The Organization of a CSN5-containing subcomplex of the COP9 Signalosome *

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CAPSULE

• **Background:** The COP9 signalosome is a conserved eukaryotic protein complex that regulates protein degradation.

• **Result:** We identified pairwise and combinatorial interactions necessary for the formation of a CSN5-containing subcomplex which binds RBX1.

• **Conclusion:** Three distinct types of protein-protein interactions stabilize the COP9 signalosome.

• **Significance:** This structure enables binding of RBX1 and supports the significance of detected CSN subcomplexes and monomers.

ABSTRACT

The COP9 signalosome (CSN) is an evolutionarily conserved multi-protein complex that interfaces with the ubiquitin-proteasome pathway and plays critical developmental roles in both animals and plants. While some subunits are present only in a ~320 kDa complex-dependent form, other subunits are also detected in configurations distinct from the eight-subunit holocomplex. To date, the only known biochemical activity intrinsic to the complex, deneddylation of the Cullin subunits from Cullin-RING ubiquitin ligases, is assigned to CSN5. As an essential step to understanding the structure and assembly of a CSN5-containing subcomplex of the CSN, we reconstituted a CSN4-5-6-7 subcomplex. The core of the subcomplex is based on a stable heterotrimeric association of CSN7, CSN4 and CSN6, requiring coexpression in a bacterial reconstitution system. To this heterotrimer, we could then add CSN5 in vitro to reconstitute a quaternary complex. Using biochemical and biophysical methods, we identified pairwise and combinatorial interactions necessary for the formation of the CSN4-5-6-7 subcomplex. The subcomplex is stabilized by three types of interactions: MPN-MPN between CSN5 and CSN6, PCI-PCI between CSN4 and CSN7, and interactions mediated through the CSN6 C-terminus with CSN4 and CSN7. CSN8 was also found to interact with the CSN4-6-7 core. These data provide a strong framework for further investigation of the organization and assembly of this pivotal regulatory complex.

INTRODUCTION

The COP9 signalosome (CSN) is a highly conserved eukaryotic protein complex, comprising eight subunits, named CSN1-CSN8 in order of decreasing molecular mass (1). The complex was originally described as a repressor of light-dependent growth in *Arabidopsis* (2,3). Subsequent identification and characterization of
the CSN from mammalian cells, insects and yeast demonstrated the complex to be a general modulator of signal transduction (4).

CSN has diverse functions in cellular and developmental processes (4-6). The best studied function is regulation of protein degradation. In this context, CSN regulates cullin-RING E3 ligase (CRL) activity by removal of the ubiquin-like protein, Nedd8, from the cullin subunit of cullin-containing E3 ligases. This deneddylation activity, localizes to the JAMM (JAB1/MPN/Mov34)/MPN+ domain of CSN5 (7,8). However, genomic studies in *Drosophila* suggest that CSN5-dependent deneddylation is only one aspect of CSN function (9). For example, a recent study showed that CSN inhibited CRL4 ubiquitin ligase activity in a non-enzymatic manner (10).

CSN deneddylation activity is dependent on the integrity of the complete CSN complex, composed of six PCI (proteasome, COP9 signalosome and eukaryotic initiation factor3) domain-containing and two MPN (Mpr1-Pad1- N-terminal) domain-containing subunits. The PCI architecture is built from helical bundle and winged-helix subdomains, where the helical bundle employs HEAT repeats (11,12). The PCI structural motif is thought to mediate protein-protein interactions (13). While the deneddylation activity resides within CSN5, CSN6, the second MPN domain subunit, lacks enzymatic activity, though it has a role in complex assembly and stability (14).

In mammals (15,16) and fission yeast (17), CSN5 is necessary for CSN complex integrity. However, several reports of studies in *Arabidopsis* and *Drosophila* indicate that loss of CSN5 does not prevent formation of a stable CSN, lacking CSN5 (18,19). Hence, the exact forms of a “functional” CSN are not always clear. While some of the eight subunits, such as CSN8, are present only in a ~320 kDa core-“complex-dependent” form, others, such as CSN5 and CSN7, are also detected in configurations distinct from the eight-subunit holocomplex. Moreover, several studies have implicated changing equilibria between these different configurations (19-21). This dynamic nature has consequences for experiment interpretation. It is still unclear in many cases which activities and phenotypes of CSN are due to the intact complex, and which are due to in the individual subunits or subcomplexes (9).

Further understanding of CSN function should be aided by understanding how this important complex is organized structurally. Indeed, several studies have investigated this question through the use of yeast two-hybrid, mass-spectrometry and electron microscopy (8,22-24). Previously, we elucidated the atomic resolution structure of the CSN7 PCI domain, and showed that CSN6 interacted with CSN7’s C-terminal tail (25). Here we document detailed pairwise and combinatorial interactions between CSN subunits as an essential step to understanding the structure and assembly of a CSN5-containing subcomplex of the CSN.
EXPERIMENTAL PROCEDURES

