric pool of EGFP-CCT4 is present in the membrane fraction.

The *CCT4-1* strain was previously shown not to grow at the non-permissive temperature and although not suggested, the data indicated that the growth defect may have been a result of having an impaired function of monomeric CCT4. Here, we show that EGFP-CCT4 rescues the growth of the *CCT4-1* strain at the non-permissive temperature, which supports a monomeric function of CCT4 in yeast.

In the context of the CCT oligomer, CCT4 has the highest affinity for ATP (Reissmann et al., 2012) and a yeast mutant with an impaired ATP-binding site of endogenous CCT4 ($CCT4^{D91E}$) is lethal (Amit et al., 2010; Reissmann et al., 2012). The growth defects in yeast upon impairing the ATP-binding site of the individual CCT subunits correlates well with the subunits affinity for ATP (Reissmann et al., 2012). Therefore, the $CCT4^{D91E}$ lethality is probably a consequence of having an impaired CCT oligomer. We showed that EGFP-$CCT4^{D91E}$ rescues the growth of the yeast strain *CCT4-1* at the non-permissive temperature. Thus, the growth rescue is indicated to be an effect of monomeric EGFP-$CCT4^{D91E}$.

Moreover, we showed that EGFP-$CCT4^{D91E}$ locates to the cell periphery in the *CCT4-1* yeast strain, which is consistent with the plasma membrane localization of monomeric EGFP-CCTδ in mammalian cells. Notably, the peripheral localization of EGFP-$CCT4^{D91E}$ in the *CCT4-1* yeast strain is different to the diffuse cytoplasmic staining of EGFP-$CCT4^{D91E}$ in a yeast wild-type strain. Thus, although $CCT4^{D91E}$ has an impaired ATP-binding site, it can locate to the cell periphery when there is no competing wild-type apical domain of CCT4 available from endogenous CCT4. Thus, the apical domain of CCT4 is essential for the peripheral

localization of CCT4 and the ATP-binding or hydrolysis is suggested to be required for an efficient peripheral localization of CCT4.

Taken together, the results presented here show a subunit-specific function of monomeric CCTδ at the plasma membrane and that the apical domain of CCTδ is essential for mediating this function. In addition, the ATP-binding equatorial domain of CCTδ is indicated to be important for the plasma membrane localization of CCTδ, although the apical domain of CCTδ alone could induce the protrusion phenotype. We also show that a monomeric CCTδ function appears conserved in eukaryotic cells, here shown in yeast, although the function *per se* may be different between species.

Paper II – A Novel Function of the Monomeric CCTepsilon Subunit Connects the Serum Response Factor Pathway to Chaperone-mediated Actin Folding

Two studies have suggested that the subunits of CCT may have individual functions when monomeric and have shown that these functions may be related to the actin cytoskeleton. A study showed that yeast strains, each with an impaired ATP-binding site of a specific subunit of CCT, had in comparison to each other different growth phenotypes at the non-permissive temperature and displayed subunit-specific sensitivity to the actin sequestering drug Lat-A (Amit et al., 2010). In addition, the yeast strains also displayed a strain-specific abnormal level of actin patches. Although the different growth defects of the yeast strains may be explained by differences between the subunits in their binding and hydrolysis of ATP, which may be correlated to a defect in protein folding by the CCT oligomer, the differences in Lat-A sensitivity and abnormal actin patches is indicative of impaired monomeric functions. Another study demonstrated that the reduction of specific subunits of CCT by siRNA altered cell shape, where cells with a reduced level of CCTε adopted a narrow elongated shape (Brackley and Grantham, 2010). As CCTε was also shown to localize to actin bundles, it was suggested that monomeric CCTε may function to stabilize actin filaments.

