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Deletion Mutants in COP9/Signalosome Subunits in Fission Yeast *Schizosaccharomyces pombe* Display Distinct Phenotypes

Kirsten E. Mundt,* Cong Liu,† and Antony M. Carr*§

*Genome Damage and Stability Centre, School of Biological Sciences, University of Sussex, Falmer, Sussex, BN1 9RR, United Kingdom; and Department of Medical Microbiology, School of Basic Medical Sciences, West China University of Medical Sciences, Chengdu 610041, People's Republic of China

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The COP9/signalosome complex is highly conserved in evolution and possesses significant structural similarity to the 19S regulatory lid complex of the proteasome. It also shares limited similarity to the translation initiation factor eIF3. The signalosome interacts with multiple cullins in mammalian cells. In the fission yeast *Schizosaccharomyces pombe*, the Csn1 subunit is required for the removal of covalently attached Nedd8 from Pcu1, one of three *S. pombe* cullins. It remains unclear whether this activity is required for all the functions ascribed to the signalosome. We previously identified Csn1 and Csn2 as signalosome subunits in *S. pombe*. *csn1* and *csn2* null mutants are DNA damage sensitive and exhibit slow DNA replication. Two further putative subunits, Csn4 and Csn5, were identified from the *S. pombe* genome database. Herein, we characterize null mutations of *csn4* and *csn5* and demonstrate that both genes are required for removal of Nedd8 from the *S. pombe* cullin Pcu1 and that their protein products associate with Csn1 and Csn2. However, neither *csn4* nor *csn5* null mutants share the *csn1* and *csn2* mutant phenotypes. Our data suggest that the subunits of the signalosome cannot be considered as a distinct functional unit and imply that different subunits of the signalosome mediate distinct functions.

INTRODUCTION

Elements of the COP9/signalosome complex were first identified genetically in *Arabidopsis* through the isolation of COP mutants that result in constitutive photomorphogenesis (Chamovitz *et al.*, 1996; Wei and Deng, 1999). The majority of the COP loci was subsequently found to correspond to subunits of a multiprotein complex (the COP9 complex) that is required for the nuclear accumulation of a transcriptional regulator, COP1. COP1 is a ring finger protein that accumulates in the nucleus when plants are germinated in the dark, but which is cytoplasmic when germination occurs in the light. Within the nucleus, COP1 binds to the HY5 transcription factor and promotes its degradation, thus negatively regulating a program of gene expression required for photomorphogenesis (Osterlund *et al.*, 2000). In *Drosophila melanogaster*, the homologous complex is essential for embryo development (Freilich *et al.*, 1999), and this has led to the

suggestion that the COP9/signalosome has a role in integrating different signaling pathways during differentiation.

An analogous human complex was identified biochemically during attempts to purify the regulatory lid component of the proteasome. The human complex copurifies with a kinase activity that is capable of phosphorylating c-Jun and IκB (Seeger *et al.*, 1998) and may be encoded by inositol 1,3,4-trisphosphate 5/6-kinase (Wilson *et al.*, 2001). Overexpression of some, but not all, subunits in human cells leads to differential effects on the stability of c-Jun (Naumann *et al.*, 1999). These observations have led to a model in which the signalosome is involved in the integration and/or modulation of multiple signal transduction pathways, and may be directly involved in regulating the transcriptional output of these pathways. A unified nomenclature for the individual subunits in different species has been agreed: the subunits are all named Csn (for COP9/signalosome) and numbered from 1 to 8 (Deng *et al.*, 2000).

The mammalian Csn5 subunit had previously been identified as c-Jun-interacting protein, Jun-activation domain binding protein JAB1 (Claret *et al.*, 1996). Csn5 also interacts with a number of nuclear hormone receptors (Chauchereau *et al.*, 2000; Li *et al.*, 2000) and with the cytoplasmic domain

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§ Corresponding author. E-mail address: a.m.carr@sussex.ac.uk.

of the integrin LFA-1. Integrin adhesion receptors transduce signals that regulate of gene expression and activation of LFA-1 leads to an increase in the nuclear pool of Csn1 that is concomitant with an increase in transcription from an AP1-dependent promoter (Bianchi *et al.*, 2000). Csn5 is also found to interact with the CDK inhibitor protein p27. Overexpression of *Csn5* decreases the nuclear abundance of p27, but the mechanism for this is unclear (Tomoda *et al.*, 1999). In *Arabidopsis*, two isoforms of Csn5 exist (also known as Ajh1 and Ajh2 with, respectively, 62 and 63% identity to human Csn5). Both *Arabidopsis* proteins exist in monomeric and complexed form, with complex formation dependent on Csn1. Although complexed Csn5 isoforms localizes predominantly to the nucleus in *Arabidopsis*, the monomeric forms are mainly cytoplasmic (Kwok *et al.*, 1998). In addition, Csn5 is the only subunit of the signalosome complex with an apparent homolog in *Saccharomyces cerevisiae*. This is further evidence that there might be a signalosome-independent function for Csn5.

Human signalosome subunits interact with multiple cullins (Lyapina *et al.*, 2001). Cullins are components of the SCF ubiquitin ligases that contain core subunits of cullin, Skp1, and a ring finger protein, and which associate with multiple F-box proteins and ubiquitin-conjugating enzymes. In fission yeast, one of the three cullins (Pcu1) requires covalent attachment of Nedd8 (a small ubiquitin-like protein) to Lys 713 for function. In *csn1* null mutants, Pcu1 is present exclusively in the Nedd8-modified form, whereas *csn*⁺ cells have ~50% of Pcu1 Nedd8 modified (Lyapina *et al.*, 2001). This observation has led to the suggestion that Nedd8 modification of Pcu1, and probably other cullins, may underlie the *csn1-d* and *csn2-d* phenotypes. We set out to determine whether *S. pombe* Csn4 and Csn5 were capable of interacting with Csn1 and with Csn2, to characterize the corresponding deletion mutants (*csn4-d* and *csn5-d*), and ascertain whether hypermodification of cullins correlated with phenotypes associated with *csn1-d* and *csn2-d*.

MATERIALS AND METHODS

Cell Biology

Protocols for checkpoint measurements, cell scoring, and irradiation are previously described (Edwards and Carr, 1997). γ -Irradiation was performed on exponentially growing cells in liquid culture, by using a Gammacell 1000 (Nordion, Ontario, Canada) ¹³⁷Cs source (12.5 Gy/min). A known number of cells was plated after different doses of irradiation and colonies deriving from surviving cells were counted after 3 to 4 d, expressing survival as a percentage of colonies formed by mock-treated cells on equivalent plates. For UV survival analysis a known number of cells was plated and irradiated with different doses by using a germicidal UVC lamp (0.2 J/m²s). Survival was scored as for γ -irradiation. Survival in hydroxyurea was performed on asynchronous cultures. Aliquots were removed at hourly intervals after addition of 10 mM hydroxyurea and plated after dilution. Survival was assessed by counting the number of colonies formed compared with the untreated control (*t*₀).