Subcloning and mutations

All genes are from *Arabidopsis thaliana*. The following constructs were cloned into the pET Duet vector (Novagen) multiple cloning site I: CSN7 (CSN7182, CSN7187, CSN7192, CSN7197, CSN7mut), CSN4full, using BamHI and NotI by creating an N-terminal His-tag for all CSN7 and CSN4 versions. CSN6 (CSN6175, CSN6176-312 afterwards noted as CSN6ΔMPN, CSN6312) and CSN7Full were inserted into multiple cloning site II, using NdeI and KpnI. CSN5b and RBX1 was cloned in frame after a HisTag GST into multiple cloning site I of a pCDF vector. For co-expression of the CSN4-6-7 heterotrimeric complex, CSN7202 and CSN4 (CSN4235, CSN4235-395) were subcloned into multiple cloning site I of pCDF vector (Novagen), where the 6xHis has been removed. Positive clones were identified by colony PCR and sequenced. Clones of CSN7202, CSN7235 in multiple cloning site I, CSN6Full in multiple cloning site II of pET Duet vector and CSN1, CSN8 constructs in pET28 vector were described previously by Dessau et al. (25).

Expression and purification

All constructs were expressed in *E. coli* T7 Express (New England Biolabs), an enhanced derivative of BL21Plus (Stratagene). Transformed bacteria were inoculated sequentially by increasing volumes of LB growth media at 37º C. Kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml) were used for CSN1 and CSN8 expression while for co-expression of CSN4-6-7 versions, 25 µg/ml streptomycin, 100 µg/ml ampicillin and 34 µg/ml chloramphenicol, for CSN7-6 constructs 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were used, while for RBX1 and CSN5, 25 µg/ml streptomycin and 34 µg/ml chloramphenicol were used. The cells were grown for 2-3 h in a final volume of 4L. The temperature was lowered to 16º C when OD600 reached 0.3. Protein expression was induced by 200 µM IPTG at OD600=0.5-0.6. Cells were harvested after 12-16 h by centrifugation (9700 g) for 10 min at 4º C. Cells were suspended at 1:10 w/v ratio in lysis buffer, Buffer L (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.1% Triton X-100, 10 mg lysozyme, 1500 U DNase and 5% glycerol). Cells were lysed using a microfluidizer (Microfluidics), and PMSF was added immediately to the lysate (0.2 mM). Cell debris was removed by 1 h centrifugation (18,000 g) at 4º C and the supernatent was loaded onto a pre-equilibrated metal chelate column (Ni2+-NTA, Novagen) (Buffer A: 50mM sodium phosphate pH 8.0, 300 mM NaCl and 5% glycerol) at flow rate of 1 ml/min. The column was washed with buffer A, containing 5 mM imidazole followed by a step elution by buffer A supplemented with 50-125 mM imidazole. Fractions containing CSN were pooled and subjected to digestion by TEV protease (26). An additional step of Q-sepharose column was carried out for CSN4-6312-7202 and RBX1 before TEV digestion. CSN4-6312-7202 or RBX1 containing fractions were pooled and diluted fivefold with Dilution buffer (10mM sodium phosphate, 10% glycerol) and loaded onto a pre-
equilibrated Q-sepharose column (25 mM sodium phosphate buffer, 50 mM NaCl). Protein was eluted using a shallow gradient (120 ml) of NaCl (25 mM-1 M). TEV proteolysis was carried out for 12-14 h at 4°C. Subsequently, the sample was loaded again onto a Ni-NTA column with unbound fractions collected and pooled. Imidazole (5 mM) was needed for CSN4-6-7 to elute the digested protein due to non-specific binding of these proteins to the column. Protein was concentrated to 5 ml using spin concentrators (Amicon Ultra). SEC was then carried out using a preequilibrated Superdex 200 HiLoad prep grade column (Amersham Biosciences). Protein was eluted with buffer G (20 mM Tris pH 7.2, 150 mM NaCl and 2 mM DTT). Purity of the protein was checked on SDS-PAGE, fractions were pooled, concentrated into aliquots, and flash frozen in liquid N₂.

**Pull-down assays**

Pull-down assays were performed for truncations of CSN4-6-7, CSN7-6 and mutations of CSN7-6 complexes. 50 ml of culture was grown as described above. Cells were lysed using French press (Aminco) with lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 0.1% Triton X-100, 10 mg lysozyme, 1500U DNase and 5% glycerol) with addition of PMSF (0.2 M). Cell debris was removed by centrifugation for 20 min. The supernatant (1.5 ml) was added to a tube containing 50 μl of Ni²⁺-NTA-beads and kept in end-to-end rotation for 1 at 4°C. Beads were then washed with Buffer A (Buffer A: 50mM sodium phosphate pH 8.0, 300 mM NaCl and 5% glycerol) followed by buffer A, containing 5 mM imidazole. Bound proteins were eluted with buffer A containing 125 mM imidazole and checked on SDS-PAGE.

**CSN7 tail preparation**

CSN7’s C-terminus tail (residues 170-197) was subcloned into pCDF vector containing maltose binding protein and TEV recognition site upstream to the MCS I. It was then overexpressed as 8xHis-MBP fusion protein in E.coli as described above. The protein was purified using standard Ni²⁺-NTA chromatography, ion-exchange chromatography (Q-sepharose, Amersham Biosciences) and was then cleaved with TEV protease, followed by Ni²⁺-NTA chromatography. Fractions containing CSN7’s tail were pooled and concentrated.