A study showed that actin treadmilling and subsequently the globular actin (G-actin) concentration was responsible for an increased serum response factor (SRF) mediated transcription (Sotiropoulos et al., 1999). The SRF-transcription was later correlated with the transcription activator protein MRTF-A that responds to the levels of cytoplasmic G-actin

(Miralles et al., 2003). In serum-starved cells, MRTF-A is sequestered by G-actin in the cytosol while being exported from the nucleus in an actin dependent manner (Miralles et al., 2003; Vartiainen et al., 2007). As MRTF-A is a co-activator of SRF mediated actin transcription, MRTF-A retained in the cytosol by G-actin results in a low transcription of actin (Vartiainen et al., 2007). However, upon serum stimulation, the Rho family proteins are activated and the cytosolic concentration of G-actin is depleted as a result of actin filament polymerization (Jaffe and Hall, 2005; Sotiropoulos et al., 1999). Thus, MRTF-A is liberated from cytosolic actin. Serum stimulation also results in the export of actin from the nucleus to the cytosol in an oxidative MICAL-2 dependent process (Lundquist et al., 2014). Therefore, MRTF-A is free to translocate to the nucleus and activate the transcription of SRF-controlled genes, including actin (Vartiainen et al., 2007) and several actin binding proteins such as cofilin, profilin and gelsolin (Sun et al., 2006) (Figure 7).

In this study, we addressed if the levels of monomeric subunits of CCT are able to influence SFR-driven transcription. Subunits of CCT were reduced by siRNA and SRF-driven transcription was measured using an SRF-dependent luciferase reporter gene assay. We showed that reduced levels of CCTε induce the SRF-mediated transcription, which was in contrast to the other seven subunits of CCT that showed no or only a minor induction of SRF-mediated transcription, although the knockdown efficiency among the subunits of CCT was similar. Thus, this suggests a role of monomeric CCTε in influencing either the assembly state of actin or the SRF pathway. Since actin synthesis is regulated via SRF-mediated transcription (Vartiainen et al., 2007), it is surprising that here and in a previous study (Brackley and Grantham, 2010) the total levels of actin remain unchanged in cells depleted of CCTε. However, it may be that the activation of SRF-mediated transcription only results in a relatively low induction of actin transcription, thus changes to the total level of actin is not detected by western blot analysis.

As SRF-mediated transcription is triggered by the reduction of G-actin levels as a result of actin polymerization, we wanted to study if changes in G-actin levels were responsible for the increased transcription of luciferase in our luciferase-based SRF-reporter gene assay. Although the ratio of G-actin:filamentous actin (G:F ratio) was reduced when CCTε was targeted by siRNA, the ratio was similarly reduced when targeting CCTδ.

