Interactions of the COP9 Signalosome with the E3 Ubiquitin Ligase SCF^{TIR1} in Mediating Auxin Response

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The COP9 signalosome is an evolutionary conserved multiprotein complex of unknown function that acts as a negative regulator of photomorphogenic seedling development in *Arabidopsis*. Here, we show that plants with reduced COP9 signalosome levels had decreased auxin response similar to loss-of-function mutants of the E3 ubiquitin ligase SCF^{TIR1}. Furthermore, we found that the COP9 signalosome and SCF^{TIR1} interacted in vivo and that the COP9 signalosome was required for efficient degradation of PSIAA6, a candidate substrate of SCF^{TIR1}. Thus, the COP9 signalosome may play an important role in mediating E3 ubiquitin ligase–mediated responses.

The COP9 signalosome is a multiprotein complex that was discovered during the characterization of the photomorphogenic *cop/det/fus* mutants from *Arabidopsis* (*1*, *2*). The COP9 signalosome is required for the proteasome-mediated degradation of HY5, a positive regulator of photomorphogenesis (*3*), and each of its eight subunits is related to one of the eight subunits that form the "lid" subcomplex of the 26*S* proteasome (*2*, *4*). Thus, it may be that the COP9 signalosome is involved in protein degradation via the ubiquitin-proteasome pathway (*2*, *5*).

We generated transgenic *Arabidopsis* plants with reduced levels of the COP9 signalosome subunit CSN5 (JAB1 or AJH) using the antisense and cosuppression strategy (Fig. 1A) (*6*–*8*). In the *cop/det/fus* mutants that affect individual subunits of the COP9 signalosome, loss of one subunit results in loss of the entire protein complex (*2*), and similarly, the reduction of CSN5 in the CSN5 transgenic plants leads to a reduction of COP9 signalosome levels (Fig. 1A). At the physiological level, this is reflected by the photomorphogenic phenotype of the dark-grown CSN5 transgenic seedlings (Fig. 1, A and B) (*6*).

On the basis of the pleiotropic phenotype of the *cop/det/fus* mutants, it has been suggested that in addition to photomorphogenesis, many other developmental processes may be affected in these mutants (*9*). Because the lethal phenotype of mutants that affect subunits of the COP9 signalosome has so far prevented insightful developmental studies, we used the weaker nonlethal phenotype of the CSN5 transgenic plants to study COP9 signalosome-mediated processes in plant development. The most striking phenotype of adult CSN5 transgenic plants was a strong increase in the number of secondary inflorescences (8 to 12 compared to 3 to 4 in the wild type) accompanied by a general reduction in plant size and internode length (Fig. 1, C through E). The outgrowth of secondary inflorescences in wild-type plants is inhibited by the phytohormone auxin, which is produced in the shoot apex of the primary inflorescence, in a physiological process known as apical dominance (*10*). Because the CSN5 transgenic plants had lost apical dominance, these plants may have become insensitive to the inhibitory auxin signal. The CSN5 transgenic plants also had strongly reduced cell size and wavy leaf morphology, phenotypes that are observed in a number of auxin-response mutants, notably the group of *axr* mutants (Fig. 1, F through H) (*11*–*14*).

We examined auxin-related phenotypes in the CSN5 transgenic plants in more detail. Whereas root growth in wild-type seedlings is inhibited by exogenous auxin application, CSN5 transgenic seedlings were more resistant to auxin (Fig. 2, A and B). Furthermore, CSN5 transgenic seedlings had fewer lateral roots, reduced root hair elongation, and reduced gravitropism response when compared to the wild type (Fig. 2, C through E). These physiological responses are known to be controlled by auxin and are affected in a qualitatively similar manner in the auxin-response mutants *axr1-3* and *tir1-1* (Fig. 2, A through E) (*11*, *15*).

The *AUX/IAA* genes form a gene family that encodes short-lived regulatory proteins (*16*, *17*). Auxin triggers the rapid and specific transcription of most members of the *AUX/IAA* gene family (*17*). The induction of these genes is reduced in several auxin-response mutants and was also compromised in the CSN5 transgenic plants (Fig. 2F) (*17*, *18*). Thus, it is likely that similar molecular mechanisms form the basis of the auxin-reponse phenotypes observed in the CNS5 transgenic plants and other auxin mutants.