**CD spectroscopy**

Measurements were performed using a Chirascan CD spectrometer (Applied Photophysics). Cuvette pathlength used was 0.1 mm or 1mm and sample concentrations were 0.37 mg/l to 2 mg/ml. Protein buffer contained 10 mM sodium phosphate, pH-8.0, 200 mM NaCl. The purity and monodispersity of samples were checked by SDS-PAGE and analytical size-exclusion chromatography. Each spectrum was averaged from four repeated scans, ranging between 180 to 300 nm at a scan rate of 1.25 nm/sec. Raw data were corrected by subtracting the contribution of the buffer to the signal. Subtracted data were then smoothed and converted to mean residue molar ellipticity units.
**CSN subunit interaction assays**

Interacting proteins were incubated in 1:1 molar ratio (100 µM) for 1 h at room temperature. Samples were loaded onto a pre-equilibrated analytical Superdex®200 SEC column (Amersham Biosciences) with buffer G. Individual proteins loading concentrations were 100 µM and proteins were monitored at 280 nm.

**SEC–MALS**

SEC–MALS experiments were performed using an analytical column Shodex KW404-4F 5µm 4.6x300mm (Shodex) equilibrated with 20 mM Tris pH 7.2 and 150 mM NaCl on an HPLC (Agilent) connected to a 18-angle light-scattering detector (DAWN HELEOS, Wyatt Technology) followed by a differential refractive-index detector (Optilab rEX, Wyatt Technology). 100 µl of the purified protein concentration of 1 mg/ml was loaded onto the analytical column Shodex KW404-4F 5µm 4.6x300mm. Data were analyzed with the ASTRA software package (Wyatt Technology).

**Analytical Ultracentrifugation**

Sedimentation velocity analysis of CSN4-6-7 protein sample were carried out at 40,000 rpm and 20 °C in an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) with a UV-visible optics detection system, using an An60Ti rotor and 12-mm double sector centerpieces. Proteins were equilibrated in 20 mM Tris, 150 mM NaCl, 2 mM dithiothreitol, pH 7.2. The sedimentation velocity runs were done at different protein concentrations ranging from 2 to 4µM. Sedimentation profiles were registered every min at 280 nm. The sedimentation coefficient distributions were calculated by least-squares boundary modeling of sedimentation velocity data using the c(s) method as implemented in the SEDFIT program. The corresponding standard s-values (s20,w) were obtained from the experimental s-values upon correction for density, viscosity, and protein concentration, using the SEDNTERP program.

**Yeast two hybrid assay**

The full-length cDNA clones of Arabidopsis CSN6a (27), fused to the activation domain in the pJG vector, as well as the full length cDNA clones of Arabidopsis CSN3 (28), CSN4 (29), CSN5a (30) CSN6a (27), CSN7 (31), and CSN7 tail (25) fused to the LexA binding domain in the pEG202 vector have been previously described. To generate the CSN6a deletions clones, the coding region of CSN6a1-136, CSN6a1-175, CSN6a137-317, CSN6a176-317, and CSN6a1-303 were PCR amplified and cloned into the pJG vector. Primers are indicated in Supplementary Table I. The LexA fusion constructs were transformed into yeast strain EGY48, while activation domain fusion constructs were transformed into yeast strain L40, which contains a β-galactosidase reporter gene. The plate and liquid assays were performed as described previously (32).

**Conservation Analysis**

Arabidopsis CSN7 amino acid sequence was used as query to search the National Center for Biotechnology Information nonredundant database using BLAST. A manual search of individual sequenced genomes (Ensemble databases) was performed. Eleven CSN7 ortholog
sequences that were found and sorted manually were submitted to ClustalW2 on the EMBL-EBI server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for multiple sequence alignment. This multiple sequence alignment was then submitted to the Consurf server (33), which uses maximum likelihood method for calculation.

RESULTS

CSN7-6 interaction

From our previous study (25), it was clear that CSN7’s C-terminal tail is responsible for the interaction with CSN6, and that the interaction was maintained even when amino acids 203 to 225 were truncated from CSN7. We therefore asked what is the minimum length of CSN7’s C-terminal tail required for CSN6 interaction. To answer this question, we constructed additional truncations of CSN7 (Figure 1A). CSN7225 and truncated forms CSN7202, CSN7197, CSN7192, CSN7187 and CSN7182 were co-expressed with CSN6. CSN7225 and CSN7202 strongly interacted with CSN6 yielding complexes that remained intact even after treatment with 2M NaCl and 2M urea (25). CSN7197 and CSN7192 also interacted with CSN6, but with apparent diminished affinity as manifested in a reduction in the ration of CSN6 to CSN7 (Figure 1B). The interaction between CSN7 and CSN6 was further diminished as the tail was shortened with little interaction between CSN6 and CSN7187 or CSN7182. The identities of the CSN7 truncations were confirmed by immunoblot (Supplementary Figure 1).

To further identify specific residues involved in the CSN7-6 interaction, we employed scanning alanine mutagenesis of CSN7. Alanine mutations were introduced as triplets from CSN7 residues 168 to 194 in CSN7197. These mutated CSN7 constructs were co-expressed with CSN6. As seen in Figure 1C, mutation of residues 168–173, or residues 183–194 did not abolish the interaction with CSN6. However, no CSN6 was detected when coexpressed with CSN7 containing alanine triplets introduced between residues 174-182, indicating that this region is essential for interaction with CSN6 (Figure 1C).