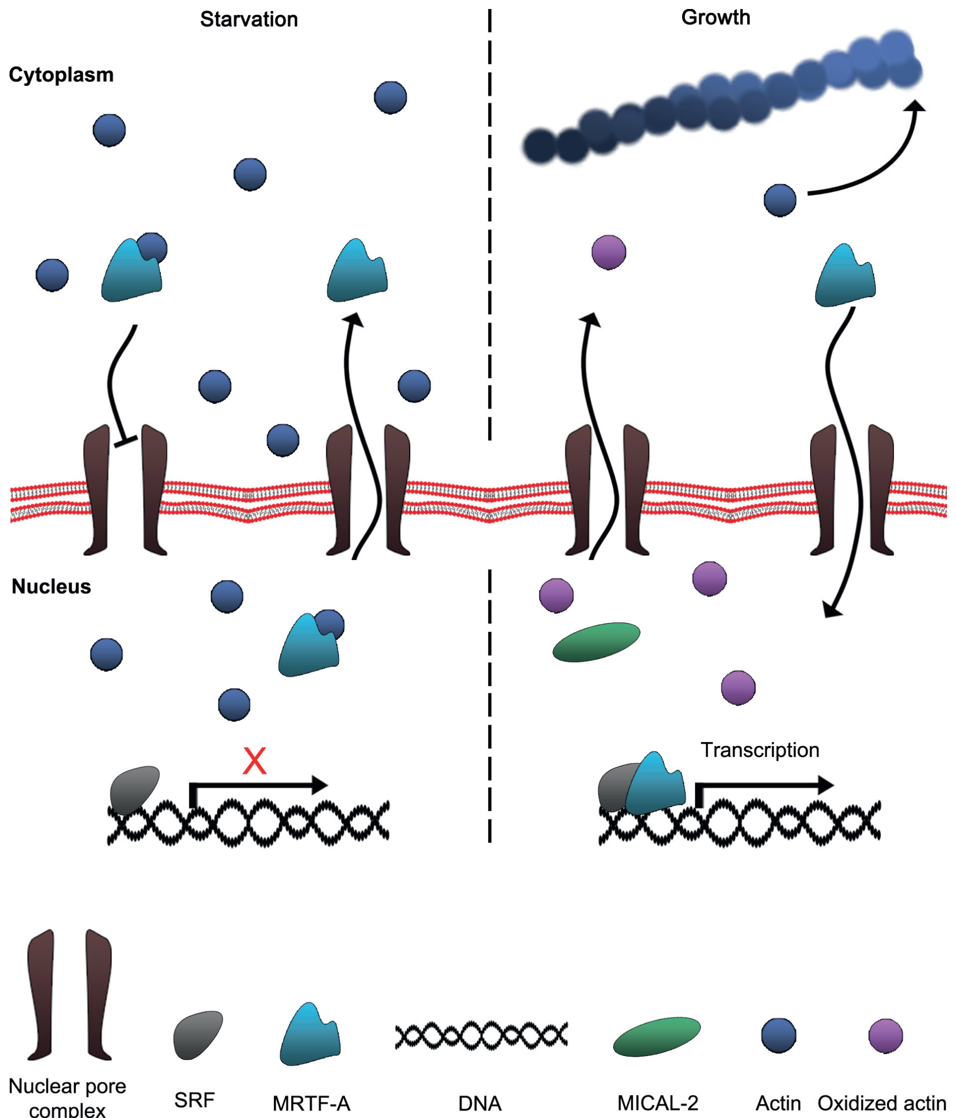

Figure 7 Nuclear shuttling of MRTF-A and SRF-mediated transcription in response to growth stimulation. Starved cells have an excess of cytosolic G-actin that sequesters MRTF-A and inhibits the translocation of MRTF-A to the nucleus (Left). In parallel, nuclear actin assists in exporting MRTF-A to the cytoplasm in order to inhibit SRF-mediated transcription. Upon growth stimulation and actin filament polymerization (Right), cytosolic G-actin is depleted and MRTF-A is free to translocate to the nucleus. In parallel, actin is exported to the cytosol, resulting in the interaction between MRTF-A and SRF and an activated transcription.

The increased SRF-transcription upon reducing the levels of CCTε by using siRNA can therefore not be due to actin polymerization as the G:F ratio was equally reduced in both cases. Thus, these data indicate that CCTε, unlike the other subunits of CCT, has a role in the SRF pathway.

Since SRF-mediated transcription is under the control of the co-transcriptional activator MRTF-A, we decided to address the possibility that CCTε may inhibit the SRF-based transcription by directly interacting with MRTF-A. We therefore produced full-length C-terminally-tagged MRTF-A, which was shown to be functional as it translocated to the nucleus upon serum stimulation, to use in *in vitro* binding assays. Crude lysates from cells transfected with MRTF-A were used to immunoprecipitate MRTF-A, where MRTF-A was shown to co-precipitate endogenous CCTε but not endogenous CCTα or CCTη. This suggests that monomeric CCTε and not the CCT oligomer or other monomeric subunits of CCT bind to MRTF-A. To confirm the interaction between MRTF-A and CCTε, we considered using crude lysates from cells transfected with MRTF-A and subunits of CCT fused to EGFP, which renders the subunits monomeric **(Paper I)**, in an EGFP-precipitation assay. To ensure the suitability of using an EGFP-CCTε fusion protein, we studied the cell shape of EGFP-CCTε expressing cells as the reduction of CCTε has previously been shown to induce a narrow elongated cell phenotype (Brackley and Grantham, 2010). Consistent with the narrow cell phenotype upon reducing the levels of CCTε, we showed that cells overexpressing monomeric EGFP-CCTε have an increased cell area. Moreover, we observed that EGFP-CCTε localizes at the cell periphery. A study showed that cells treated with AlF_4 had an increased formation of lamellipodia (Hahne et al., 2001). By treating cells with AlF_4, we showed that cells have endogenous CCTε localized to the cell periphery, which is consistent with the localization of EGFP-CCTε. Together, the results indicate that the use of an EGFP probe to render CCTε monomeric is a suitable approach as EGFP-CCTε behaves as endogenous CCTε. By immunoprecipitating EGFP-CCTε, we showed that EGFP-CCTε in contrast to EGFP-CCTα or EGFP alone co-precipitates MRTF-A, which supports a monomeric and subunit-specific interaction between CCTε and MRTF-A.

