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Short communication

# Affinity purification reveals the association of WD40 protein constitutive photomorphogenic 1 with the hetero-oligomeric TCP-1 chaperonin complex in mammalian cells

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## Abstract

Constitutive photomorphogenic 1 (COP1), a protein composed of a RING finger, a coiled-coil domain and seven WD40 repeats, functions as an E3 ubiquitin ligase that targets key transcription factors for ubiquitination and degradation in both higher plants and mammalian cells. While COP1 is required for light-mediated development in plants, its mammalian counterpart has been implicated in tumorigenesis. We previously showed that COP1 forms high-molecular-weight complexes in mammalian cells. Here we report our attempts in characterizing the components of the mammalian COP1 complexes by affinity purification combined with mass spectral analysis. We find that both transiently and stably expressed COP1 associates with the hetero-oligomeric TCP-1 chaperonin complex (TRiC), heat shock protein 70 (Hsp70) and BAG-family molecular chaperone regulator-2 (BAG2). In addition, stably expressed COP1 binds to major vault protein (MVP) and translocated promoter region (Tpr). The TRiC/Hsp70 complex is known to interact with and assist in the folding of a number of WD40 proteins in *Saccharomyces cerevisiae*. The association of WD40 protein COP1 with TRiC/Hsp70 in mammalian cells suggests that facilitating the folding of WD40 proteins may be a conserved function for TRiC/Hsp70 from yeast to mammals.

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**Keywords:** COP1; TRiC; Hsp70; WD40; Protein folding

## 1. Introduction

Constitutive photomorphogenic 1 (COP1) was first discovered in Arabidopsis through genetic screens as an essential negative regulator of light-mediated seedling development (Deng, Caspar, & Quail, 1991). Mutations in the *COP1* gene uncouple light-induced developmen-

tal pathways from light signals and cause vast changes in gene expression profiles (Deng et al., 1991; Ma et al., 2002). An interesting characteristic of the Arabidopsis COP1 protein (atCOP1) is that its subcellular localization is regulated by light (Von Arnim & Deng, 1994). AtCOP1 localizes predominantly to the cytoplasm in the presence of light, but becomes nuclear enriched when transferred to the dark. Within the nucleus, atCOP1 is thought to promote the degradation of several photomorphogenesis promoting transcription factors such as HYH, LAF1 and HFR1, and a major photoreceptor, phyA, through its inherent E3 ubiquitin ligase activ-

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ity (Ang et al., 1998; Duek, Elmer, van Oosten, & Fankhauser, 2004; Holm, Ma, Qu, & Deng, 2002; Jang et al., 2005; Osterlund, Hardtke, Wei, & Deng, 2000; Saijo et al., 2003; Seo et al., 2003; Seo, Watanabe, Tokutomi, Nagatani, & Chua, 2004; Yang et al., 2005).

COP1 homologues have been identified in vertebrates, including fish, frogs, birds and mammals (Yi, Wang, Wei, & Deng, 2002). While a COP1 homologue exists in the mosquito genome, no COP1 homologues have been identified in yeast, *Drosophila* or *C. elegans*. We previously showed that similar to atCOP1, mammalian COP1 also shuttles between the nucleus and the cytoplasm (Yi et al., 2002). Moreover, like its plant counterpart, mammalian COP1 binds to ubiquitinated proteins and is itself ubiquitinated in vivo (Yi et al., 2002). So far, two COP1 ubiquitination substrates, proto-oncogene c-Jun and tumor suppressor p53, have been identified in mammalian cells (Dornan, Wertz et al., 2004; Wertz et al., 2004), implying a role of COP1 in tumorigenesis. Recently, COP1 has consistently been found overexpressed in a number of breast and ovarian cancer samples, especially among those containing wild-type p53 (Dornan, Bheddah et al., 2004).

