The molecular chaperone CCT modulates the activity of the actin filament severing and capping protein gelsolin in vitro

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Abstract The oligomeric molecular chaperone CCT is essential for the folding of the highly abundant protein actin, which in its native state forms actin filaments that generate the traction forces required for cell motility. In addition to folding proteins, CCT can provide a platform for protein complex assembly and binds actin filaments assembled in vitro. Some individual subunits of CCT, when monomeric, have been shown to be functionally active, and in particular, the CCTepsilon subunit is involved in the serum response factor pathway that controls actin transcription. Thus, there is a complex interplay between CCT and actin that extends beyond actin folding. CCT has recently been shown to bind gelsolin, an actin filament severing protein that increases actin dynamics by generating filament ends for further actin polymerization. However, the biological significance of the CCT:gelsolin interaction is unknown. Here, using a co-immunoprecipitation assay, we show that CCT binds directly to gelsolin in its calcium-activated, actin-severing conformation. Furthermore, using actin filaments retained from fixed and permeabilized cells, we demonstrate that CCT can inhibit the actin filament severing activity of gelsolin. As our work and that of others shows gelsolin is not folded by CCT, the CCT:gelsolin interaction represents a novel mode of binding where CCT may modulate protein activity. The data presented here reveal an additional level of interplay between CCT and actin mediated via gelsolin, suggesting that CCT may influence processes depending on gelsolin activity, such as cell motility.

Keywords Molecular chaperone · CCT · Actin · Gelsolin · Cytoskeleton

Introduction

Chaperonin containing Tcp-1 (CCT), also known as Tcp-1 ring complex (TRiC), forms an oligomer that consists of eight distinct subunits (named α - θ in mammals) each occupying a fixed position within the two back-to-back chaperonin rings (Kalisman et al. 2012; Kim et al. 1994). CCT was originally identified as a molecular chaperone required for the folding of the highly abundant proteins actin and tubulin (e.g., Sternlicht et al. 1993). The binding of these proteins to CCT occurs via interactions with specific CCT subunits, indicative of CCT having a specialized role in the folding of these proteins (Llorca et al. 2001; Llorca et al. 2000; Llorca et al. 1999). More recently, CCT has been shown to interact with a more extended range of proteins (e.g., Dekker et al. 2008; Yam et al. 2008), some of which will represent obligate substrates, some non-obligate substrates, and some regulatory proteins (reviewed by Brackley and Grantham 2009). However, actin and tubulin represent the major folding substrates of CCT (Grantham et al. 2006; Sternlicht et al. 1993), thus linking CCT to important biological functions mediated by the cytoskeleton, such as cell migration, chromosome segregation, and intracellular transport (reviewed by Fletcher and Mullins 2010).

In the case of actin, CCT has an extended role, which includes the CCT ϵ subunit in its monomeric form. CCT ϵ has been shown to co-localize with actin bundles and depleting CCT ϵ results in a narrow, elongated cell phenotype, suggesting that CCT ϵ may participate in stabilizing actin bundles (Brackley and Grantham 2010). CCT ϵ was recently shown to influence the serum response factor signaling pathway by

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binding to the co-transcription factor myocardin-related co-transcription factor-A (MRTF-A) and affecting MRTF-A nucleo-cytoplasmic shuttling, suggesting that CCT ϵ connects the cell's capacity to fold actin with actin transcription (Elliott et al. 2015).

It has also been demonstrated that the CCT oligomer affects actin filament dynamics by reducing the initial rate of polymerization of actin filaments and the rate of barbed-end elongation in vitro (Grantham et al. 2002). Thus, CCT is able to directly influence the dynamics of assembling actin. Additionally, the CCT oligomer was shown to bind the cytosolic isoform of gelsolin via a co-immunoprecipitation assay using lysates from cultured mammalian cells and in in vitro translation assays (Brackley and Grantham 2011). This reveals an indirect way in which CCT may influence actin dynamics, as it is well established that gelsolin severs, caps, and nucleates actin filaments (e.g., Way et al. 1989; Yin et al. 1981) and reviewed by Nag et al. (2013).