We then continued to study which domains of MRTF-A and CCTε were responsible for mediating their interaction. We showed that the apical domain, which is the domain with the least conserved amino acid sequence between the subunits of CCT (Kim et al., 1994), of CCTε ($CCT\varepsilon^{AD}$) binds to MRTF-A. Consistent with the apical domain of CCTε binding to

MRTF-A, the induced SRF-mediated transcription of luciferase upon targeting endogenous CCTε by siRNA was in contrast to EGFP-CCTα supressed by the overexpression of EGFP-CCTε^{AD}. Furthermore, we showed that only the C-terminal half of MRTF-A, which has been suggested to be involved in its cytoplasmic retention (Miralles et al., 2003), binds to full-length CCTε and CCTε^{AD}.

In order to study the effect on the nuclear translocation of MRTF-A upon changing the intracellular levels of CCTε, we overexpressed EGFP-CCTε and studied the location of MRTF-A over-time by using immunofluorescence. We showed that the overexpression of EGFP-CCTε in contrast to EGFP and EGFP-CCTη delayed the nuclear translocation of MRTF-A upon serum stimulation, which is possibly a consequence of a transient interaction between MRTF-A and CCTε in the cytosol. Consistent with a transient interaction between EGFP-CCTε and MRTF-A, the maximum number of cells with a nuclear staining of MRTF-A was shown to be similar for EGFP-CCTε as to EGFP and EGFP-CCTη. Together, these data suggest a novel role of monomeric CCTε in delaying SRF-mediated transcription by sequestering MRTF-A in the cytosol.

The CCT oligomer has been shown to be highly dynamic (Roobol et al., 1999a), thus alternating between an oligomeric assembly and a pool of free monomeric subunits. Based on our findings, we suggest a model wherein cells have developed a sensor-system that delays actin transcription when the levels of the CCT oligomer are reduced and the cells are unable to fold actin, as increased levels of monomeric CCTε would delay the nuclear translocation of MRTF-A.

Paper III – The Molecular Chaperone CCT Modulates the Activity of the Actin Filament Severing and Capping Protein Gelsolin In vitro

A previous study that identified the actin filament severing protein gelsolin as a CCT binding partner showed that the binding kinetics of gelsolin to CCT was slow and that gelsolin accumulated over time on CCT (Brackley and Grantham, 2011). This was in stark contrast to the folding substrate actin that rapidly binds and subsequently releases from CCT. Thus, the findings indicated that the biological significance of the binding of gelsolin to CCT is not for gelsolin to be folded by CCT. Indeed, although bacteria lack CCT, gelsolin can successfully

be produced as a native protein in bacteria (Nag et al., 2009). This underscores the fact that CCT is not essential for the folding of gelsolin. Instead, CCT may have a regulatory effect on gelsolin and CCT may indirectly influence actin filament dynamics via gelsolin. A study showed that CCT is able to influence actin dynamics *in vitro* by reducing the initial rate of actin filament polymerization (Grantham et al., 2002). Thus, the binding of gelsolin to CCT may be an additional way for CCT to control actin filament dynamics. It has been shown that the actin filament severing state of gelsolin is induced by the conformational changes that calcium mediates to the structure of gelsolin (reviewed by Nag et al., 2013). We sought to assess if CCT preferentially interacts with the inactive or the active actin filament severing conformation of gelsolin and if the interaction between CCT and gelsolin is direct.

Using bacteria as host for protein production, we were able to obtain a large amount of gelsolin for purification via a N-terminal HIS-tag. The ability of purified recombinant gelsolin to adopt different conformations upon adding or chelating calcium, indicative of a native state of gelsolin, was confirmed by non-denaturing PAGE. Gelsolin appeared as several smeared bands in the presence of calcium, indicating an open and flexible state of gelsolin, while the absence of calcium produced a single fast migrating entity of gelsolin, suggesting gelsolin to have obtained a closed and less flexible state.