Biochemistry

Proteins were identified in crude total extracts prepared by mechanical lysis in trichloroacetic acid, which prevents proteolysis. This is described in Caspari *et al.* (2000).

Gel Filtration Analysis

Gel filtration analysis was performed as previously described (Caspari *et al.*, 2000). Briefly, extracts were prepared by dripping the cells resuspended in buffer B (50 mM NaH₂PO₄/Na₂HPO₄ pH 7, 150 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, 5 mM EGTA, 60 mM β -glycerolphosphate, 0.1 mM NaF, 10 μ g/ml apoprotinin, 10 μ g/ml leupeptin, 25 mM *p*-aminobenzamidine, 10 mM phenylmethylsulfonyl fluoride) into a mortar filled with liquid nitrogen and grinding them with a pestle. Frozen cell homogenate was allowed to thaw then was clarified (2 min 2000 rpm; Eppendorf), and the resulting supernatant was spun for 30 min at 30,000 rpm in a Sorvall AH650 at 4°C. The extract (~1 mg of total protein) was then passed through an equilibrated Superdex 200HR 10/30 column (Pharmacia) and 0.5-ml fractions were collected. The column was calibrated using the low- and high-molecular weight gel filtration calibration kit (Pharmacia).

Coimmunoprecipitation

For coimmunoprecipitation experiments, soluble, whole cell extracts were prepared in buffer B by grinding cells in liquid nitrogen as described above. Extract (400 μ l, concentration 5 mg/ml) was precleared by incubation with prewashed protein G beads rotating for 30 min at 4°C. The supernatant was transferred to a fresh tube and 4 μ l of primary rabbit anti-MYC antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:2, or 4 μ l of mouse anti-hemagglutinin (HA) antibody (BabCO, Richmond, CA), diluted 1:5, was added for 1 h, rotating at 4°C. The extract was centrifuged for 5 min at 14,000 rpm in a microfuge and transferred to a fresh tube containing 15 μ l of prewashed protein G beads. The immunocomplexes were left to absorb to the protein G beads by rotating incubation at 4°C for 30 min and subsequently washed once in buffer B and twice in buffer B containing 250 mM NaCl. SDS sample-buffer (2 \times , 100 μ l) was added to the washed pelleted beads, boiled for 5 min, and separated by 8% SDS-PAGE. Standard protocols were used for SDS-PAGE and Western blot analysis. Detection was by chemiluminescence.

Immunofluorescence Microscopy of Csn1-MYC, Csn2-MYC, Csn4-MYC, and Csn5-MYC

For immunofluorescence staining cells were processed essentially as previously described (Hagan and Hyams, 1988). Cells were fixed in mid-exponential growth by adding the culture to freshly prepared 30% paraformaldehyde solution to a final concentration of 3.75% and shaking for 10 min at 30°C. Cells were subsequently washed two times in PEM buffer [100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) pH 6.9; 1 mM EGTA, 1 mM MgSO₄] and once in PEMS buffer (PEM + 1.2 M sorbitol). The cell wall was digested with 1.25 mg/ml zymolase (ICN Biomedicals, Cleveland, OH) in PEMS for 70 min at 37°C and membranes subsequently permeabilized with 1% Triton X-100 in PEMS. After two washes in PEM the cells were blocked in PEMBAL (PEM + 1% bovine serum albumin, 0.1% Na₂S₂O₃, 100 mM lysine hydrochloride pH 6.9) for >30 min. Cells were incubated overnight with primary antibody 12CA5 anti-HA antibody (BabCO) at a dilution of 1:250 or primary rabbit anti-MYC antibody (RDI) at a dilution of 1:150. They were washed three times in PEM and incubated with secondary Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:250 or fluorescein isothiocyanate-conjugated anti-rabbit antibody (DAKO, Bucks, United Kingdom) at 1:150 for 2–6 h. After washing in PEM, phosphate-buffered saline (PBS), and PBS with 4,6-diamidino-2-phenylindole (DAPI) (1 μ g/ml), the cells were resuspended and stored in PBS + 0.1% Na₂S₂O₃ at 4°C.

Cells were viewed after mounting in 90% glycerol containing 1 mg/ml *para*-phenylene diamine on a Leitz microscope and pictures were acquired using the MetaMorph imaging system (Universal Imaging, Downingtown, PA).

Genomic Tagging of Csn2, Csn4, and Csn5 and Gene Deletion

We genomically tagged the C terminus of *csn2*, *csn4*, and *csn5* with 13-MYC epitopes by using the one-step polymerase chain reaction-tagging technology previously described by Bahler *et al.* (1998). Replacements of the complete open reading frames of *csn4* and *csn5* with *ura4⁺* were performed as described in Bahler *et al.* (1998). Recombination at the correct locus was checked by Southern blot analysis and back-crossing of tagged alleles with the respective deletion mutants.

Strains Used and Constructed in This Analysis

The full list of strains is in Table 1. All *csn-d* mutants have the open reading frame replaced by the *ura4⁺* gene. All *csn-MYC* and *csn-HA* strains include a transcription termination site and a kanamycin resistance cassette after the stop codon. The diploid strain was created by mating the *csn2-HA ade6.M210* and *csn2-MYC ade6.M216*. These two *ade6* alleles exhibit intra-allelic complementation, and a diploid can be obtained by selecting for growth on adenine after zygote formation but before meiosis. The pREP41-HA-Nedd8 plasmid was introduced into relevant strains by standard techniques and cells maintained in minimal media without thiamine.