The COP1 protein contains three recognizable domains: an N-terminal RING-finger motif, followed by a coiled-coil domain, and seven WD40 repeats at the carboxyl terminus (Deng et al., 1992; Yi et al., 2002). All three modules are commonly found motifs involved in protein–protein interactions. The COP1 RING-finger, composed of conserved zinc-binding cysteine and histidine residues (C3HC4), binds to E2 ubiquitin conjugating enzymes and enables COP1 to mediate the transfer of ubiquitin from E2s to its substrates, typical of an E3 ubiquitin ligase (Bianchi et al., 2003; Dornan, Wertz et al., 2004; Von Arnim & Deng, 1993). The coiled-coil domain has been implicated in COP1 homodimerization and its interaction with other proteins (Holm & Deng, 1999; Wertz et al., 2004). The WD40 repeats (also known as Trp-Asp or WD repeats) at the carboxyl terminus of the COP1 protein are defined by seven repeating units containing a conserved core of approximately 40 amino acids that usually end with tryptophan-aspartic acid (WD) (Holm et al., 2002). WD40 proteins are known to adopt a circularized  $\beta$ -propeller conformation—a highly symmetrical structure made up of repeats that each consists of a small four-stranded anti-parallel  $\beta$ -sheet (Smith, Gaitatzes, Saxena, & Neer, 1999). The hydrophobic interactions between the  $\beta$ -sheets create a rigid platform, but solvent-exposed residues in the loops between the  $\beta$ -sheets are free to sequentially and/or simultaneously interact with multiple proteins (Smith et al., 1999). Numerous proteins have been found to bind to

the COP1 WD40 domain, the majority of which belong to the bZIP transcription factor family, including HY5, HYH in plants, c-Jun and JunD in mammals (Ang et al., 1998; Bianchi et al., 2003; Holm et al., 2002).

Because COP1 forms large protein complexes in mammalian cells, we attempted to purify the COP1 complexes by affinity purification using HEK293 cells transiently or stably expressing FLAG-tagged COP1. We found that multiple copies of the COP1 proteins associate with hetero-oligomeric chaperonin TriC in both transient and stable expression systems. The interaction between COP1 and TriC is also detectable at the endogenous level. These results suggest that newly synthesized WD40 protein COP1 may require TriC for its proper folding.

## 2. Materials and methods

### 2.1. DNA constructs and antibodies

A single EST clone (GenBank accession no. [BI461685](#)) containing the full-length *HsCOP1* open reading frame was obtained from ResGen and a ClaI site was introduced immediately upstream of the start codon of *HsCOP1* cDNA using primer 5'-GGAGGGAGTatCGATGTCTGGTAGCCG-3'. ClaI and PstI sites were used to clone *HsCOP1* cDNA into pBlueScript II KS (+) vector. *HsCOP1* cDNA was cut out from pBlueScript II KS (+) vector using SalI and PstI and subsequently placed into pFast-HTb vector digested with the same sites. pFast-HTb-*HsCOP1* was digested with ClaI and XhoI and the resulting fragment containing the *HsCOP1* cDNA was inserted into the ClaI and SalI sites of the pFLAG-CMV II vector. Direct sequencing was used to ensure in-frame insertions of these constructs.

Purified anti-COP1 antibody was described previously (Yi et al., 2002). Anti-Flag (Sigma) and anti-CCT $\alpha$  (Stressgen) antibodies were used according to manufacturer's recommendations.

### 2.2. Cell culture, transfection and generation of stable cell lines

HEK293 cells were cultured in high glucose DMEM with 10% FBS in a humidified chamber with 5% CO<sub>2</sub> at 37 °C. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To generate stable lines, 293 cells were transfected with Flag-hCOP1 or Flag-CMV-2 vector together with pBABE. Puromycin in the final concentration of 3  $\mu$ M was used for selection.