In order to confirm that the conformational changes of recombinant gelsolin in the presence of calcium produce a functionally active gelsolin, we used *in vitro* cultured cells as a source of actin filaments and studied the actin filament severing activity of gelsolin in the presence and absence of calcium. The actin filaments are accessible to gelsolin after cell permeabilization and the actin filament severing activity of gelsolin can be measured as a reduction of phalloidin stained actin bundles (Cooper et al., 1987). Recombinant calcium-activated gelsolin was shown to be able to sever actin filaments, which was in contrast to calcium-free gelsolin. Thus, recombinant gelsolin is able to switch between an active and inactive state and is concluded to be in a native-like state, although a minor conformational difference when comparing bacterial expressed gelsolin to wild-type gelsolin cannot be excluded.

To study the interaction between CCT and gelsolin, we used a reducible cross-linker. Calcium-saturated gelsolin was shown to co-precipitate with immunoprecipitated CCT, which was in contrast to calcium-free gelsolin. Assuming that CCT only binds a single substrate at a time, as is the case for actin and tubulin (Llorca et al., 2000), we estimated that 30% of

immunoprecipitated CCT oligomer co-precipitated calcium-saturated gelsolin. The benefit of immunoprecipitating CCT instead of gelsolin is that the quantification of a single co-precipitating band of gelsolin could be done more accurately than quantifying several bands corresponding to the CCT subunits that will be in close proximity to the antibody heavy-chains. Since CCT was shown to only co-precipitate calcium-activated gelsolin and not calcium-free gelsolin, we concluded that the result from using a cross-linker shows a *bona fide* interaction between CCT and calcium-bound gelsolin. Moreover, gelsolin was shown to be the only co-precipitating protein from the precipitation of sucrose fractionated CCT supplemented with pure recombinant gelsolin. Thus, CCT was concluded to interact directly with calcium-saturated gelsolin.

We then sought to study if the calcium-dependent interaction between CCT and gelsolin affected the calcium-activated actin filament severing activity of gelsolin. For this assay, sucrose fractionated CCT was further purified using ATP-affinity chromatography. Calcium-activated recombinant gelsolin was pre-incubated with CCT or with BSA as a control and then incubated with fixed and permeabilized cells. We showed that CCT inhibits the gelsolin-mediated actin filament severing, as measured by the reduction of actin bundle staining, in a dose-dependent manner. In contrast, BSA did not protect the actin filaments from being severed by gelsolin, excluding the possibility of molecular crowding as a cause of the actin filament protection seen when gelsolin was incubated with CCT.

It has previously been shown that CCT co-sediments with actin filaments (Grantham et al., 2002) and that CCTε co-localizes with actin bundles (Brackley and Grantham, 2010). Therefore, we wanted to exclude the possibility that purified CCT protects the actin filaments from being severed by gelsolin by coating the actin bundles. Purified CCT was added to fixed and permeabilized cells and stained with a fluorescent antibody. The antibody staining of CCT did however not appear as filamentous, which indicates that the reduced actin filament severing activity of gelsolin is due to the interaction between gelsolin and CCT.

As the purified CCT contained contaminating hsc70, we showed that hsc70 does not affect the actin filament severing activity of gelsolin. Thus, the actin filament protection was dependent on CCT. To ensure that the actin filament severing activity of gelsolin is not due to unspecific proteolytic degradation as a result of protein impurities from the gelsolin purification, microtubule structures were studied after being incubated with calcium-saturated

gelsolin. We showed that purified gelsolin does not affect the microtubule structures and concluded that it is gelsolin that causes the severing of the actin filaments.