Proper auxin response in *Arabidopsis* is mediated by the E3 ubiquitin ligase SCF^{TIR1} (*15*). Molecular and genetic evidence suggests that AUX/IAA proteins are SCF^{TIR1} substrates (*19*). The rapid turnover of AUX/IAA proteins by SCF^{TIR1} appears to be an integral feature of auxin response (*16*), and their increased half-life in the SCF^{TIR1} loss-of-function mutants is the basis of their auxin phenotype (*19*, *20*). To test whether the COP9 signalosome was involved in regulating the turnover of AUX/IAA proteins, we used a transgenic line that expresses the luciferase reporter in frame with *PSIAA6*, an *AUX/IAA* from pea (*20*). Using luciferase activity as a measure of PSIAA6 abundance, we found that the PSIAA6LUC degradation rate was reduced in protein extracts prepared from CSN5 transgenic lines compared to those from the wild type (Fig. 2G). At the same time, the stability of a native luciferase protein was not affected, indicating that it was the PSIAA6 moiety that specifically promoted the degradation of PSIAA6LUC. Because protein degradation appears to be a common feature to most AUX/IAA proteins (*16*), it is likely that the COP9 signalosome may also be required for their degradation and that increased AUX/IAA levels cause the auxin-response phenotype of the CSN5 transgenic plants.

The fact that the COP9 signalosome and SCF^{TIR1} were required for AUX/IAA protein degradation, and recent evidence that the cullin subunit of SCF-type E3 ubiquitin ligases from other eukaryotes can interact with the COP9 signalosome (*21*), prompted us to investigate a physical interaction between the COP9 signalosome and SCF^{TIR1}. Indeed, we observed copurification of the SCF^{TIR1} subunit AtCUL1 in immunoaffinity-purified fractions of the COP9 signalosome (Fig. 3A). In a reciprocal experiment, an AtCUL1 antibody immunoprecipitated the COP9 signalosome and the SCF^{TIR1} subunit ASK1 (Fig. 3B). Finally, immunoprecipitation of TIR1, the F-box domain subunit that confers substrate-specificity to SCF^{TIR1} (*15*), yielded the entire COP9 signalosome in addition to the SCF^{TIR1} components AtCUL1 and ASK1 (Fig. 3C), suggesting that the COP9

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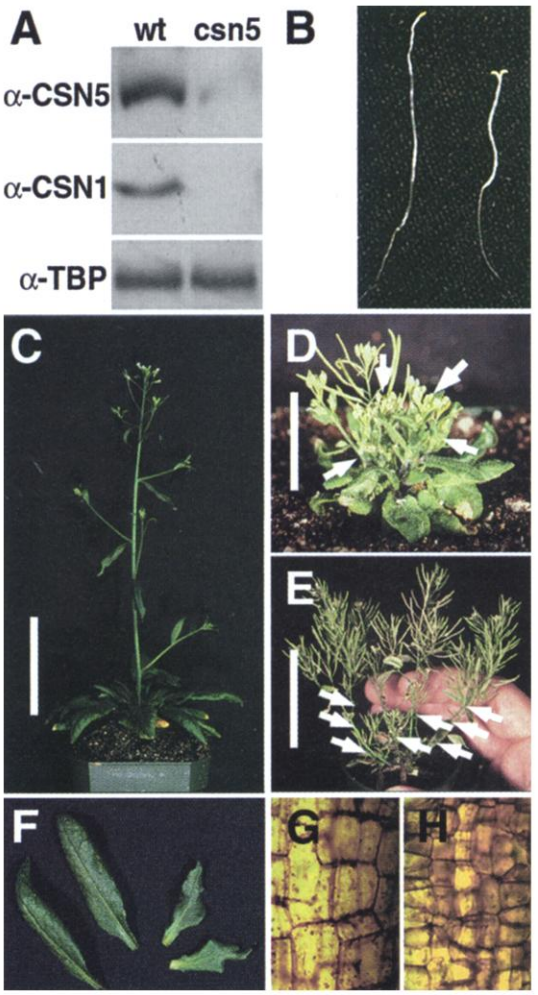

Fig. 1. CSN5 transgenic plants had a photomorphogenic phenotype in the dark and reduced apical dominance. (**A**) Immunoblot with protein extracts from a wild-type and a CSN5 transgenic (CSN5 tg) seedling using CSN5 and CSN1 antibodies (*6, 27, 28*). Seedlings from CSN5 antisense and cosuppression lines with similar phenotypes had similar immunoblot profiles; the result from a representative CSN5 antisense line (line J1L1 #20) is shown here. The reduction of CSN1 expression is an example for a reduction of COP9 signalosome levels (*2, 28*). The TATA-box binding protein (TBP) blot served as a loading control. (**B**) Seven-day-old dark-grown wild-type (left) and CSN5 tg (antisense J1L1 #20, right) seedlings. (**C**) Four-week-old wild-type plant. (**D**) Four-week-old CSN5 tg plant (antisense line J1L1 #20). (**E**) Eight-week-old CSN5 tg plant (cosuppression line X1#7). All plants are Columbia ecotype. Bar in (C) and (E) is 5 cm and in (D) is 2 cm. Arrows in (D) and (E) indicate secondary inflorescences. (**F**) Leaves from a 4-week-old wild-type (left) and a CSN5 tg plant (cosuppression line X1#7, right). (**G** and **H**) Toluene blue–stained (0.01%) longitudinal section of stems from wild-type (G) and CSN5 tg (cosuppression line X1#7) (H) plants. Sections were taken from 3-week-old plants at the midpoint of their primary inflorescence. Both images were taken at the same magnification.