### 2.3. Flag purification

For transient expression, 24 h after transfection, 293 cells expressing Flag-hCOP1 or empty vector were harvested and washed with PBS. The cell pellets were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.4% NP-40, 10% glycerol, 1 mM PMSF, 1× EDTA-free protease inhibitor cocktail purchased from Roche), incubated on ice for 30 min and vortexed regularly. The lysates were then cleared by centrifugation at  $16,500 \times g$  for 30 min. The supernatants were collected and centrifuged again at  $16,500 \times g$  for 30 min. The supernatants were collected and subjected to immunoprecipitation with anti-Flag M2 affinity resin (Sigma) overnight at 4 °C. The resin was first washed with high salt wash buffer (20 mM Tris-HCl, pH 7.4, 550 mM NaCl, 0.4% NP-40, 10% glycerol, 1 mM PMSF, 1× EDTA-free protease inhibitor cocktail), then washed three times with lysis buffer, and at last washed twice with elution buffer

(25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% NP-40, 10% glycerol, 1 mM EDTA). Proteins were eluted with 1 mg/ml Flag peptide in elution buffer. Eluted proteins were precipitated with TCA, resuspended in 2× SDS sample buffer and subjected to SDS-PAGE. Coomassie or silver stain was used to visualize the protein bands. The protein bands of interest were excised, digested by trypsin and analyzed by MALDI-MS and LC-MS.

For Flag-hCOP1 stable expression lines, purification was performed as described above, except that the elution step was eliminated. After washing, resins were directly boiled in 3× SDS sample buffer and then subjected to SDS-PAGE.

### 2.4. Immunoprecipitation

Cell lysates were incubated with the indicated antibody at 4 °C for 4 h to overnight. Immunocomplexes

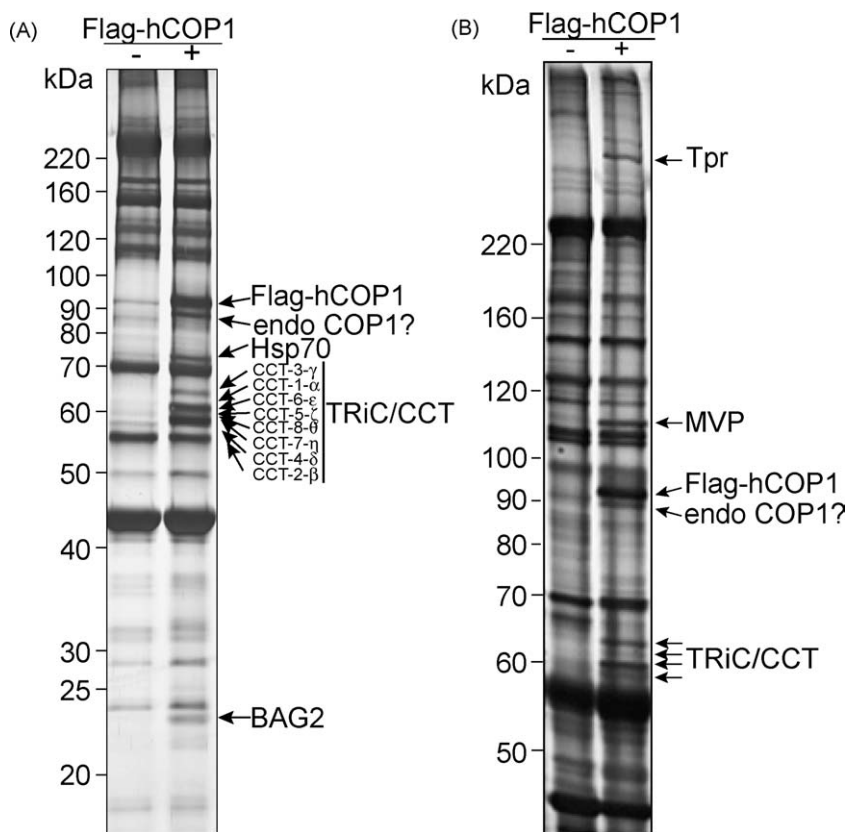


Fig. 1. Transiently and stably expressed mammalian COP1 associates with the TRiC/CCT chaperonin complex. (A) Silver stained SDS-PAGE gel from Flag-peptide elution after affinity purification using Flag-antibody coupled beads. Flag purification was performed using HEK293 cells transiently transfected with Flag-hCOP1 or empty vector as specified above each lane. (B) Lysates from HEK293 cells stably expressing empty Flag vector (-) or Flag-hCOP1 (+) were subjected to affinity purification using Flag-antibody coupled beads. After extensive washing, Flag beads were directly boiled in SDS sample buffer, separated by SDS-PAGE, and visualized by silver stain. Unique protein bands are indicated by arrows, followed with their identities revealed by mass spectrometry analysis.