Taken together, we showed that the actin filament severing is mediated by gelsolin and that the severing activity is inhibited specifically by CCT as a result of the direct interaction between CCT and gelsolin. Consistent with the finding that the actin filament severing activity of gelsolin may be regulated by interacting proteins, a recent study showed that activated protein kinase receptor (PKR), which is up regulated during viral infection (Ank et al., 2006), binds to gelsolin and functions to reduce viral up-take by decreasing actin dynamics as a result of inhibiting gelsolin activity (Irving et al., 2012). In conclusion, our findings show that the role of CCT extends beyond the folding and assembly of substrates to also influence actin filaments dynamics via its binding to the calcium-activated actin filament severing protein gelsolin.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

We have shown that two of the subunits of CCT have a monomeric function. This includes an unknown function of endogenous monomeric CCTδ at the plasma membrane. In the overexpression of monomeric CCTδ, mammalian cells were shown to have an induction of cellular protrusions that were mainly identified as retraction fibres from retracting lamellipodia. We showed that the substrate-binding apical domain of CCTδ is essential for the localization of CCTδ to the plasma membrane and for inducing the protrusion phenotype. In addition, the ATP-binding domain of CCTδ was indicated to be important for inducing the phenotype. From yeast data, the efficiency of the membrane localization of CCT4 was shown to be dependent on the ATP-binding site of CCT4. In our second study, we showed that monomeric CCTε binds to the transcription activator MRTF-A and is able to delay the translocation of MRTF-A to the nucleus. We suggested that the binding of CCTε to MRTF-A serves as a sensing mechanism of the assembly state of CCT, where the SRF-target gene actin is not transcribed unless there is oligomeric CCT ready to fold the newly synthesised actin.

Besides showing novel monomeric functions for two of the subunits of CCT, we demonstrated that the CCT oligomer binds to the calcium-activated actin filament severing protein gelsolin and thereby inhibits the ability of gelsolin to sever actin filaments. Thus, the CCT oligomer is able to influence actin filament dynamics via gelsolin.

Together these findings demonstrate that the CCT oligomer, monomeric CCTε and monomeric CCTδ are able to influence the actin cytoskeleton and cellular processes linked to the actin cytoskeleton and that CCT has a function besides the folding and assembly of substrate proteins (Figure 8).

CELL SPREADING & MOTILITY

As the data presented here connects the CCT oligomer and the CCT monomers to cell shape and actin filament dynamics, the data will be discussed in relation to cell spreading and cell motility as these are processes dependent on the actin cytoskeleton.

Upon growth stimulation and cell spreading, actin polymerization is favoured and the concentration of G-actin is reduced. In parallel, the oligomeric assembly of CCT may be favoured over free single subunits by a so-far unknown mechanism based on additional factors (Roobol et al., 1999a). As a result, MRTF-A is free to translocate to the nucleus and initiate actin transcription due to its loss of binding to monomeric CCTε and actin **(Paper II)**. The reduction of monomeric CCTδ has previously been shown to increase cell spreading (Brackley and Grantham, 2010). We have shown that overexpression of monomeric CCTδ induces the formation of membrane protrusions **(Paper I)**. As cell spreading and cell shape dynamics at the leading edge are important for cell locomotion, the unknown function of monomeric CCTδ at the plasma membrane may be important for cell motility.

Cell migration *per se* is dependent on increased actin dynamics at the leading edge, where gelsolin increases the number of actin filaments that are available for actin polymerization (Figure 9). We demonstrated that CCT inhibits the actin filament severing activity of gelsolin **(Paper III)**, although it remains elusive where this inhibition takes place in the cell. Therefore, to understand the biological relevance of CCT to inhibit the actin filament severing activity of gelsolin, it is necessary to design experiments that study the subcellular location of where CCT and gelsolin may interact. Whereas CCT is seen evenly distributed throughout the cell (Brackley and Grantham, 2010), gelsolin seems to be partly enriched at the cell-periphery (Cooper et al., 1988). In addition, the intracellular ratio of CCT and gelsolin is different and estimated to be 0.04% (Boudiaf-Benmammar et al., 2013) and 0.1% respectively (Cunningham et al., 1991). Thus, there may be local concentration differences between CCT and gelsolin, which could have an impact on the subcellular location of where the activity of gelsolin is inhibited by CCT.