signalosome and SCF^{TIR1} interact in vivo. In the yeast two-hybrid system, we detected direct interactions between AtCUL1 and CSN2, as well as between the SCF^{TIR1} subunit AtRBX1 and the COP9 signalosome subunits CSN1 and CSN6 (Fig. 3D).

AXR1 is a component of an enzyme cascade that conjugates the ubiquitin-related protein RUB1 to the AtCUL1 subunit of SCF^{TIR1} (*22*). A recent observation suggests that the COP9 signalosome promotes RUB1 deconjugation (*21*). Indeed, we found that *Arabidopsis* COP9 signalosome mutants preferentially accumulated RUB1-conjugated AtCUL1, whereas wild-type extracts contained unmodified and RUB1-modified AtCUL1 (Fig. 3E). Thus, the essentially antagonistic steps of AXR1-mediated RUB1 conjugation and its subsequent COP9 signalosome–promoted deconjugation are both required for proper auxin response and act together toward the degradation of SCF^{TIR1} substrates (Fig. 2). The requirement of AXR1 and the COP9 signalosome for proper auxin response was further confirmed in a genetic interaction study. When we introduced a CSN5 cosuppressing transgene (csn5 tg) into a homozygous *axr1-3* mutant background, the auxin-response phenotypes of the *axr1-3*/csn5 tg seedlings were enhanced compared to the parents (Fig. 4A). Furthermore, *axr1-3*/csn5 tg plants had reduced fertility (Fig. 4B), a phenotype that was observed in neither of the parent lines but in a strong mutant allele of *AXR1* (*11*), suggesting a synergistic genet-