Further analyses of the amino acid conservation among diverse CSN7 orthologs revealed that within this region tryptophan 177 is completely conserved (Figure 1D). To determine if this residue is directly involved in the CSN7-6 interaction, we introduced point mutations changing this tryptophan to either alanine or aspartate. As seen in Figure 1E, the interaction with CSN6 was strongly reduced in both the point mutations. Almost no CSN6 was recovered when coexpressed with CSN7197-W177D, and little CSN6 was recovered when coexpressed with CSN7197-W177A. We conclude that tryptophan 177 is a critical residue in the interface of CSN7 and CSN6 (Figures 1D-E).

We then investigated what might be the structure of the CSN7 tail, now clearly established as the binding interface for CSN6. Previous work had shown the full length tail (170-225) to be unstructured (25). Having now more precisely defined the CSN6 binding site, we purified
CSN7’s truncated C-tail, residues 170 to 197, and measured the CD spectrum in the concentration range of 0.3 to 2 mg/ml. Disordered structure of the truncated tail was observed at 0.3 mg/ml, whereas increasing concentrations indicated increasing fractions of helical secondary structure (Figure 1F). Such behaviour is consistent with intrinsically disordered protein that upon association with protein undergoes a structured transition, facilitating binding with its target, here expected to be CSN6 (34).

**CSN4-6-7 heterotrimeric complex**

CSN4 is a PCI protein that interacts with both CSN7 and CSN6 (22-24). Having specifically defined the region of CSN7 necessary for the interaction with CSN6, we next asked how this heterodimer interacts with CSN4. When expressed alone in *E. coli*, CSN4 expression is limited. However, when co-expressed with CSN6 and CSN7, expression was robust and a stable, soluble heterotrimeric complex of CSN4-6-7 could be detected.

However, we could not purify this complex to homogeneity, due to apparent partial degradation of CSN6. Indeed, SDS-PAGE analyses revealed that approximately 1.6 kDa is truncated from CSN6 (Supplementary Figure 2). Subsequent N-terminal sequencing revealed an intact amino terminus of CSN6 in this preparation, suggesting that CSN6 is digested from the C-terminus. Further deletion analysis showed that removal of only five amino acids from the C-terminus of CSN6, i.e. CSN6^{312}, resulted in a degradation-resistant protein when co-expressed with CSN4 and CSN7^{202}, yielding a heterotrimeric complex subunit with an apparent stoichiometric ratio of 1:1:1 (Figure 2A).

Multi-angle light scattering results confirm that the complex is intact with a molecular mass of 101 kD (Figure 2B), as expected for a 1:1:1 complex. Further, sedimentation velocity studies indicate that the complex is homogeneous and monodisperse (Figure 2C) with a sedimentation coefficient of 4.88 s and a frictional ratio (f/f₀) of 1.58 at 2 µM concentration. These values are stable for at least a two fold concentration range.

**CSN4 interacts with both CSN6 and CSN7**

Further experiments were carried out to determine whether CSN4 directly interacts with CSN6 or CSN7. A His-tagged version of CSN4 was co-expressed in *E. coli* with either CSN7 or CSN6 separately, and Ni²⁺-NTA beads were used to pulldown CSN4 from the protein lysate. As seen in Figure 3A, CSN6 clearly co-purified with His-CSN4. The CSN4 interaction with CSN7 was not clearly observed by SDS-PAGE, however immunoblot analysis using α-CSN7 or α-CSN6 antibodies confirmed that both proteins were present in the pulldown with CSN4 (Figure 3B). The results indicate that CSN4 interacts with both CSN6 and CSN7, though the interaction with CSN6 may be of higher affinity.

Based on earlier protein-interaction studies (22-24), we hypothesized that the interaction between CSN4 and CSN7 in the heterotrimeric CSN4-6-7 complex is mediated
through PCI-PCI domain interactions. This hypothesis was tested by co-expressing CSN7 and CSN6 together with a CSN4 construct lacking the PCI domain. Constructs of CSN4 were designed to separate CSN4 into PCI and ΔPCI forms (Figure 3C). CSN4ΔPCI did not facilitate the formation of the heterotrimeric complex of CSN4ΔPCI-6-7. On the other hand, CSN4PCI interacts with CSN7 and CSN6 to form the CSN4-6-7 complex, indicating that the PCI domain of CSN4 acts as the scaffold for CSN4-6-7 interaction (Figure 3D).

Previously, based on our crystallographic study, we identified surface residues on different faces of the CSN7 HB and WH subdomains, and mutated these residues, mapping interaction interfaces for CSN8 (25). In order to further map CSN4 and CSN6 interactions with CSN7, these CSN7 mutants, namely D12A, E44A, H71 located in the HB subdomain and K144A, E153A located in the WH subdomain, were tested in copurification assays (Figure 3E). When CSN7202, mutated for these various residues, was coexpressed with CSN4 and CSN6, pulldown experiments indicated that the CSN4-6312-7202 heterotrimeric complex was disrupted when either single or double CSN7 mutants containing the E44A and/or H71A mutations were employed, whereas the CSN7202-CSN6 complex formation was not affected (Figure 3F) in the presence of these mutations. We therefore conclude that CSN4 interacts with the CSN7 HB subdomain.