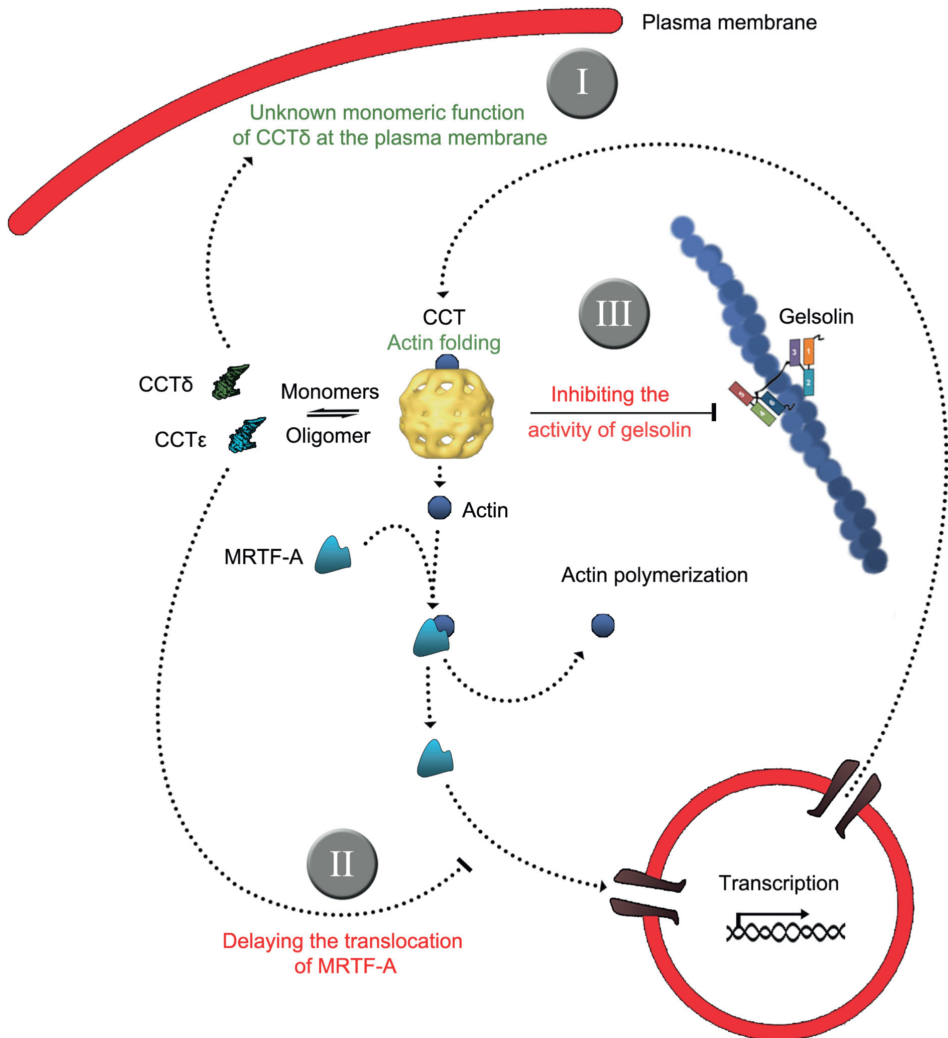

Figure 8 Summary of the findings on the relation between CCT and the actin network We propose that the cell has developed a safeguarding system that connects the oligomeric state of CCT to SRF-mediated transcription, where monomeric CCTε will delay nuclear translocation of MRTF-A **(paper II)**. Thus, unless CCT is oligomeric and ready to fold newly translated transcripts of SRF-target genes, such as actin, the transcription will not be initiated. We have also shown that the CCT oligomer inhibits the actin filament severing activity of gelsolin **(Paper III)** and that monomeric CCTδ has an unknown function at the cell membrane **(Paper I)**.