Table 1

Mass spectrometry identification of several members of Hsc/Hsp70 protein family as mammalian COP1 associating proteins

Protein name	Accession number	Number of matching peptides	Number of unique matching peptides	Matched peptides
Heat shock 70 kDa protein 8	P11142	21	12	DAGTIAGLNVLRL GPAVGIDLGTTYSCVGVFQHGK HWPFMVVNDAGRPK LDKSQIHDIVLVGGSTR MKEIAEAYLGK MVNHFAIEFK NQVAMNPTNTVFDAL RFDDAVVQSDMK SFYPPEEVSSMVLTK SINPDEAVAYGAAVQAAILSGDK SQIHDIVLVGGSTR TVTNAVVTVPAYFNDSQR ARFEELNADLFR <sup>a</sup> DNNLLGK <sup>a</sup> FEELNADLFR <sup>a</sup> IINEPTAAAIAYGLDKK <sup>a</sup> STAGDTHLGGEDFDNR <sup>a</sup> LLQDFENGK <sup>b</sup> LLQDFENGKELNK <sup>b</sup> ITITNDKGR <sup>c</sup> TTPSYVAFTDTER <sup>c</sup>
Heat shock 70 kDa protein 1	P08107	7	4	NQVALNPQNTVFDAL DAGVIAGLNVLRL AAAIGIDLGTTYSCVGVFQHGK LDKAQIHDIVLVGGSTR IINEPTAAAIAYGLDR <sup>d</sup> ITITNDKGR <sup>c</sup> TTPSYVAFTDTER <sup>c</sup>
Heat shock 70 kDa protein 6	P17066	7	2	AEDEAQRDR VEILANDQGNR LLQDFENGK <sup>b</sup> LLQDFENGKELNK <sup>b</sup> IINEPTAAAIAYGLDR <sup>d</sup> ITITNDKGR <sup>c</sup> TTPSYVAFTDTER <sup>c</sup>
Heat shock 70 kDa protein 2	P54652	9	0	ARFEELNADLFR <sup>a</sup> DNNLLGK <sup>a</sup> FEELNADLFR <sup>a</sup> IINEPTAAAIAYGLDKK <sup>a</sup> STAGDTHLGGEDFDNR <sup>a</sup> LLQDFENGK <sup>b</sup> LLQDFENGKELNK <sup>b</sup> ITITNDKGR <sup>c</sup> TTPSYVAFTDTER <sup>c</sup>

<sup>a</sup> Matches heat shock 70 kDa protein 2 and 8.<sup>b</sup> Matches heat shock 70 kDa protein 2, 6 and 8.<sup>c</sup> Matches heat shock 70 kDa protein 1, 2, 6 and 8.<sup>d</sup> Matches heat shock 70 kDa protein 1 and 6.

were precipitated with Protein A or Protein G resins by rotating at 4 °C for 2 h, washed five times with lysis buffer, resuspended in SDS sample buffer and subjected to Western blot analysis.

### 2.5. Gel filtration chromatography

Gel filtration analysis was carried out as previously described (Yi et al., 2002).

### 3. Results and discussion

To uncover the composition of COP1-containing protein complexes in mammalian cells, we expressed Flag-tagged hCOP1 in HEK293 cells and used Flag-antibody-coupled beads (M2) to purify Flag-hCOP1 and its associated proteins. Twenty-four hours after transfection, HEK293 cells transfected with Flag-hCOP1 or empty vector were harvested, lysed and incubated with Flag-antibody-coupled beads. After extensive washes, proteins were eluted from the beads with Flag peptide and were separated on SDS-PAGE followed by silver staining. Compared to the control, two unique protein bands around 90-kDa were consistently obtained from Flag-hCOP1 expressing cells (Fig. 1A). Peptides identified by mass spectral analysis from both bands match with the amino acid sequence of human COP1 (data not shown). Western blot analysis indicated that the upper band represents Flag-hCOP1, whereas the lower band is most likely endogenous COP1, since both bands reacted with a COP1 antibody, whereas a Flag antibody only recognized the upper band.