**MPN domain of CSN6 is not required for CSN4-6-7 complex integrity**

Having dissected the interactions of the two PCI-subunits in this complex, we next turned to understand the contribution of CSN6. CSN6 contains an N-terminal MPN domain and a newly identified S6CD domain in the carboxyl half of the protein (35). We first employed the yeast two-hybrid assay to determine which domains of CSN6 contribute to its interaction with CSN7. As expected, CSN6 interacted in this assay with CSN5, CSN7, and the C-terminal tail of CSN7, but not with the PCI-domain of CSN7 (Figure 4A). While the MPN domain alone of CSN6 interacted with CSN5, it did not interact with CSN7 or with CSN7’s C-tail. On the other hand, the S6CD-containing C-terminal half of CSN6 did interact with CSN7.

To further confirm these interactions in vitro, two constructs of CSN6 were constructed, CSN6MPN which contains residue 1 to 175 including the MPN domain, and CSN6ΔMPN, encoding residues 176-312 (Figure 4B). These constructs of CSN6 were co-expressed with CSN7 alone, or with CSN7 and CSN4 together. Co-purification assays reveal a robust interaction between CSN6ΔMPN and CSN7, and with CSN7 and CSN4 (Figure 4C). However, no interaction was observed with the construct CSN6MPN indicating that the MPN domain is dispensable for the formation of the heterotrimeric complex.

**Interaction of CSN4-6-7 complex with other subunits**

The SEC assay is a convenient method to check subunit interactions (25). Various interaction studies had indicated that CSN7
interacts with both CSN8 and CSN1 (22,23,25),
while a mass-spectrometry-based model of the
entire complex did not support such an interaction
(24). To determine if CSN8 or CSN1 can interact
with the CSN4-6^{312}-7^{202} heterotrimeric complex to
form a ternary complex, CSN4-6^{312}-7^{202} was
incubated with either CSN8 or CSN1, and then
analyzed by SEC. As seen in Figures 5A-B,
CSN4-6^{312}-7^{202} interacts with CSN8, forming a
heterotetrameric complex, CSN4-6^{312}-7^{202}-8.
However, the CSN4-6^{312}-7^{202} complex did not
show interaction with CSN1 (Figures 5C-D).

Yeast two hybrid, mass spectrometry and
cryo-EM studies indicated a stable tetrameric
complex of CSN4-5-6-7 (8,22-24). The SEC assay
was used to check the interaction of CSN5 with
CSN4-6^{312}-7^{202}. A CSN4-5-6^{312}-7^{202} complex was
observed, when analyzed by SEC (Figures 6A-B),
whereas no direct interaction of CSN5 was
observed with CSN7^{197} alone (Figures 6C-D).

To map the interface responsible for CSN5
or CSN8 binding to the CSN4-6-7 complex, we
purified a CSN4-6^{\text{AMPN}}-7^{202} complex to
homogeneity and its interaction was checked by
SEC-MALS with CSN5 and CSN8. CSN5 did not
bind to a CSN4-6^{\text{AMPN}}-7^{202} complex indicating that
the MPN domain of CSN6 is required for CSN4-5-6-7
complex formation (Figure 6E). In contrast,
CSN8 interacts with a CSN4-6^{\text{AMPN}}-7^{202} complex,
as observed by the increase of molecular mass in
SEC-MALS, forming a CSN4-6^{\text{AMPN}}-7^{202}-8
heterotetrameric complex (Figure 6F). We
reconstituted a heteropentameric complex,
comprising CSN4-5-6-7-8, by incubation of
purified CSN4-6-7 with purified CSN5 and CSN8.
This material was then run on a SEC column and
the SDS-PAGE of the elution peak shows all
subunits coelute (Figure 6G).

**RBX1 interacts with the CSN4-5-6-7 subcomplex**

Both catalytic and non-catalytic activities of
the CSN vis à vis protein degradation require
binding to CRLs (10,36,37). Protein interaction
studies indicated that this interaction is partially
mediated through the binding of CSN6 to RBX1
(38). We therefore asked whether this interaction
is maintained in the CSN4-5-6-7 subcomplex of
the CSN.

The SEC assay was used to check the
interaction of RBX1 with CSN4-5-6^{312}-7^{202}. RBX1
from *Arabidopsis* was expressed in *E.coli*, purified
to near homogeneity, and incubated with the
CSN4-5-6^{312}-7^{202} tetrameric complex, isolated as
in the previous section. As seen in Figure 7, RBX1
alone elutes in low molecular weight fractions.
Following incubation with the CSN subcomplex,
RBX1 co-eluted with the CSN subunits. We
conclude that the CSN4-5-6-7 subcomplex retains
a functional organization, configured for potential
binding to a CRL.

**DISCUSSION**

We have identified robust pairwise and
combinatorial interactions necessary for the
formation of a CSN5-containing subcomplex of
the CSN, and showed that this subcomplex is
sufficient for mediating interactions with the CRL
subunit RBX1. The core of the subcomplex is
based on a stable heterotrimeric association of
CSN7, CSN4 and CSN6. Notably, Sharon, et al., (23) observed that recombinant CSN complexes can be dissociated into two principal stable subcomplexes, one containing CSN7, CSN6 and CSN4. These results are also consistent with earlier protein-protein interaction studies (22, 23, 25, 39).