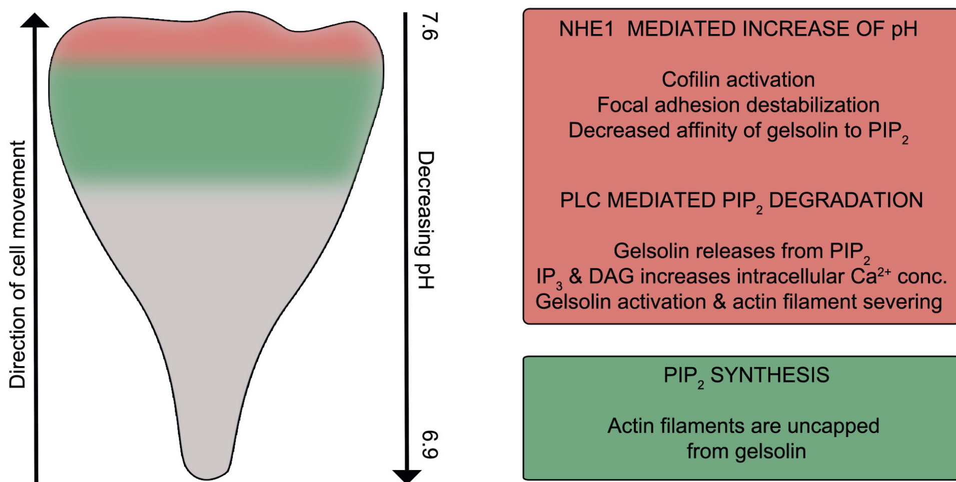

Figure 9 Gelsolin activation and cell migration. A local intracellular increase in pH as a result of the activation of the H^+ exporter NHE1 at the leading edge by growth factors destabilizes focal adhesions, activates actin filament turnover via cofilin (reviewed by Casey et al., 2010; Srivastava et al., 2007) and decreases the affinity between gelsolin and membrane bound PIP_2 (Lin et al., 1997). In parallel, growth factor activated PLC competes with gelsolin in binding PIP_2 (Sun et al., 1997) and hydrolyses PIP_2 to IP_3 and DAG (reviewed by Lemmon and Schlessinger, 2010), which results in an intracellular increase in calcium (reviewed by Berridge et al., 2000) and an increased cytosolic level of gelsolin. Increased calcium levels activates free gelsolin to sever actin filaments (Area marked in red)(reviewed by Nag et al., 2013), resulting in gelsolin capping the actin filaments (Harris and Weeds, 1984). Behind the leading edge (Marked in green), PIP_2 is synthesized (Luo et al., 2004) and uncaps gelsolin from the actin filaments by binding to gelsolin (Allen, 2003; Janmey et al., 1987). The affinity of PIP_2 for gelsolin during increased calcium concentrations increases by 8-fold (Lin et al., 1997). The severing and capping of the actin filaments results in increased actin filament dynamics by increasing the number of filament ends that are available for polymerization.

As the formation of lamellipodia is dependent on the activity of gelsolin (Azuma et al., 1998), it may be that gelsolin is inhibited by CCT at other subcellular locations away from the leading edge where the activity of gelsolin is not required. Since the activation of gelsolin is a dynamic process that occurs during cell migration, it may be an advantage to study the co-localization of CCT and gelsolin using time-lapse microscopy on motile cells. A previous study measured the dissociation of actin from gelsolin by using fluorescence resonance energy transfer (FRET), where purified actin and gelsolin were injected to living cells

following their coupling with a donor and acceptor fluorophores respectively (Allen, 2003). However, calcium levels changed the emission of the gelsolin-coupled acceptor fluorophore independently of any FRET process following the excitation of the donor fluorophore. Therefore, fluorescence quenching by resonance energy transfer (FqFRET) was used instead of FRET. Gelsolin was coupled with a quenching fluorophore (QSY9) that absorbs the emitted fluorescence of the donor fluorophore (Rhodamine) without fluorescing and the dissociation of actin from gelsolin was measured as a decreased fluorescence of the donor fluorophore. As a control for the cellular distribution of gelsolin, gelsolin was coupled with a second fluorophore in complex with unlabelled actin. By applying the same method when studying the co-localization of CCT and gelsolin in a motile cell, FqFRET may be used to study a decreased emission of rhodamine coupled to CCT in the presence of gelsolin coupled to the quenching fluorophore QSY9. As a control for protein distribution, CCT would be coupled with a second fluorophore. The co-localization of CCT and gelsolin would be expected to occur during processes that are dependent on the active actin filament severing state of gelsolin, such as during cell locomotion (Azuma et al., 1998).

LAST WORD

In summary, this thesis has presented data that has shown a novel function of the CCT oligomer, as well as identified specific functions of the monomeric subunits CCTδ and CCTε of CCT. Clearly, these findings show that the molecular chaperone CCT has roles beyond the folding of proteins, which may have a broad impact on cell biology.

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