Accession Numbers

Csn1a SPBC215.03C, AL033534, g7492977
Csn2 AF314168_1
Csn3 SPAC821.02C, CAB60708, g7491244
Csn4 SPAC22A12.03C, CAB16573, g2414596
Csn5 SPAC1687.13C, T37756, g7492119
Csn7a SPAC1952.12C, T37940, g7490883
Csn7b SPAC1751.03, BAA31742, g6455949

RESULTS

Csn1, Csn2, Csn4, and Csn5 Interact In Vivo

Our previous work demonstrated an interaction between Csn1 and Csn2 and suggested that these proteins formed a

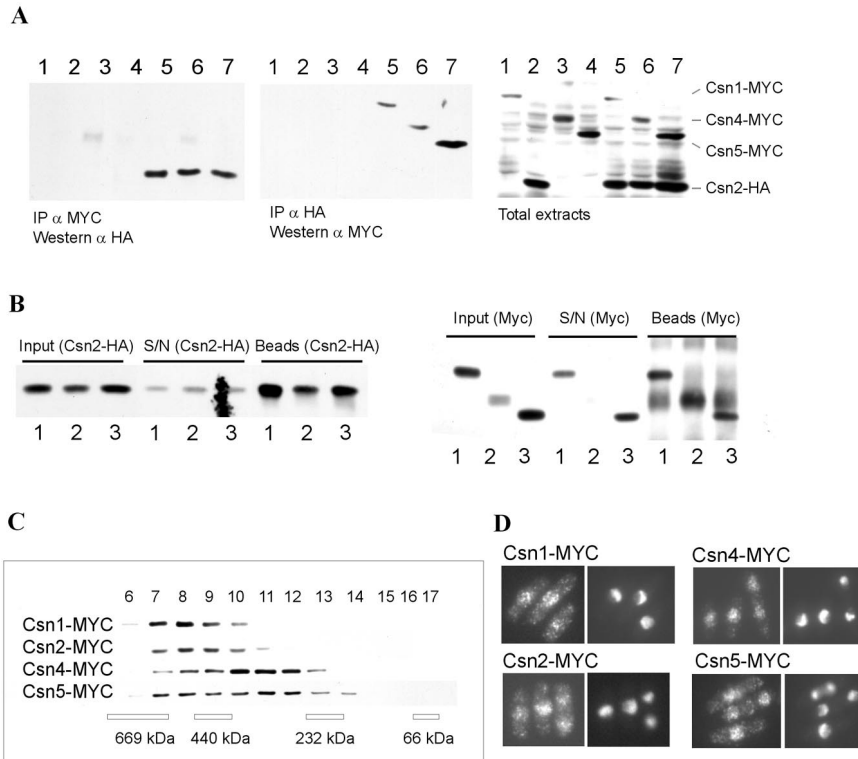
soluble protein complex of >500 kDa, consistent with the existence of a signalosome-like particle in fission yeast. The sequence similarity of Csn4 and Csn5 to signalosome subunits in higher eukaryotes predicts that these are subunits of a signalosome-like complex in fission yeast. To verify this, we added a C-terminal tag to both the *csn4* and *csn5* genes, which encodes the MYC epitope, and tested coimmunoprecipitation of Csn4 and Csn5 with Csn2-HA (Figure 1A). We were able to ascertain that these tagged proteins retained function (Figure 4B). With the anti-MYC antibody, immunoprecipitated MYC-tagged Csn1, Csn4, and Csn5 proteins were all found to be associated with HA-tagged Csn2 (Figure 4B, lanes 5–7). Csn2-HA was not precipitated in the absence of MYC-tagged Csn subunits (Figure 4B, lanes 2–4). Vice versa, coimmunoprecipitation of MYC-tagged Csn1, Csn4, or Csn5 protein with the anti-HA antibody (Figure 4B, lanes 5–7) required the presence of Csn2-HA. In addition, we also Flag-tagged Csn5 and used the anti-Flag antibody to coprecipitate Csn1 and Csn4 and demonstrated that Flag-tagged Csn5 could be immunoprecipitated with Csn1-MYC and Csn4-MYC (our unpublished data). Together, these data demonstrate that Csn1, Csn2, Csn4, and Csn5 interact in vivo. We have attempted to quantify the extent of coimmunoprecipitation (Figure 1B). Western blots of similar amounts of crude extract and depleted extract were compared after coprecipitation of Csn2-HA with either Csn1-MYC, Csn4-MYC, or Csn5-MYC and vice versa. The results are reasonably reproducible and suggest that, for example, although all of Csn2-HA is precipitated by anti-HA antibody (our unpublished data) the MYC-tagged partner is depleted by >50%. Such experiments do not allow us to conclude with certainty the proportions of protein in different complexes, but they are broadly consistent with size exclusion data discussed below.

Because Csn1 and Csn2 are associated with a complex of >500 kDa (as judged by gel filtration analysis) our coimmu-

Table 1. Localization of Csn subunits

Genotype	Phenotype ^a	Relative protein level	Predominant localization
Csn1-MYC	No	+++	Nuclear
Csn1-MYC <i>csn2-d</i>	Yes	++++	Dispersed/masked
Csn1-MYC <i>csn4-d</i>	No	+++	Dispersed/masked
Csn1-MYC <i>csn5-d</i>	No	+++	Dispersed/masked
Csn2-MYC	No	+	Nuclear
Csn2-MYC <i>csn1-d</i>	Yes	+++	Dispersed/masked
Csn2-MYC <i>csn4-d</i>	No	++	Dispersed/masked
Csn2-MYC <i>csn5-d</i>	No	++	Dispersed/masked
Csn4-MYC	No	+	Nuclear
Csn4-MYC <i>csn1-d</i>	Yes	++	Dispersed/masked
Csn4-MYC <i>csn2-d</i>	Yes	++	Dispersed/masked
Csn4-MYC <i>csn5-d</i>	No	+	Dispersed/masked
Csn5-MYC	No	+++	Nuclear
Csn5-MYC <i>csn1-d</i>	Yes	++++	Dispersed/masked
Csn5-MYC <i>csn2-d</i>	Yes	++++	Dispersed/masked
Csn5-MYC <i>csn4-d</i>	No	++++	Dispersed/masked

^a As characterized for *csn1-d* and *csn2-d*, including sensitive to DNA damage by UV and γ -irradiation, synthetic lethality with *rad3ts*, morphological defects.



Logarithmically growing cells were prepared for indirect immunofluorescence as described and costained with DAPI. Staining always corresponded to the nucleus and is most diffuse during mitosis (for example, the binucleate cell, bottom right).

noprecipitation analysis suggests that Csn4 and Csn5 should be associated with the same complex. Indeed, gel filtration analysis of Csn4 and Csn5 demonstrates that these proteins are associated with a protein complex of >500 kDa, consistent with their being associated with a Csn1- and Csn2-containing complex (Figure 1C). It should be noted, however, that the majority of both Csn4 and Csn5 is present in fractions representing lower molecular weights. This may indicate that not all of the Csn4 and Csn5 is associated with a core signalosome complex. *Arabidopsis* Csn5 has been found in both a nuclear (complexed) form and a cytoplasmic (non-complexed) form. We therefore used immunolocalization by indirect fluorescence to establish the localization of Csn4 and Csn5 in *S. pombe*. As we previously reported for Csn1 and Csn2, both Csn4 and Csn5 are predominantly localized to the nucleus (Figure 1D). From the data described above we conclude that Csn4 and Csn5 are bona fide subunits of the Csn1- and Csn2-containing signalosome complex in fission yeast.