To reconstitute such a complex in E. coli, coexpression of both CSN4 and CSN6 with CSN7 was required, as only CSN7 appears stable alone and the expression or either CSN4 or CSN6 alone was severely limited. To this heterotrimer, we could then add CSN5 in vitro to reconstitute a quaternary complex.

From the different deletion constructs analyzed here, we propose a model whereby this subcomplex is organized via distinct interaction surfaces (Figure 6G). Our model shows that CSN6 is a core protein in the subcomplex, interacting with CSN4, CSN5 and CSN7. The non-MPN part of the CSN6 is in association with CSN4 and CSN7, whereas the CSN6 MPN domain is responsible for the interaction with CSN5. This central positioning of CSN6 fits earlier genetic studies in Arabidopsis which showed that complete loss of CSN6 results in loss of entire CSN complex (14).

Previously, we reported that CSN7 comprises an N-terminal PCI domain and a C-terminal tail extending from the residue 170-225, and that this tail was essential for CSN6 stability in vitro, and CSN function in vivo (25). Here, we further narrowed the CSN6 binding site to the CSN7 tail proximal to the PCI domain. While CSN7\textsuperscript{202} was found sufficient to interact with CSN6 at a 1:1 ratio, the apparent stability of this interaction declined as the CSN7’s tail was truncated and little or no interaction was observed with CSN6 with truncation of CSN7\textsuperscript{187} or CSN7\textsuperscript{182}. Scanning alanine mutagenesis on CSN7\textsuperscript{197} revealed that the region between amino acids 174 and 182 is important for interaction with CSN6. Within this region, W177 was identified as essential for the CSN7–CSN6 interaction. The importance of this residue in mediating the CSN7-CSN6 interaction is further underscored by its evolutionary conservation. While the overall level of amino acid conservation in the C-terminal tail of CSN7 is not high, W177 is completely conserved in all orthologs.

The interaction between CSN7 and CSN4 is dependent on PCI-PCI interactions. Further, the CSN4 PCI domain is also required for association with the non-PCI protein, CSN6. These interactions then conceivably leave the C-terminal half of CSN4 (amino acids 235-395) available for interaction with extra-complex proteins. In this vein, this end of CSN4 would be similar to the distal regions of the CSN7 tail, that bind a variety of proteins such as RNR2 and PCIP1(40), and the non-PCI region of CSN1 which has transcriptional transactivation activity (41). The C-terminal half of CSN6 is required for its interaction with both CSN4 and CSN7, leaving its MPN domain available for interaction with CSN5. How this fits with a recent report where the MPN domain is not essential for CSN5-mediated deneedlylation is not clear (35). Our preparation of a heterotrimer of CSN4, CSN6 and CSN7 may best fit with earlier
findings that both Arabidopsis and Drosophila CSN5 appeared to be non-essential for complex formation (18,19), in contrast, to another report which showed that CSN5 does appear to be essential for complex formation (14).

The CSN4-5-6-7 subcomplex is stabilized by three types of interactions: MPN-MPN, PCI-PCI, and interactions mediated through the CSN6 C-terminus. How does our working model compare to current ones? While the structural model proposed by Sharon et al. (24) with two subcomplexes i.e. CSN1-2-3-8 and CSN4-5-6-7 has provided a framework for subsequent studies, it did not predict many of the binary interactions previously reported for CSN subunits, such as between CSN7 and CSN8. Here, we show that at least one subunit of the CSN1-2-3-8 subcomplex, namely CSN8, can stably associate with a CSN4-6-7 heterotrimer. Interestingly, while CSN7-CSN1 interaction has been shown by several different assays (25), we were unable to detect an interaction between CSN1 and the CSN4-6\textsuperscript{312-7\textsuperscript{202}} heterotrimer, even though a CSN4-6-1-8 was detected (24), perhaps implying that a number of subcomplex permutations are biochemically possible, if not physiologically relevant. Alternatively, assembly order may be critical to formation of the native CSN.

Our results also fit well with the CSN model proposed by Enchev et al. (8) based on cryo-EM single particle analysis. These workers observed three connections between what they assign to be the small and large “segments”, where segments correspond to CSN4-5-6-7 and CSN1-2-3-8 subcomplexes, respectively. This finding suggests that the two subcomplexes then will have more than one interface, in contrast to the single CSN1-6 interface postulated by Sharon et al. Our data regarding CSN7-8 interactions in the context of the CSN4-6-7 core could then represent one of the additional interfaces. Moreover, other data, reviewed by Pick and colleagues (42), as well as more recent EM findings on the 19S proteasome lid (43,44) demonstrate that MPN-MPN domain containing subunits interact directly in each of the three PCI/MPN complexes.

Many publications have reported CSN subunits as monomers or as part of smaller complexes than the full eight subunit CSN. However, it is not clear whether these forms have biological significance or are experimental artifacts occurring during biochemical isolation. Many of these reports concern CSN5, which would also fit with our findings presented here (20,45), since it appears as if the core subcomplex is CSN4-6-7. For example, a cytoplasmic complex containing CSN4-5-6-7b-8 was also reported in mammalian cells (46).