Gelsolin consists of six domains (Burtnick et al. 1997) and has been shown to bind calcium at several internal sites, which sequentially induces conformational changes, resulting in an active open state of gelsolin (reviewed by Nag et al. 2013). Following actin filament severing, gelsolin is left capping the barbed end of the severed actin filament (Yin et al. 1981) and is recycled by being released from the filament upon binding to phosphatidylinositol biphosphate (Janmey et al. 1987). Thus, the activation of gelsolin, followed by severing and uncapping of actin filaments, increases the number of barbed filament ends available for elongation.

Interestingly, the kinetics of gelsolin binding to CCT are different to those of the obligate folding substrate actin, which rapidly binds and subsequently releases from CCT, while gelsolin binds to CCT in a slow and accumulative fashion, indicating that gelsolin is not a folding-substrate of CCT (Brackley and Grantham 2011). Gelsolin produced in bacteria can fold to an active state (Nag et al. 2009), and as bacteria lack CCT, these observations are consistent with CCT not being required for the folding of gelsolin. Thus, the biological significance of the interaction between gelsolin and CCT remains elusive. Here, we use immunoprecipitation to determine which conformation of gelsolin binds to CCT and perform in vitro actin filament severing assays to address the biological significance of the CCT:gelsolin interaction.

Methods

Cloning, expression, and purification of His-tagged mouse gelsolin

The cytosolic isoform of mouse gelsolin (NM_146120.4, amino acids M50-A780) was cloned into the destination plasmid pET43.1a (Merck Millipore) using the cloning primers listed

in Table 1, and the resulting construct was verified by sequencing. BL21(DE) cells were transformed with the plasmid containing the His₆-tagged mouse gelsolin and grown at 37 °C at 200 rpm to an OD₆₀₀ of 0.5–0.7. Cells were then incubated for 30 min at 18 °C at 200 rpm, and the expression of gelsolin was induced with 1 mM IPTG for 21 h at 18 °C at 200 rpm. Cells were collected by centrifugation at 5300g and lysed for 20 min on ice with B-PER reagent (Thermo Scientific) containing bacterial protease inhibitors diluted 1/60 (P8849, Sigma Aldrich). Lysates were clarified by centrifugation at 13,500g for 5 min at 4 °C and then supplemented with 25 mM imidazole prior to incubating with Ni-NTA resin (Invitrogen) for 30 min at 4 °C on a rotating wheel. The Ni-NTA resin was then washed in ice-cold purification buffer (50 mM HEPES pH 8, 150 mM NaCl) containing 25 mM imidazole, and gelsolin was eluted with purification buffer supplemented with 250 mM imidazole. Eluted proteins were dialyzed overnight at 4 °C against gelsolin buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10 % glycerol). Protein concentrations were determined by using the extinction coefficient of 115,280 M⁻¹ cm⁻¹ as estimated using ExpASY (Swiss Institute of Bioinformatics).

Native-PAGE

Purified gelsolin was incubated with a final concentration of 5 mM CaCl₂ or 5 mM EGTA for 1 h on ice and then resolved on a 6 % non-denaturing polyacrylamide gel at 4 °C at 90 V (Liou and Willison 1997). Proteins were visualized by staining with Coomassie Brilliant Blue.

Cell culture

BALB 3T3 cells were maintained in growth medium (DMEM supplemented with 10 % heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml plasmocin) at 37 °C with 5 % CO₂. Cells were passaged by washing with 37 °C PBS followed by an incubation in 0.25 % trypsin-EDTA solution (Sigma-Aldrich). Detached cells were collected by centrifugation at 700g at room temperature for 5 min and resuspended in growth medium.

Sucrose density gradient fractionation

Confluent BALB 3T3 cells from four petri-dishes of Ø 9 cm were washed in 37 °C PBS and detached using 1 mM EDTA in 37 °C PBS. Cells were collected by centrifugation, washed in PBS, and lysed in ice-cold lysis buffer (50 mM HEPES pH 7.2, 90 mM KCl, 0.5 % v/v IGEPAL) containing mammalian protease inhibitor, diluted 1/500 (P8340 Sigma-Aldrich). The lysate was clarified by centrifugation at 4600g for 5 min at 4 °C, and the resulting post-nuclear supernatant was loaded on a continuous gradient of 10–40 % sucrose (w/v) in sucrose