Deletion of *csn4* and *csn5*

To characterize the genetic interactions of *csn4* and *csn5*, we deleted both genes from the genome by using standard techniques. We have previously reported that both *csn1-d* and *csn2-d* are sensitive to ionizing irradiation and UV light and are delayed in progression through S phase during logarithmic growth (Mundt *et al.*, 1999). Unexpectedly, *csn4-d* and *csn5-d* cells display wild-type profiles of DNA

Figure 1. Characterization of protein interactions. (A) Coimmunoprecipitation of Csn2-HA with Csn1-MYC, Csn4-MYC, or Csn5-MYC: either anti-MYC (left) or anti-HA (middle) were used to immunoprecipitate Csn subunits from total protein extracts (25 μ g of which is probed with both anti-HA and anti-MYC: right). The precipitated proteins were detected with anti-HA or anti-MYC antibodies, respectively. Extracts prepared from lane 1, *csn1*-MYC; lane 2, *csn2*-HA; lane 3, *csn4*-MYC; lane 4, *csn5*-MYC; lane 5, *csn1*-MYC *csn2*-HA; lane 6, *csn4*-MYC *csn2*-HA; and lane 7, *csn5*-MYC *csn2*-HA. (B) Quantification of immunoprecipitation. Two milligrams of total extract from strains *csn1*-MYC *csn2*-HA (1) *csn4*-MYC *csn2*-HA (2), and *csn5*-MYC *csn2*-HA (3) was immunoprecipitated with either α -MYC (left) or α -HA (right) and equal amounts (100 μ g) of input and remaining supernatant (S/N) plus 10 or 20% (left and right, respectively) of the beads were Western blotted with either α -HA (left) or α -MYC (right). (C) Size exclusion chromatography of MYC-tagged Csn1, Csn2, Csn4, and Csn5 proteins. Extracts were prepared from the strains containing either the *csn1*, *csn2*, *csn4*, or *csn5* genes tagged with 13-MYC epitopes and fractionated on the basis of complex size by using a Sephadex 200 column. Elution positions of known control proteins are marked below. (D) Localization of MYC-tagged Csn1, Csn2, Csn4, and Csn5 proteins.

damage sensitivity (Figure 2A) and the cell cycle parameters appear normal (our unpublished data). In addition, deletion of *csn5* in a *csn1-d* background did not further exacerbate the radiation sensitivity of this mutant (Figure 2A).

Loss of either *csn1* or *csn2* leads to activation of the DNA structure checkpoint pathway as judged by assaying phosphorylation of the DNA damage-specific checkpoint kinase Chk1 and the activation of the S-phase-specific checkpoint kinase Cds1 (Mundt *et al.*, 1999). Consistent with this, we also reported that both *csn1-d* and *csn2-d* mutant cells require the presence of a number of checkpoint genes for viability, including *rad3*, which encodes the central PI3-like checkpoint kinase (Mundt *et al.*, 1999). In contrast, we find that neither *csn4-d* nor *csn5-d* requires *rad3* for survival. As shown in Figure 2B, although *csn1-d rad3^{ts}* and *csn2-d rad3^{ts}* die at the restrictive temperature, both *csn4-d rad3^{ts}* and *csn5-d rad3^{ts}* are viable. This is consistent with the lack of a cell elongation phenotype for either *csn4-d* or *csn5-d*, which have previously been documented for *csn1-d* and *csn2-d* mutants. Taken together, these observations demonstrate that the *csn4-d* and *csn5-d* mutants do not suffer from the same intrinsic DNA damage as *csn1-d* and *csn2-d* and are not defective in S-phase progression.

Complex Formation and Nuclear Localization in Deletion Mutants of COP9/Signalosome Subunits

Intrigued by the distinct phenotypes resulting from the deletion of different COP9/signalosome subunits, we went on

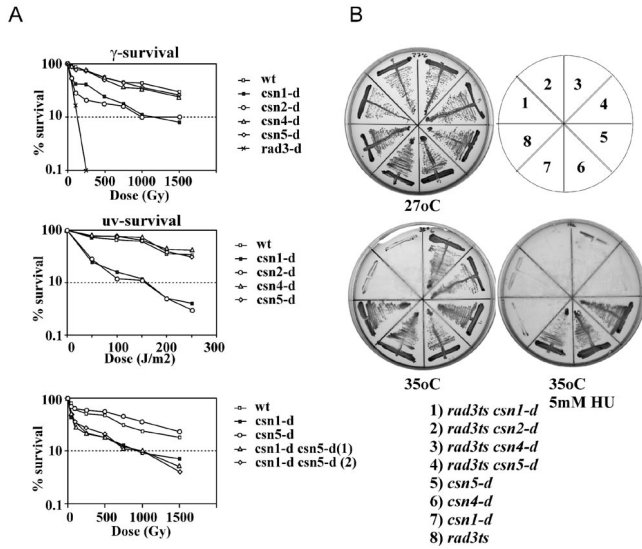


Figure 2. Analysis of *csn4-d* and *csn5-d*. (A) Survival analysis. Top, percentage survival of *csn⁺* (wt), *csn1-d*, *csn2-d*, *csn4-d*, *csn5-d*, and *rad3-d* cells after increasing doses of ionizing radiation. Middle, percentage survival of *csn⁺* (wt), *csn1-d*, *csn2-d*, *csn4-d*, and *csn5-d* cells after increasing doses of UV irradiation. Bottom, percentage survival of *csn⁺* (wt), *csn1-d*, *csn5-d*, and double-deleted *csn1-d csn5-d* (strains 1 and 2) cells after increasing doses of ionizing radiation. (B) Genetic interactions of *rad3^{ts}* with deletion mutants of signalosome subunits: analysis of growth on YEA plates at 27°C, 35°C, or in the presence of 5 mM HU at 35°C. Strains tested: *rad3^{ts} csn1-d* (1), *rad3^{ts} csn2-d* (2), *rad3^{ts} csn4-d* (3), *rad3^{ts} csn5-d* (4), *csn5-d* (5), *csn4-d* (6), *csn1-d* (7), and *rad3^{ts}* (8).

to analyze the effect of loss of *csn1*, *csn2*, *csn4*, and *csn5* on complex formation (Figure 3A) and subunit localization (Figure 3B and Table 2). The size of the Csn1-containing complex is modestly affected by the deletion of *csn2*, but either not at all, or to an undetectable extent, by deletion of *csn4* or *csn5*. It is particularly noticeable that the elution profile of Csn1-MYC spreads to lower molecular weight fractions in the *csn2-d* and *csn5-d* backgrounds. The profile of Csn2-MYC is shifted to a smaller size in *csn1-d* and, to a lesser extent in *csn4-d*, but not significantly in *csn5-d* mutants. Analysis of Csn4-MYC in *csn1-d*, *csn2-d*, and *csn5-d* reveals the most consistent result, with Csn4 not forming the higher molecular weight complex in either *csn1-d*, *csn2-d*, or *csn5-d* cells. Similarly, the amount of Csn5 in the higher molecular weight fractions is reduced in *csn2-d* and *csn4-d* and may be modestly affected in *csn1-d* cells. These data suggest that signalosome subunits Csn1 and Csn2 exist together in a multisubunit complex with Csn4 and Csn5, whereas Csn4 and Csn5 also exist in other, lower molecular weight forms.