In light of recent publications claiming a noncatalytic structural role for the CSN in regulation of CRLs (10,37) the binding of RBX1 by the subcomplexes studied here opens the possibilities that different conformations of CSN subunits may interact with CRLs to regulate their activity. Further investigation is required to test the physiological relevance of such subcomplexes.
References

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Abbreviations

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<tr>
<th>PCI</th>
<th>Proteasome-COP9-eukaryotic initiation factor 3</th>
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<tr>
<td>MPN</td>
<td>MPR1-PAD1-N-terminal domain</td>
</tr>
<tr>
<td>JAMM</td>
<td>JAB1/MPN domain metalloenzyme (or MPN+).</td>
</tr>
<tr>
<td>HEAT</td>
<td>Huntington, elongation factor 3, PR65/A, TOR</td>
</tr>
<tr>
<td>HB</td>
<td>helix-bundle</td>
</tr>
<tr>
<td>WH</td>
<td>winged-helix</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethlysulfonyl fluoride</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose-binding protein</td>
</tr>
<tr>
<td>SEC</td>
<td>size-exclusion chromatography</td>
</tr>
<tr>
<td>MALIS</td>
<td>multi-angle light scattering</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
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FIGURE LEGENDS

Figure 1. CSN7-6 interaction. (A) Schematic depiction of recombinant CSN7 C-terminal tail truncations. These constructs were coexpressed with CSN6. (B) Coomassie Blue stained SDS-PAGE showing HisTag-pulldowns of CSN7\textsuperscript{Full} and truncations with CSN6. CSN7\textsuperscript{Full}, CSN7\textsuperscript{202} showed strong interaction with CSN6, where as interaction steeply declined from CSN7\textsuperscript{197}. No interaction of CSN6 was observed with the truncations CSN7\textsuperscript{187} and CSN7\textsuperscript{182}. Values listed below each lane represent the ration of CSN6 to CSN7 as assessed by densitometry of the displayed gel. (C) Coomassie Blue stained SDS-PAGE showing HisTag-pulldowns of CSN7 C-terminal tail alanine triplet mutations coexpressed with CSN6. CSN7\textsuperscript{168-173} and CSN7\textsuperscript{183-194} showed significant interaction of CSN6, whereas mutations CSN7\textsuperscript{174-176}, CSN7\textsuperscript{177-179} and CSN7\textsuperscript{180-182} showed little or no interaction with CSN6. The lower gel shows an independent pull-down assay for denoted mutants, confirming that CSN7 triple alanine mutants expressed but did not bind CSN6. (D) Multiple sequence alignment of CSN7 is shown with 11 different orthologs. Region marked CSN7\textsuperscript{174-182} showed no interaction of the CSN6 when mutated to alanine. Residue indicated by asterisk shows W177 conservation, where CSN7-W177A disrupted CSN6 interaction when coexpressed. (E) Coomassie Blue stained SDS-PAGE showing HisTag-pulldowns of CSN7- W177 point mutations co-expressed with CSN6. Most of CSN6 was not observed when coexpressed with CSN7\textsuperscript{197-177D}, and little CSN6 was observed when coexpressed with CSN7\textsuperscript{197-W177A}. (F) CD spectroscopic analysis of the CSN7\textsuperscript{170-197} tail at differing concentrations as indicated. The inset shows a SDS-PAGE of the purified CSN7\textsuperscript{170-197} tail.

Figure 2. CSN4-6-7 heterotrimeric complex. (A) Coomassie Blue stained SDS-PAGE showing the purified ternary complex of CSN4-312-7202 was coexpressed in E.coli. (B) SEC-MALS of purified CSN4-6\textsuperscript{312-7202} ternary complex showing the mass ~100 kDa indicating the complex is intact. (C) Sedimentation analysis showing CSN4-6\textsuperscript{312-7202} complex is monodisperse having a sedimentation coefficient of 4.88 s and a frictional ratio (f/f\textsubscript{0}) of 1.58 at 2 µM concentration. The sedimentation was performed at 40000 rpm at 20° C.

Figure 3. CSN4 interactions with CSN6 and CSN7. (A) Coomassie Blue stained SDS-PAGE showing HisTag-pulldowns of CSN4-7 and CSN4-6 coexpressed in E.coli, where CSN4 was HisTagged. Arrow indicates the CSN6 band. (B) Western blot analysis of gel in panel (A), confirming the interaction of CSN4-6, CSN4-7 as probed with α-CSN6 and α-CSN7 antibodies as indicated. (C) Schematic depiction of truncations of CSN4 coexpressed with CSN6 and CSN7. (D) Coomassie Blue stained SDS-PAGE showing interaction of the heterotrimeric complex, CSN4-6\textsuperscript{312-7202} (left lane). The middle lane shows CSN4\textsuperscript{PCI}, containing the PCI domain (residues 235 to 395), in complex with CSN6 and CSN7\textsuperscript{202}. Deletion of the CSN4 PCI domain abrogates the interaction (right lane). (E) Ribbon/sticks representation of CSN7 point mutants. The HB and WH subdomains are colored magenta and yellow, respectively. (F) Coomassie Blue stained SDS-PAGE showing CSN7 point mutations coexpressed with CSN4 and CSN6\textsuperscript{312}. Mutations on E44A and H71A disrupted the CSN4-6\textsuperscript{312-7202} complex.