Table 1 Cloning primers

Primer	Enzyme	Oligonucleotide sequence 5'–3'
FW	NdeI	GCATCATATGCATCATCATCATCACATGGTGGTG GAGCACCCCGAATTCCTGAAGGCAGG
RV	NotI	CGTAGCGGCCGCTCAGGCAGCCAGCTCAGCCAAGG

buffer (50 mM HEPES pH 7.2, 90 mM KCl) containing mammalian protease inhibitor diluted 1/5000. Gradients were run in a XL-90 ultracentrifuge (Beckman) using a SW55Ti rotor for 18 h at 4 °C at 85,000g, and 400- μ l fractions were collected. The 20S sucrose fraction contained a final concentration of 180 nM CCT oligomer, as calculated by estimating a purity of 40 % and by using 1 abs=1 mg/ml and a molecular weight of 0.95 MDa.

ATP affinity chromatography purification

The CCT oligomer, enriched by sucrose gradient fractionation, was diluted with three volumes of CCT binding buffer (20 mM HEPES pH 7.2, 25 mM KCl, 10 mM MgCl₂) and loaded onto an ATP-affinity column run under gravity at 4 °C. The column was washed with four column volumes of binding buffer prior to eluting CCT with binding buffer supplemented with 10 mM ATP. Fractions containing CCT were diafiltrated and concentrated in CCT binding buffer using an Amicon Ultra 30 k (Merck Millipore) at 4 °C. The concentration of the CCT oligomer was measured by the Bradford assay method (Bradford 1976).

Immunoprecipitation

The CCT oligomer, enriched by sucrose gradient fractionation, and gelsolin were clarified by centrifugation at 21, 130g for 15 min at 4 °C. CCT and gelsolin were mixed to a final concentration of 50 and 450 nM, respectively, and supplemented with a final concentration of 2 mM MgCl₂ including either 5 mM CaCl₂ or 5 mM EGTA. The protein solutions were incubated for 30 min on ice to allow protein:protein interactions to occur. Samples were then cross-linked by incubation with a final concentration of 0.25 mM dithiobis(succinimidyl propionate) (Thermo Scientific) at room temperature for an additional 30 min. The cross-linker was quenched for 15 min at room temperature with a final concentration of 45 mM TRIS-base (pH 7.5), and the sample was diluted three times in IP buffer (50 mM HEPES pH 7.2, 90 mM KCl, 0.5 % IGEPAL, 0.05 % deoxycholate). Cross-linked proteins were incubated with the monoclonal antibody to CCT ϵ , clone ϵ AD1 (Llorca et al. 2001), on ice for 45 min and added to a 1:1 protein-G bead slurry (GE Healthcare) prewashed in IP buffer. Samples were then incubated for 45 min at 4 °C on a rotating wheel and washed four times for 5 min in IP buffer prior to being dried under vacuum.

Proteins were extracted from the beads by addition of reducing SDS-PAGE sample buffer and then resolved by SDS-PAGE on a 9 % polyacrylamide gel. Proteins were visualized by silver staining.

Actin filament severing assay

BALB 3T3 cells were plated on glass coverslips (#1.5) at a cell density of 20×10^4 cells per petri-dish of $\varnothing$ 6 cm and cultured for 1–2 days. Cells were then washed twice in 37 °C PBS and fixed at 37 °C in 4 % formaldehyde (v/v) for 10 min, followed by three additional washes in PBS. Fixed cells were permeabilized for 15 min by incubating in 0.2 % TritonX-100 (v/v) in PBS at room temperature and then washed three times in PBS. Specific proteins, each at a concentration of 100–300 nM, were preincubated with 5 mM CaCl₂ or 5 mM EGTA for 30 min on ice before being added to the permeabilized cells. The cells were then incubated with the protein solutions at room temperature for 15 min, washed three times in PBS and blocked with 3 % BSA (w/v, PBS) at room temperature for 30 min. Following blocking, cells were incubated for 20 min with FITC-conjugated phalloidin (0.1 μ g/ml) in blocking solution and washed three times in PBS. Coverslips were then mounted in Prolong Gold antifade reagent (Invitrogen). Images were taken with an exposure time of 2500 ms, and fluorescence intensity per area unit was measured for single cells using ImageJ software (National Institutes of Health).