Surprisingly, examination of the localization of the signalosome subunits by indirect immunofluorescence did not correlate with the observed differences in phenotype between *csn1* and *csn2* mutants and *csn4* and *csn5* mutants. In strains deleted for any one of the four subunits, *csn1-d*, *csn2-d*, *csn4-d*, and *csn5-d*, the remaining epitope-tagged subunits were no longer observed to localize to the nucleus and the overall staining intensity was similar to that observed in

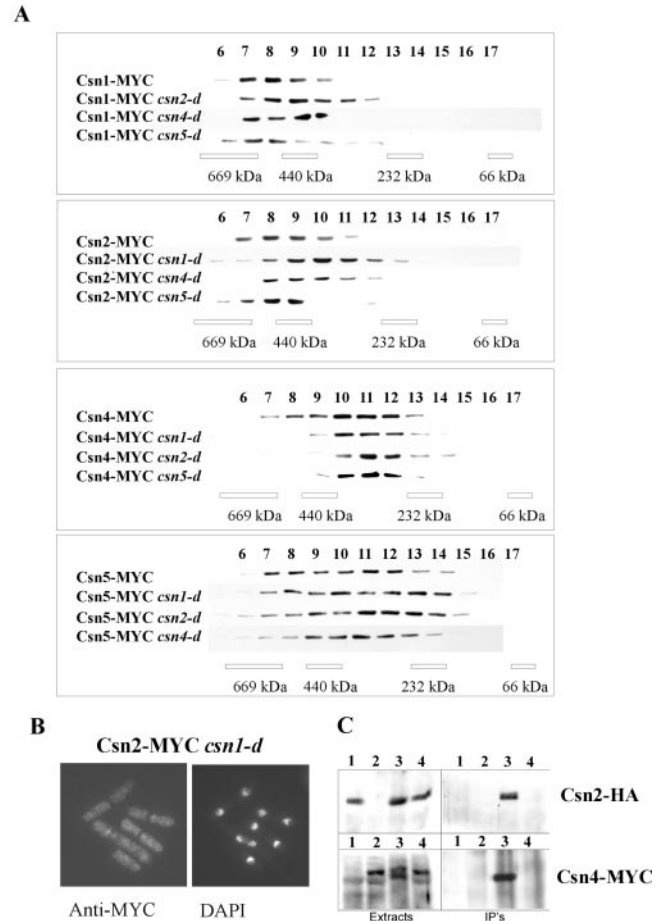


Figure 3. Subunit localization and size exclusion chromatography in *csn* mutant backgrounds. (A) Size exclusion chromatography of MYC-tagged Csn1, Csn2, Csn4, and Csn5 proteins in *csn* mutant backgrounds. Extracts were prepared from the strains indicated and fractionated on the basis of complex size by using a Sephadex 200 column. Elution positions of known control proteins are marked below. (B) Localization of Csn2-MYC in *csn1-d*: an example of mislocalization. *csn2-MYC csn1-d* cells were prepared for indirect immunofluorescence and costained with DAPI as described. (C) Coimmunoprecipitation of Csn2-HA and Csn4-MYC is dependent on *csn5*. Top, total extract (25 μ g) (left) and anti-MYC IP (right) probed with anti-HA. Bottom, total extract (25 μ g) (left) and anti-HA IP (right) probed with anti-MYC. Extracts prepared from lane 1, *csn2-HA*; lane 2, *csn4-MYC*; lane 3, *csn2-HA csn4-MYC*; and lane 4, *csn2-HA csn4-MYC csn5-d*.

control (nontagged) wild-type cells. The MYC-tagged Csn subunits are stable under these conditions (Figure 5C) so these data suggest that the proteins are either dispersed throughout the cell, or the tag is no longer accessible to the antibody under these conditions. Tubulin staining in the same cells excluded the possibility that a difference in cell wall permeability had led to this observation (our unpublished data).

The size exclusion chromatography results suggest that Csn4 requires Csn5 to associate with the Csn1/Csn2-containing complex. To test this further, we established whether the interaction between Csn2 and Csn4 required the pres-

Table 2. Strains used in this study

Strain	Genotype	Reference
"Wild-type" 501	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	Murray <i>et al.</i> (1992)
<i>csn1-d (caa1-d)</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	Mundt <i>et al.</i> (1999)
<i>csn2-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	Mundt <i>et al.</i> (1999)
<i>csn4-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>rad3-ts</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	Martinho <i>et al.</i> (1998)
<i>csn1d rad3-ts</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	Mundt <i>et al.</i> (1999)
<i>csn2-d rad3-ts</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn4-d rad3-ts</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn5-d rad3-ts</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-d csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn1-MYC</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	Mundt <i>et al.</i> (1999)
<i>csn1-MYC csn2-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn1-MYC csn4-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn1-MYC csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-MYC</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-MYC csn1-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-MYC csn4-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-MYC csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-HA</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-HA csn1-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-HA csn4-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-HA csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn4-MYC</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn4-MYC csn1-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn4-MYC csn2-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn4-MYC csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn5-MYC</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn5-MYC csn1-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn5-MYC csn2-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn5-MYC csn4-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>csn2-HA</i>	<i>ura4-D18, leu1.32, ade6.M210 h⁻</i>	This study
<i>csn2-MYC</i>	<i>ura4-D18, leu1.32, ade6.M216 h⁺</i>	This study
<i>pcu1-MYC</i>	<i>ura4-D18, leu1-32</i>	Osaka <i>et al.</i> (2000)
<i>pcu1-MYC csn1-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>pcu1-MYC csn2-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>pcu1-MYC csn4-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁻</i>	This study
<i>pcu1-MYC csn5-d</i>	<i>ura4-D18, leu1.32, ade6.704 h⁺</i>	This study

ence of the *csn5* gene. By comparing coimmunoprecipitation from extracts prepared from *csn2-HA csn4-MYC csn5⁺*, and *csn2-HA csn4-MYC csn5-d* cells we can clearly show that the Csn2-Csn4 immunoprecipitation requires the integrity of *csn5* (Figure 3C).