In addition to Flag-hCOP1 and endogenous COP1, a number of protein bands around 60 kDa were readily detectable by silver stain specifically in the Flag-hCOP1 lane (Fig. 1A). Mass spectral analysis revealed that these protein bands represent all eight subunits of TRiC (TCP-1 chaperonin complex) (Supplementary Fig. I). TRiC, also known as chaperonin complex TCP-1 (CCT), is a large cylinder-shaped complex composed of two rings stacked back to back, each containing eight homologous subunits of approximately 60 kDa (CCT-1- $\alpha$  to CCT-8- $\theta$ ; Valpuesta, Martin-Benito, Gomez-Puertas, Carrascosa, & Willison, 2002). The peptide sequences from another unique band around 70 kDa matched with several members of Hsp70 family (heat shock protein 70) (Fig. 1A; Table 1), whereas the 23-kDa-band corresponded to BAG2 (BAG-family molecular chaperone regulator-2) (Fig. 1A; Supplementary Fig. II).

In order to more closely mimic the natural state of endogenous COP1, we developed HEK293 cell lines stably expressing FLAG-COP1 and screened for lines with very low exogenous COP1 expression. Similar to the transient system, endogenous COP1 was also co-purified with Flag-hCOP1 from these stable lines (Fig. 1B), implying that the COP1 complexes contain two or more copies of the COP1 proteins in mammalian cells. This is consistent with a previous study in which the authors showed that exogenously expressed COP1 fused to two different tags co-precipitated with each other (Bianchi et al., 2003). Interestingly, dimerization/oligomerization was also reported

for Arabidopsis COP1 (Torii, McNellis, & Deng, 1998).

Reduced amount of TRiC was copurified with Flag-hCOP1 from stable lines expressing low level of COP1 (Fig. 1B). This could be because a larger fraction of COP1 was incorporated into functional complexes in the stable expression system, whereas in the transient system (24 h after transfection), the majority of COP1 was newly synthesized and still in the process of folding by TRiC. Supporting this assumption, two extra protein bands of approximately 260 and 110 kDa molecular weight co-purified with Flag-hCOP1 from the stable lines (Fig. 1B). Mass spectral analysis identified the 260-kDa-band as translocated promoter region (Tpr) and the 110-kDa-band as major vault protein (MVP) (Supplementary Fig. III; Yi et al., 2005). Tpr is a central architectural element that forms the scaffold of the nuclear basket (Krull, Thyberg, Bjorkroth, Rackwitz, & Cordes, 2004). Interestingly, a portion of the COP1 protein associates with the nuclear envelope in mammalian cells (Yi et al., 2002). The co-purification of Tpr with Flag-hCOP1 suggests that Tpr may be responsible for tethering COP1 to the nuclear envelope. MVP, also known as lung resistance-related protein (LRP), is the core component of “vault”, a 13-MDa ribonucleoprotein organelle of unknown function (Mossink, van Zon, Scheper, Sonneveld, & Wiemer, 2003). Detailed characterization of the interaction between COP1 and MVP has been described elsewhere (Yi et al., 2005).

We next performed Western blot analysis to verify the co-purification of TRiC with Flag-hCOP1 by using an antibody against CCT $\alpha$ , a subunit of TRiC. Consistent with mass spectrometry results, CCT $\alpha$  was only detected in the Flag-eluent from the cells expressing Flag-hCOP1, not from the mock purification (Fig. 2A). To test endogenous COP1 association with TRiC, we carried out immunoprecipitation with an anti-COP1 antibody or an anti-Flag antibody from untransfected HEK293 cells. As shown in Fig. 2B, the anti-COP1 antibody pulled down both endogenous COP1 and endogenous CCT $\alpha$ , whereas the anti-Flag antibody failed to precipitate either protein, confirming that the copurification of TRiC with Flag-hCOP1 was not an overexpression artifact. We also examined the gel filtration profile of CCT $\alpha$  and compared it to those of Flag-hCOP1 and endogenous COP1. Partial overlapping in the gel filtration profiles between CCT $\alpha$ , Flag-hCOP1 and endogenous COP1 connotes a specific interaction between COP1 and TRiC (Fig. 2C and D).