Immunofluorescence

Cells were fixed, permeabilized, and blocked as for the actin filament severing assay. Following blocking, cells were incubated for 1 h with either the monoclonal antibody to CCT δ , clone δ 8g (Llorca et al. 1999) or the monoclonal antibody to α -tubulin clone B-5-1-2 (Sigma-Aldrich) diluted 1/100 in blocking solution. Cells were then washed three times in PBS and incubated for 1 h with TRITC-conjugated anti-mouse secondary antibody diluted 1/200 in blocking solution. Following an additional three washes in PBS, coverslips were mounted in Prolong Gold antifade reagent (Invitrogen).

Microscopy

Images were taken using a Zeiss Axioplan 2 imaging wide-field microscope with AxioVision4 software. The objectives

Plan-NeoFluar 100×/1.30 oil ∞/0.17 and Plan - NeoFluar 40×/0.75 ∞/0.17 were used with the following Zeiss filtersets: 10 (FITC, Ex BP 450–490, FT510, Em BP 515–565) and 15 (TRITC, Ex BP 546/12, FT580, Em LP 590).

Results

His-tagged recombinant gelsolin is produced as an active enzyme

In order to study the interaction between CCT and gelsolin, cytosolic mouse gelsolin was produced in *E. coli*. Recombinant gelsolin, purified via an N-terminal His₈-tag, has previously been shown to sever in vitro polymerized actin filaments in the presence of calcium (Nag et al. 2009). Here, N-terminal His₆-tagged gelsolin was similarly purified via Ni-NTA resin. However, including 25 mM imidazole in clarified lysates and in washing buffers during purification reduced the levels of co-purifying proteins and excluded the need for further purification, as gelsolin purity was typically >85 % pure as confirmed by SDS-PAGE. The activation mechanism of native gelsolin, which leads to actin filament severing and increased actin dynamics, requires gelsolin to undergo conformational changes. These changes are induced by the binding of calcium with different affinities to several internal sites within gelsolin, which then sequentially adopts an open conformation (Burtnick et al. 2004 and Fig. 1a). Calcium-saturated and calcium-free recombinant gelsolin was resolved on a native acrylamide gel and Coomassie stained. Calcium-

saturated gelsolin migrated as a diffused cluster of bands, whereas calcium-free gelsolin formed a sharp band that migrated considerably further through the gel (Fig. 1b). These observations are therefore consistent with purified recombinant gelsolin being able to change conformation upon binding calcium. To confirm that recombinant gelsolin severs actin filaments in a calcium-dependent manner, actin filaments retained from fixed and permeabilized BALB 3T3 cells were incubated with calcium-saturated or calcium-free gelsolin. The severing activity of gelsolin was evaluated by the staining intensity of actin bundles with FITC-conjugated phalloidin. Cells incubated with calcium-saturated gelsolin showed a reduced actin bundle staining as compared to calcium-free gelsolin (Fig. 1c). Thus, purified recombinant gelsolin is concluded to be native due to its ability to change conformation and sever actin filaments in the presence of calcium.

CCT binds directly to calcium-saturated gelsolin

Gelsolin binds to the molecular chaperone CCT (Brackley and Grantham 2011) but does not require interactions with CCT in order to be functional, as active gelsolin can be produced in the absence of CCT in *E. coli* (Nag et al. 2009). The binding of gelsolin to CCT was shown to occur in cell lysates where calcium was neither added nor chelated (Brackley and Grantham 2011). We therefore addressed if the conformational state of gelsolin is important for its interaction with the CCT oligomer. To this end, we chose a calcium concentration where we expected gelsolin to be in its fully open conformation in the context of an in vitro assay (Ashish et al. 2007) as the affinity of

Fig. 1 Recombinant His-tagged gelsolin is produced as an active enzyme. **a** Gelsolin (*Gsn*) exists in an active calcium-bound open state or an inactive calcium-free closed state. **b** Incubating purified gelsolin with calcium or EGTA induces a conformational change that results in a mobility shift during Native PAGE, visualized by staining with Coomassie Brilliant Blue. **c** BALB 3T3 cells cultured for 2 days were fixed, permeabilized, and incubated with 300 nM gelsolin in the presence of calcium or EGTA. Actin bundles were visualized with FITC-conjugated phalloidin staining. Scale bar 50 μm