Cullin Modification by Nedd8 in *csn* Mutants

Approximately half of the *S. pombe* Pcu1 cullin protein is modified by Nedd8 at lysine 713 in wild-type cells (Osaka *et al.*, 2000). This modification is required for Pcu1 function. Furthermore, purification of mammalian cullin-associated proteins identified association with Csn2 (Lyapina *et al.*, 2001). Interestingly, in *csn1-d* cells, all of the Pcu1 is modified by Nedd8, suggesting that the *csn1* and by extension the signalosome, is required to remove Nedd8 from cullins (Lyapina *et al.*, 2001). Using an HA-Nedd8 expression vector, we have established an assay for Pcu1 cullin modification (Figure 4A). We find that loss of either *csn1*, *csn2*, *csn4*, or *csn5* results in hyper-Nedd8 modification of Pcu1 (Figure 4B). Because loss of *csn4* or *csn5* does not result in the

radiation sensitivity and slow S-phase phenotypes seen for loss of *csn1* and *csn2*, this establishes that the inability to remove Nedd8 from Pcu1 (and presumably other cullins) is not the cause of these phenotypes.

The reported interaction between Pcu1 and signalosome subunits raises the possibility that the signalosome is part of a stable complex with Pcu1. We have used the size exclusion chromatography assay to establish whether there is evidence that this is the case. Under the same conditions that clearly demonstrated the existence of an *S. pombe* signalosome complex (Figure 3A), we did not find that the elution profile of Pcu1-MYC was dependent on the integrity of the *csn1*, *csn2*, or *csn4* genes (Figure 4C).

Lack of Evidence for Csn2 Dimerization

Yeast offers a particularly useful system to study dimerization of proteins because diploid strains can be constructed containing two distinct tagged copies of a single gene locus. It has previously been suggested that *Arabidopsis* Csn2 may dimerize to form the core of the signalosome complex. We

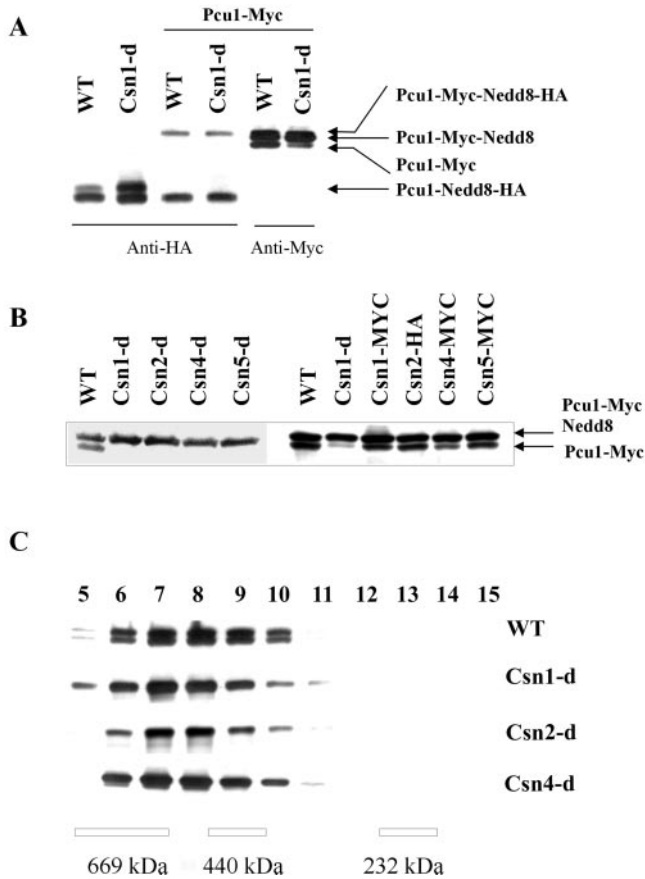


Figure 4. Pcu1 modification by Ned8. (A) Extracts from cells containing a Rep41-HA-Nedd8-expressing plasmid. The left two lanes contain extracts from *pcu1*⁺ cells. The remaining four lanes contain extracts from cells with a MYC-tagged *pcu1* locus. When probed with HA, the band corresponding to Pcu1-Nedd8-HA shifts to a higher molecular weight when Pcu1 protein also contains a MYC tag. When probed with MYC antibody, three forms of tagged Pcu1 are visible as indicated. (B) Total protein extracts from the *pcu1*-MYC (WT) and *pcu1*-MYC *csn*-null and tagged mutants (as indicated) were probed with anti-MYC to visualize the modification status of Pcu1. All signalosome subunits were required to maintain the wild-type levels of Pcu1-Nedd8. *csn1*-MYC, *csn2*-HA, and *csn5*-MYC retained essentially full function in this assay, whereas *csn4*-MYC appeared to have intermediate level of function. (C) Size exclusion chromatography of Pcu1-Myc extracts from *csn*⁺, *csn1*-d *csn2*-d, and *csn4*-d cells by using a Sephadex 200 column. Elution positions of known control proteins are marked below.

thus investigated the potential dimerization of Csn2 in fission yeast by constructing a diploid strain carrying both *csn2*-MYC and *csn2*-HA alleles and by preparing extracts from this strain. Soluble, whole cell extract contained the expected proportions of Csn2-HA and Csn2-MYC. Despite efficient immunoprecipitation of either protein by using the appropriate MYC or HA antibody, we did not detect coimmunoprecipitation of reciprocally tagged Csn2 protein (Figure 5A). Thus, we conclude that in fission yeast, at least, Csn2 is not present in multiple copies in a single protein complex. Although both Csn2-MYC and Csn2-HA are func-