TRiC was originally thought to specialize in the folding of actins and tubulins (Frydman et al., 1992; Gao, Thomas, Chow, Lee, & Cowan, 1992; Gao, Vainberg,

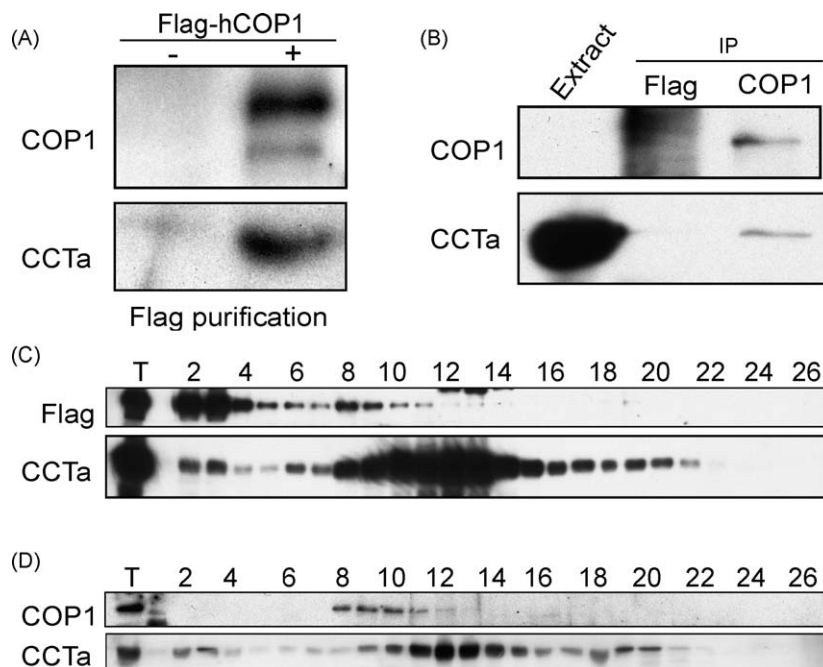


Fig. 2. Co-purification and co-fractionation of TRiC/CCT with both Flag-hCOP1 and endogenous COP1. (A) Western blot analysis of purification samples from HEK293 cells expressing Flag-hCOP1 (+) or empty Flag vector (-) by anti-COP1 and anti-CCT $\alpha$  antibodies. (B) Endogenous COP1 interacts with endogenous TRiC/CCT. HEK293 cells were immunoprecipitated with anti-COP1 antibodies, and subjected to Western blot with anti-COP1 and anti-CCT $\alpha$  antibodies. (C) Gel filtration analysis of TRiC/CCT and Flag-hCOP1. The total extracts from COS7 cells transiently transfected with Flag-hCOP1 were fractionated by Superose 6 column. Fractions were concentrated with StrataClean resin and subjected to immunoblotting with anti-Flag and anti-CCT $\alpha$  antibodies as indicated on the left. The fraction numbers are indicated on the top of the panel. T, total extracts. (D) Gel filtration analysis of TRiC/CCT and endogenous COP1 protein. The total extracts of 8.5-day mouse embryos were fractionated by Superose 6 column. Fractions were concentrated with StrataClean resin and subjected to immunoblotting with anti-COP1 and anti-CCT $\alpha$  antibodies as indicated on the left. The fraction numbers are indicated on the top of the panel. T, total extracts.