gelsolin binding calcium *in vivo* will be influenced by the presence of actin (reviewed by Nag et al. 2013). To ensure maximal recovery of CCT, we utilized the monoclonal antibody ϵ AD1 which recognizes the apical domain of CCT ϵ and has been shown to immunoprecipitate CCT independently of the chaperonin nucleotide cycle, thus rendering it highly efficient for recovering CCT oligomer (Llorca et al. 2001). CCT and gelsolin were cross-linked after a binding incubation step in the presence or absence of calcium, and then complexes were immunoprecipitated. Calcium-saturated gelsolin was shown to co-immunoprecipitate with CCT, whereas under the same cross-linking conditions, calcium-free gelsolin was not co-immunoprecipitated (Fig. 2). We estimated by densitometric analysis that approximately 30 % of the recovered CCT oligomers have gelsolin bound, assuming that one molecule of gelsolin will bind to one CCT oligomer as is the case for actin and tubulin (e.g., Llorca et al. 2001). The immunoprecipitation of CCT was shown more efficient in calcium-free conditions, yet no gelsolin was co-immunoprecipitated (Fig. 2a), further supporting a calcium-dependent binding of gelsolin to CCT. Furthermore, no additional proteins from the CCT-enriched sample were co-immunoprecipitated, consistent with gelsolin interacting directly with CCT (Fig. 2a).

Gelsolin-mediated severing of actin filaments is inhibited by CCT

Gelsolin is active and severs actin filaments in the presence of calcium (reviewed by Nag et al. 2013; Yin and Stossel 1979), the same conditions in which gelsolin binds to CCT (Fig. 2). We therefore asked if CCT altered the ability of active gelsolin to sever actin filaments. Actin filaments retained from fixed and

permeabilized cells were incubated with calcium-saturated gelsolin and increasing molar-ratios of CCT to gelsolin. In the presence of CCT, actin filament severing by gelsolin was inhibited in a dose-dependent manner (Fig. 3a, b). No severing inhibition was observed with increasing concentrations of BSA, excluding an effect of molecular crowding being responsible for the observed decreases in severing activity in the presence of CCT. Although the molecular chaperone Hsc70 co-purified with CCT (verified by mass-spectrometry), no inhibitory effect upon actin filament severing was observed in the presence of purified Hsc70 (Fig. 3c). Thus, the inhibition of gelsolin severing activity is specific to CCT and is not a general chaperone effect.

The CCT oligomer has previously been shown to co-sediment with actin filaments assembled *in vitro* (Grantham et al. 2002), and CCT ϵ was found to co-localize with actin bundles in cultured mammalian cells (Brackley and Grantham 2010). To exclude the possibility that during the actin filament severing assays shown here, CCT oligomer interacts directly with the actin filaments, thus preventing gelsolin severing, fixed cells were incubated with CCT and stained with the monoclonal antibody δ 8g. However, no filamentous staining was observed, indicating that the added CCT oligomer does not bind actin bundles under these conditions (Fig. 3d). Thus, the inhibition of gelsolin severing activity is most probably a result of CCT:gelsolin interactions. To ensure that the severing effect seen on actin filaments was not a result of unspecific proteolytic degradation, the integrity of microtubules, which are not targets of gelsolin, was investigated. Cells incubated with calcium-saturated gelsolin showed no reduced microtubule staining as compared to untreated cells, thus excluding unspecific proteolytic degradation of the actin filaments occurring in the actin filament severing assays (Fig. 3e).

Fig. 2 CCT binds directly to calcium-saturated gelsolin. **a** Gelsolin (*Gsn*) preincubated with calcium or EGTA was incubated with CCT followed by cross-linking and immunoprecipitation with an antibody to CCT ϵ . Resolved proteins were visualized by silver staining. The positions of

gelsolin (arrowhead), CCT (bracket), antibody heavy chain (*H*), and antibody light chain (*L*) are indicated. **b** Quantification of co-immunoprecipitated gelsolin. SEM ($n=4$) is presented. * $p<0.05$ using the Mann-Whitney test