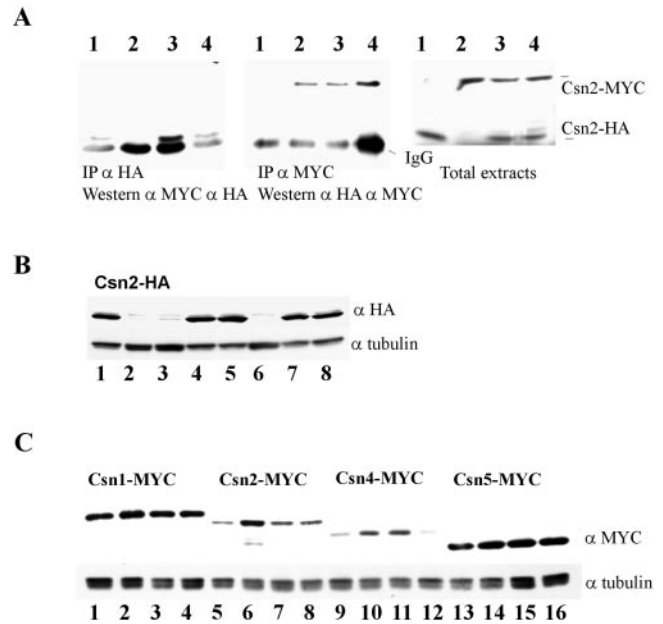


Figure 5. Csn protein levels in *csn* mutants. (A) Coimmunoprecipitation of Csn2-HA with Csn2-MYC. Either anti-HA (left) or anti-MYC (middle) was used to immunoprecipitate Csn2 subunits from total protein extracts (25 μ g of which is probed with both anti-HA and anti-MYC; right). The precipitated proteins were detected with anti-HA and anti-MYC antibodies. Lane 1, Csn2-HA; lane 2, Csn2-MYC; and lanes 3 and 4, diploid Csn2-MYC Csn2-HA. (B) Stability of Csn2-HA in *csn1*-d, *csn4*-d, and *csn5*-d: Western blots of total protein extracts were probed with anti-HA antibody and anti-tubulin antibody (as a loading control). Extracts were prepared from lane 1, *csn2*-HA; lane 2, *csn2*-HA *csn1*-d (isolate A); lane 3, *csn2*-HA *csn1*-d (isolate B); lane 4, *csn2*-HA (isolate A) after back-cross into *csn1*⁺; lane 5, *csn2*-HA (isolate B) after back-cross into *csn1*⁺; lane 6, nontagged *csn2*⁺; lane 7, *csn2*-HA *csn4*-d; and lane 8, *csn2*-HA *csn5*-d. Note the weak nonspecific band just above the Csn2-HA-specific band. (C) Stability and protein levels of MYC-tagged Csn1, Csn2, Csn4, and Csn5 in *csn1*-d, *csn2*-d, *csn4*-d, and *csn5*-d. Western blots of total protein extracts were probed with anti-MYC antibody and anti-tubulin antibody (as a loading control). Extracts were prepared from lane 1, *csn1*-MYC; lane 2, *csn1*-MYC *csn2*-d; lane 3, *csn1*-MYC *csn4*-d; lane 4, *csn1*-MYC *csn5*-d; lane 5, *csn2*-MYC; lane 6, *csn2*-MYC *csn1*-d; lane 7, *csn2*-MYC *csn4*-d; lane 8, *csn2*-MYC *csn5*-d; lane 9, *csn4*-MYC; lane 10, *csn4*-MYC *csn1*-d; lane 11, *csn4*-MYC *csn2*-d; lane 12, *csn4*-MYC *csn5*-d; lane 13, *csn5*-MYC; lane 14, *csn5*-MYC *csn1*-d; lane 15, *csn5*-MYC *csn2*-d; and lane 16, *csn5*-MYC *csn4*-d.

tional, judged by the lack of phenotype associated with the respective alleles, we cannot formally exclude the possibility of steric hindrance between the HA-MYC tags preventing heterodimerization. If this was the case, such steric hindrance presumably does not occur between HA-HA or MYC-MYC. If such HA-HA and MYC-MYC steric hindrance does occur, dimerization cannot be important for the signalosome functions we assay.

Loss of *csn1* Leads to Instability of HA- but not MYC-tagged Csn2

Protein stability, and thus the steady-state levels, of subunits of complexes are sometimes affected by the presence

or absence of the other subunits. Indeed, it has been reported that loss of Csn1 in *Arabidopsis* significantly reduces levels of other components of the signalosome. During our analysis, we observed that Csn2-HA levels in a *csn1-d* background were greatly reduced compared with those seen in *csn1*⁺ cells (Figure 5B). This suggests that Csn2-HA becomes unstable in the absence of *csn1* (Figure 5B, lanes 2 and 3). After a long exposure (our unpublished data), low levels of HA-tagged protein can still be detected compared with a nontagged wild-type strain (Figure 5B, lane 6). As a further control, we crossed two independently isolated Csn2-HA *csn1-d* strains (Figure 5B, lanes 2 and 3) back into a *csn1*⁺ background. This rendered the protein stable and detectable (Figure 5B, lanes 4 and 5). The deletion of *csn4* and *csn5* had no obvious effect on the stability of Csn2-HA (Figure 5B, lanes 7 and 8). To verify and extend this result, we tested all four 13-MYC-epitope-tagged subunits under circumstances where one of each of the remaining subunits was deleted. Contrary to the result described above, we found that Csn2-MYC was more abundant, and thus apparently stabilized in the *csn1-d* background (Figure 3B). This result could be interpreted in two ways: either Csn2 is protected from degradation by the presence of the C-terminal MYC tags, or Csn2 is promoted for degradation (but only in the absence of its partner subunits) by the presence of C-terminal HA tags. This phenomenon of increased abundance in specific null mutant backgrounds was also apparent with Csn4-MYC in both the *csn1-d* and *csn2-d* backgrounds. In contrast, deletion of *csn1*, *csn2*, or *csn4* did not affect the protein stability of Csn5, suggesting that Csn5 protein stability is not dependent on the Csn1/Csn2-containing signalosome complex (Figure 3B). It is intriguing that although all four subunits were tagged with 13-MYC epitopes, their apparent abundance in the *csn*⁺ background (Figure 3B, lanes 1, 5, 9, and 13) differs greatly and Csn1-MYC and Csn5-MYC appear to exist in excess over Csn2-MYC and Csn4-MYC.

DISCUSSION

Csn4 and Csn5 Are Bona Fide Subunits of COP9/Signalosome in Fission Yeast

The Csn1 subunit of the signalosome in fission yeast was initially identified through a genetic screen for suppression of Chk1-induced G2 arrest coupled with radiation sensitivity. Csn2 was identified as a two-hybrid interacting protein with Csn1. Csn4 and Csn5 were identified as putative subunits based on, respectively, 23 and 41% identity with their human counterparts. Herein, we have demonstrated that the Csn4 and Csn5 proteins interact with Csn2 in vivo. Consistent with our previous data, we also demonstrate that endogenous Csn1-MYC interacts with Csn2. Using a diploid strain, carrying both an HA- and an MYC-tagged allele of Csn2, we have further investigated the possibility that Csn2 dimerizes. Under identical conditions used to coimmunoprecipitate the other subunits, we could not find any association of Csn2-HA with Csn2-MYC and vice versa. Csn2-HA and Csn2-MYC are functional in all the assays we have available. This strongly suggests that the Csn2 subunit is not present in more than one copy in the same protein complex. However, we should bear in mind the caveat that

this specific combination of HA and MYC tags could prevent heterodimerization of Csn2.