Figure 4. The MPN domain is not required for assembly of the CSN7-6 and CSN4-6-7 complexes. (A) Yeast two hybrid analysis showing interaction of CSN subunits with CSN6 truncations. Left panel illustrates the CSN6 deletions used in the prey constructs. Boxes designated with >1, >2, >3, >4 indicates the four α helices, while the box at the C-terminus indicates a conserved region of CSN6 spanning amino acids 305-317. Right panel shows the corresponding β-galactosidase activities of the interaction with CSN subunits 3,4,5,7 and CSN7 C-terminal tail (CSN7\textsuperscript{Pro-225}). The legend at the bottom right corner indicates the relative β-galactosidase activities values in Miller units. (B) Schematic depiction of CSN6 truncations coexpressed with CSN7\textsuperscript{202} and with both CSN7\textsuperscript{202} and CSN4. (C) Coomassie Blue stained SDS-PAGE showing: (left lane) heterotrimeric CSN4-6-7 complex; (second lane) removal of residues 176-317 from CSN6 (denoted CSN6\textsuperscript{AMPN}) abrogates interaction with CSN7; (third lane) purified CSN7\textsuperscript{202}-CSN6\textsuperscript{AMPN} and (right lane) CSN4-CSN6\textsuperscript{AMPN}-CSN7\textsuperscript{202} complexes.
Figure 5. Assembly of CSN subcomplexes, involving CSN8 or CSN1. (A) SEC elution profile showing the peak shift indicating the CSN4-6\textsuperscript{312-7202-8} tetrameric complex formation. (B) Coomassie Blue stained SDS-PAGE of SEC fractions from panel (A) showing interaction of CSN4-6\textsuperscript{312-7202-8} complex. (C) SEC elution profile indicates that CSN1 does not stably interact with the CSN4-6\textsuperscript{312-7202} complex. (D) Coomassie Blue stained SDS-PAGE of SEC fractions from panel (C). CSN1 does not interact with the CSN4-6\textsuperscript{312-7202} complex.

Figure 6. Assembly of CSN subcomplexes: implications of domain deletions. (A) SEC elution profile of CSN5 interaction with CSN4-6\textsuperscript{312-7202} which shows a shift in peak indicating 4-5-6\textsuperscript{312-7202} heterotetrameric complex formation. (B) Coomassie Blue stained SDS-PAGE of SEC fractions from panel (A) showing interaction of CSN5 with CSN4-6\textsuperscript{312-7202}. The extreme right lanes show relevant CSN protein standards. (C) SEC elution profile of CSN5 interaction with CSN7\textsuperscript{197} is shown. No shift in the elution peak is observed. CSN5 was incubated with CSN7\textsuperscript{197} for 1 h at room temperature and analyzed. (D) Coomassie Blue stained SDS-PAGE of SEC fractions from panel (C) showing no interaction between CSN5 and CSN7\textsuperscript{197}. The extreme right lanes show relevant CSN protein standards. (E) SEC-MALS showing CSN5 does not interact with CSN4-6\textsuperscript{ΔMPN-7202} complex. CSN5 was incubated with CSN4-6\textsuperscript{ΔMPN-7202} complex for 1 h at room temperature and analyzed. (F) SEC-MALS showing interaction of CSN8 with CSN4-6\textsuperscript{ΔMPN-7202} complex. Molecular mass was increased after interaction which is ~100 kDa. CSN8 was incubated with CSN4-6\textsuperscript{ΔMPN-7202} complex for 1 h at room temperature and analyzed. (G) Coomassie Blue stained SDS-PAGE of a reconstituted, purified CSN4-5-6-7-8 complex. (H) A working model of a CSN subcomplex, summarized by schematic depiction of the CSN4-5-6-7-8 complex and its interactions with other CSN subunits. Ovals indicate PCI domains while rectangles denote MPN domains. The extension extending from CSN4 depicts the HEAT/TPR repeat structure N-terminal to its PCI domain. The CSN7 tail is drawn explicitly with the conserved Trp to which CSN6 binds.

Figure 7. Assembly of a CSN4-5-6-7 subcomplex with RBX1. (A) SEC elution profile of RBX1 interaction with CSN4-5-6\textsuperscript{312-7202}, showing co-elution of RBX1 with the CSN subcomplex (fractions 19-21). The elution of RBX1 alone is found at a greater elution volume (fractions 30-31). The three SEC experiments (RBX1 alone, CSN subcomplex alone, RBX1-CSN subcomplex mixture) are denoted. The amount of CSN subcomplex used was identical. (B) Coomassie Blue stained SDS-PAGE of fractions from the RBX1-CSN subcomplex mixture SEC run, in panel (A).
Figure 2

(A) PAGE analysis of CSN4, CSN6, and CSN7.

(B) SEC analysis showing the elution time distribution.

(C) Sedimentation analysis showing the sedimentation coefficient distribution.
Figure 3
Figure 4
**Figure 5**
Figure 6
Figure 7