Chow, & Cowan, 1993; Yaffe et al., 1992). However, recent work has demonstrated that it also mediates folding and complex assembly of non-cytoskeletal substrates, including G- $\alpha$ -transducin, VHL tumor suppressor and Cdc20 (Camasses, Bogdanova, Shevchenko, & Zachariae, 2003; Farr, Scharl, Schumacher, Sondek, & Horwich, 1997; Feldman, Thulasiraman, Ferreyra, & Frydman, 1999; McClellan, Scott, & Frydman, 2005). In the case of VHL, TRiC has been established as the chaperonin complex responsible for its proper folding and integration into the VHL-elongin B/C (VBC)-Cullin 2 complex (Czyzyk-Krzyszka & Meller, 2004; McClellan et al., 2005). Importantly, several tumorigenic mutations in VHL disrupt its association with TRiC and result in misfolded protein products, indicating that TRiC-mediated folding is critical for functional VHL protein (Feldman, Spiess, Howard, & Frydman, 2003). Cdc20 binds to and activates the anaphase-promoting complex (APC) at the end of mitosis and is essential for exit from mitosis during cell cycle progression (Irniger, 2002). Proper folding of the Cdc20 protein by TRiC is a prerequisite

for its ability to integrate into and subsequently activate APC complex (Camasses et al., 2003). Curiously, Cdc20 together with several other WD40 proteins were identified as interaction partners of TRiC in a global proteomic analysis of protein complexes in yeast (Ho et al., 2002). Further studies confirmed that these proteins, when newly synthesized, transiently bind to TRiC and require TRiC to achieve their native conformation, suggesting that TRiC may be the primary chaperonin responsible for the folding of WD40 proteins in yeast (Valpuesta et al., 2002; Siegers et al., 2003). Several studies also showed that TRiC directly interacts with Hsp70 and requires its cooperation in assisting protein folding (McClellan et al., 2005; Melville, McClellan, Meyer, Darveau, & Frydman, 2003; Siegers et al., 2003). Since COP1 contains WD40 repeats, association of newly synthesized COP1 with TRiC/Hsp70 may indicate that COP1 requires folding by TRiC/Hsp70 preceding integration into functional protein complexes.

The physiological significance of BAG2 co-purifying with COP1/TRiC/Hsp70 is unclear. However, BAG2

belongs to a family of proteins that have been shown to interact, through their BAG domains, with the ATPase domain of Hsp70, and suppress its chaperon activities (Takayama, Xie, & Reed, 1999). Therefore, BAG2 may be involved in regulating the folding of COP1 by the TRiC/Hsp70 complex.

Recently, another group had reported the identification a COP1-containing protein complex from HEK293 cells using a similar affinity purification approach (Wertz et al., 2004). However, Western blot analysis with antibodies against DET1, DDB1, Cul4A and Roc1, the reported components of the COP1-containing complex, failed to detect any of these proteins in the COP1 complexes purified under our conditions (data not shown). The apparent discrepancy could be because of our more stringent purification conditions, and/or because in their study, Flag-DET1, instead of Flag-COP1, was used as the bait (Wertz et al., 2004). The same group also reported the co-purification of p53 with COP1 from U2-OS cells (Dornan, Wertz et al., 2004). Western blot analysis again failed to detect p53 in the COP1 complexes purified in this study (data not shown). This could be attributable to differences in cell type. In contrast, a previously identified COP1-interacting partner, c-Jun, was readily detectable in our purified COP1 complexes (Bianchi et al., 2003; Yi et al., 2005).

In summary, this study has uncovered a novel interaction between WD40 protein COP1 and the chaperonin TRiC/Hsp70. In light of the recent discoveries of TRiC/Hsp70's involvement in the protein folding and complex assembly, particularly of several WD40 proteins, it is tempting to speculate that TRiC/Hsp70 may also be required for the folding of the COP1 protein and its subsequent integration into protein complexes. It has become increasingly clear that mammalian COP1 plays pivotal roles in multiple cellular processes (Yi & Deng, 2005). Therefore, further research is required to establish whether TRiC/Hsp70-mediated COP1 folding occurs and how it affects assembly of COP1 complexes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biocel.2005.12.019](https://doi.org/10.1016/j.biocel.2005.12.019).

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