Extensive size exclusion chromatography analysis indicated that, although Csn4 and Csn5 elute in similar size fractions to Csn1 and Csn2, significant quantities are also found in smaller molecular weight fractions. This could be indicative of the existence of either a smaller size subcomplex of the signalosome or of distinct additional complex(es) containing these proteins. Although this is consistent with data reported in *Arabidopsis*, we cannot exclude the possibility that the epitope tags affects the stability of the signalosome complex, although they clearly retain function as judged by the ability to promote the deneddylation of Pcu1. By analyzing size distribution of different subunits in various null mutant backgrounds, we are able to clearly show that the fraction of Csn4, which exists in a complex of >500 kDa, requires the presence of Csn1, Csn2, and Csn5. Indeed, we are also able to show that Csn5 is required for the coimmunoprecipitation of Csn4 with Csn2. The distribution of Csn2 in these experiments is clearly affected when Csn1 and Csn4 are absent, although a less dramatic change is evident when Csn5 is absent. Conversely, only subtle changes in molecular weight distribution are evident for Csn1 and Csn5 when other subunits are deleted.

Genetic Analysis Distinguishes csn4-d and csn5-d from csn1-d and csn2-d

Despite the association of Csn4 and Csn5 with Csn1 and Csn2 proteins, the deletion of *csn4* and *csn5* from the genome did not phenocopy *csn1* and *csn2* null mutants. *csn4-d* and *csn5-d* are not sensitive to UV or ionizing irradiation. Furthermore, *csn5-d* did not exacerbate the sensitivity of the *csn1-d* mutant. Unlike *csn1-d* and *csn2-d*, neither *csn4-d* nor *csn5-d* is synthetic lethal with *rad3* mutants or with other checkpoint mutants (our unpublished data), suggesting that the intrinsic DNA damage generated in *csn1* or *csn2* null cells does not occur in *csn4* or *csn5* mutants. This is consistent with the lack of the cell elongation phenotype characteristic of *csn1-d* and *csn2-d* mutants, which correlates with S-phase delay and the constitutive activation of DNA-integrity checkpoint kinases. Despite trying a number of assays, we have yet to detect a clear phenotype associated with loss of either Csn4 or Csn5. This indicates two things. First, the different subunits of the signalosome do not act as a single functional unit in fission yeast, but presumably mediate a number of processes, some of which are dependent on specific subunits and may reflect distinct subcomplexes. Second, rapidly growing cells under laboratory conditions do not necessarily represent all the cellular requirements demanded of yeast cells in nature.

Nuclear Localization of Signalosome Subunits

Using indirect immunofluorescence, we find that Csn1, Csn2, Csn4, and Csn5 localize predominantly to the nucleus. It has been speculated that the signalosome may interact with the core 20S subunit of the proteasome, because it has structural similarity to the 19S regulatory lid complex. The proteasome in fission yeast has been shown to localize to the nuclear periphery (Wilkinson *et al.*, 1998). We do not detect any accumulation of COP9/signalosome subunits in this

region. We cannot exclude the colocalization of a subpopulation of the signalosome with the proteasome.

We analyzed the effects of the deletion of the different signalosome subunits on nuclear localization. We had anticipated a correlation among nuclear localization, complex formation (as judged by size exclusion chromatography), and the pleiotrophic phenotypes associated with loss of *csn1* and *csn2*. However, the emerging picture, summarized in Table 1, was more complex and does not yet allow a straightforward interpretation. When any one subunit was deleted from the genome, we were unable to detect specific nuclear localization of any of the remaining MYC-tagged subunits. Western blot analysis indicated that the proteins were present in the cells. In particular, the loss of nuclear staining for Csn1-MYC and Csn2-MYC in *csn4-d* or *csn5-d* mutant backgrounds was surprising because it did not correlate with the radiation sensitivity associated with *csn1* and *csn2* deletion mutants. Although it is possible that nuclear localization of Csn1 and Csn2 is not important for their function, it is also possible that Csn1 and Csn2 proteins are dispersed throughout the cell, and that sufficient material is present in the nucleus to retain function.

Cullin Modification in Signalosome Mutants

The Nedd8 modification of Pcu1 lys 713 is required for its essential function. Intriguingly, in the absence of *csn1*, Pcu1 is hyper-Nedd8 modified. This suggested that the lack of regulation of cullin modification by Nedd8 may underlie the phenotypes associated with *csn1* and *csn2* deletion mutants. However, we have shown that Pcu1 is hyper-Nedd8 modified in *csn4* and *csn5* null mutants. Because neither of these mutants exhibit any obvious phenotype, this precludes a causative relationship between Pcu1 modification status and the *csn1-d* or *csn2-d* phenotypes. *S. pombe* contains two further cullin homologs, Pcu3 and Pcu4. Although we have not assessed their Nedd8 modification status in the signalosome mutants, it is likely that they are also hypermodified.

It remains unclear what the biochemical function(s) of the signalosome subunits is. The accumulating evidence suggests a close involvement in aspects of ubiquitin-dependent protein degradation. Csn1 and Csn2 are apparently involved in a specific pathway or pathways that are required for a normal DNA damage response and the proper execution of S phase. Csn4 and Csn5 are not required for this set of functions. We have been unable to identify any biological endpoints for the loss of Csn4 and Csn5 function and thus we are not able to assess whether their function(s) require Csn1 and Csn2. However, based on our size exclusion chromatography and immunolocalization data, we speculate that the different subunits, or subsets of subunits, participate in distinct functions. Clearly, in our model these subcomplexes must aggregate, presumably for a purpose that is, as yet, undetermined but that may affect the global regulation of SCF ubiquitin ligase complexes. A complete and satisfactory explanation will have to await the characterization of the other missing subunits of the fission yeast signalosome-like complex and the determination of its structure and function.

A Cautionary Tail of Tags

In *Arabidopsis*, mutations in *csn1* (*COP11*) lead to instability of Csn8 (*COP9*) protein (Wei *et al.*, 1994). We observed that

Csn2-HA becomes unstable in the *csn1-d* background. Unexpectedly, while attempting to verify this observation by using the *csn2-myc* cells we found that the effect of *csn1-d* on Csn2-MYC stability was opposite: Csn2-MYC was stabilized and not destabilized, as was observed with the Csn2-HA protein. This difference could possibly be due to protection of the MYC-tagged protein from degradation by the long C-terminal extension, which would subsequently lead to Csn2-MYC accumulation. However, it is also formally possible that the HA-tag promotes degradation of Csn2-HA protein when it is not in the requisite complex. We must await the generation of a suitably sensitive antibody to the endogenous protein before resolving this point, but it is noticeable how much of the literature relies on HA- or MYC-tagged proteins to support conclusions concerning protein abundance.

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