Fig. 3 Gelsolin-mediated severing of actin filaments is inhibited by CCT. **a** BALB 3T3 cells plated for 1 day were fixed, permeabilized, and incubated with 100 nM calcium-saturated gelsolin (*Gsn*) and an increasing molar ratio (1, 2, and 3) of CCT or BSA. BSA was dissolved in CCT binding buffer, and the proteins were incubated in a 1:1 volume ratio. Actin bundles were visualized with FITC-conjugated phalloidin staining. **b** Quantification of actin bundle staining. SEM (counting 100 cells for each condition, $n=3$ to 7) is presented. X =molar ratio to gelsolin. $*p<0.05$ using the Mann-Whitney test. **c** Cells incubated with 100 nM calcium-saturated gelsolin and 195 nM Hsc70. Hsc70 was diluted in CCT binding buffer, and the proteins were incubated in a 1:1 volume ratio. ATP-affinity purified CCT was resolved on a polyacrylamide gel and visualized by Coomassie Brilliant Blue staining. **d** Cells were fixed

and permeabilized as in **a**, followed by an incubation for 15 min at room temperature with or without 300 nM CCT (*CCT* or *Ctr*) and with a final concentration of 5 mM calcium. CCT was incubated in a 1:1 volume ratio with gelsolin buffer. Cells were then washed once in PBS, and an additional fixation step was performed by incubating with 4 % (v/v) formaldehyde for 10 min at room temperature followed by washing three times in PBS. Cells were then stained with a monoclonal antibody to CCT δ . **e** Cells were fixed and permeabilized as in **a**, followed by an incubation for 15 min at room temperature with or without 300 nM gelsolin (*Gsn* or *Ctr*) and with a final concentration of 5 mM calcium. Cells were then washed three times in PBS and stained with a monoclonal antibody to α -tubulin. Scale bar 50 μ m

Discussion

The interplay between CCT and actin extends from the folding of actin (e.g., Llorca et al. 1999; Sternlicht et al. 1993) to include influencing actin dynamics (Grantham et al. 2002) and actin bundle binding (Brackley and Grantham 2010). Here, we focus on the functional significance of the CCT:gelsolin interaction (Brackley and Grantham 2011) and

establish CCT as factor for regulating the actin filament severing activity of gelsolin.

CCT interacts with several categories of binding partners: the obligate folding substrates such as actin and tubulin, less stringent folding substrates, and proteins that regulate CCT activity (reviewed by Grantham 2010). Furthermore, CCT has also been shown to act as a platform for protein complex assembly (Feldman et al. 1999). Recombinant gelsolin

expressed in *E. coli* is functionally active (Fig. 1c and Nag et al. 2009), and since bacteria lack CCT, these findings further support previous data suggesting that gelsolin does not need CCT to reach its native state and is therefore not an obligate folding substrate of CCT (Brackley and Grantham 2011).

We demonstrate that it is specifically the calcium-activated conformation of gelsolin that binds directly to CCT (Fig. 2), consistent with either CCT having a regulatory role in gelsolin activity or CCT facilitating conformational changes in gelsolin. However, as CCT has an inhibitory effect on gelsolin-mediated actin filament severing (Fig. 3a, b), this is indicative of CCT regulating actin severing by sequestering active gelsolin. This would add an additional level of control for gelsolin-mediated actin filament severing beyond changes in cellular Ca^{2+} concentrations. Consistent with the activity of gelsolin being influenced by regulatory proteins, a recent report has demonstrated that the protein kinase receptor (PKR), a sensor-protein that is upregulated during viral infection (Ank et al. 2006), interacts with gelsolin and inhibits actin filament severing (Irving et al. 2012).

Overexpression of gelsolin in fibroblast cells increases cell motility (Cunningham et al. 1991), whereas fibroblasts from gelsolin knock-out mice have increased numbers of stress fibers and display reduced motility during wound healing assays (Witke et al. 1995). These results are consistent with invasion studies in cancer cell lines where reducing gelsolin levels reduces invasiveness (Van den Abbeele et al. 2007). Thus, it is possible that CCT may be able to influence cell migration via sequestering gelsolin, and it will therefore be important to understand how CCT availability to perform specific functions, such as actin and tubulin folding and gelsolin regulation, is accommodated. For example, in a rapidly growing cell, increased levels of CCT may be required predominantly for actin and tubulin folding with less CCT available for modulating gelsolin activity. In conclusion, the CCT/gelsolin interaction does not only represent an indirect way for CCT to influence actin dynamics by adding to the complexity of the interplay between the actin cytoskeleton and CCT but also reveal a novel mode of action for CCT in regulating protein activity.

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