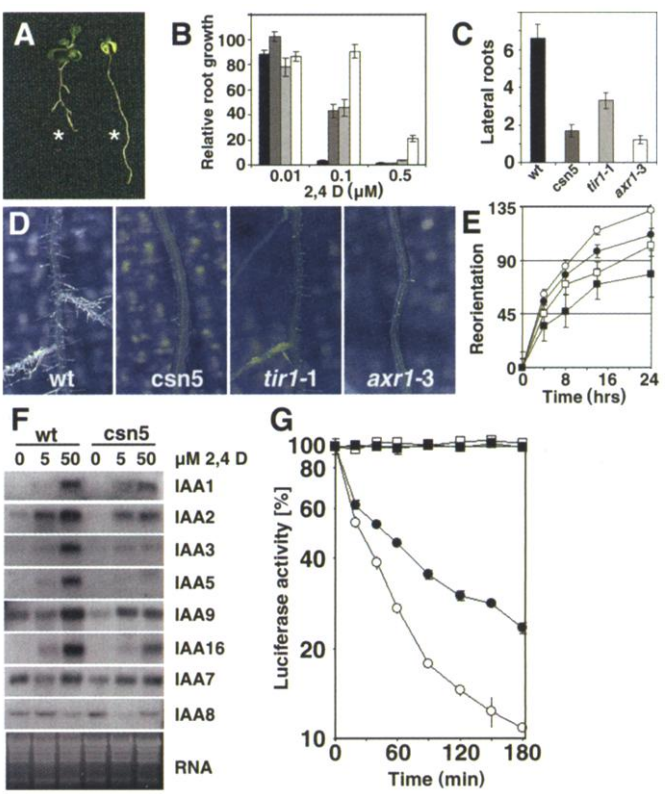

Fig. 2. Auxin-response phenotypes of CSN5 transgenic seedlings. (**A**) Nine-day-old wild-type (left) and CSN5 transgenic (right) seedlings. The asterisk indicates the position of the root tip at the time of transfer to auxin-containing media. (**B**) Relative root growth of seedlings on 2,4-dichlorophenoxyacetic acid (2,4-D)–containing medium compared to root growth on unsupplemented medium (100%). Wild type, black bars; CSN5 tg (antisense line J1L1 #20), dark-gray bars; *tir1-1*, light gray bars; *axr1-3*, white bars (*29*). (**C**) Number of lateral roots and (**D**) images of roots from 12-day-old seedlings. All images were taken at the same magnification. wt, wild-type; csn5, CSN5 transgenic (antisense line J1L1 #20). (**E**) Root tip reorientation in degrees after a change in the gravitropic vector by 90°. ○, wild-type; ●, CSN5 tg (antisense line J1L1 #20); □, *tir1-1*; ■, *axr1-3*. Experimental procedures from (A) to (E) are as previously described (*11*). (**F**) Northern hybridization of 25 μg total RNA prepared from wild-type and CSN5 tg plants with *AUX/IAA* probes as indicated (*30, 31*). (**G**) The PSIAA6LUC degradation rate is reduced in CSN5 transgenic plants (*31*). ○, PSIAA6LUC in wild-type background; ●, PSIAA6LUC in CSN5 transgenic (antisense line J1L1 #20) background; □, LUC in wild-type background; ■, LUC in CSN5 transgenic (antisense line J1L1 #20) background.

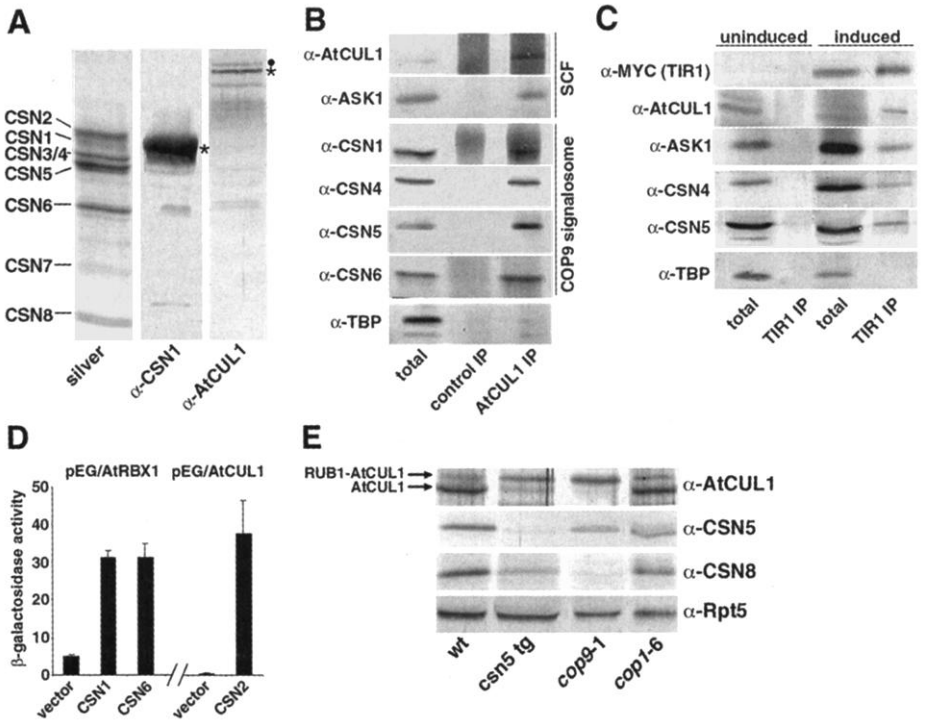

Fig. 3. COP9 signalosome and SCF^{TIR1} interact in vivo and COP9 signalosome mutants accumulate preferentially RUB1-modified AtCUL1 (*31*). (**A**) Silver-stained gel of an immunoaffinity-purified COP9 signalosome from cauliflower (CSN subunits are indicated, left lane) (*28*). Immunoblots of the purified COP9 signalosome were probed with CSN1 and AtCUL1 antibodies (*15, 27*). Asterisks show the position of CSN1 and AtCUL1, respectively, ● indicates the position of the RUB1-conjugated AtCUL1. (**B**) The AtCUL1 antibody coimmunoprecipitates SCF^{TIR1} components and the COP9 signalosome. *Arabidopsis* seedling extract (total) was used for immunoprecipitation with the AtCUL1 antibody (AtCUL1 IP) and the corresponding preimmune serum (control IP) followed by an immunoblot analysis. Four COP9 signalosome subunits that coimmunoprecipitated with AtCUL1 are shown. (**C**) MYC-tagged TIR1 protein immunoprecipitates SCF^{TIR1} subunits and the COP9 signalosome (*15*). (**D**) *Arabidopsis* CSN1, CSN2, and CSN6 interact with AtCUL1 and AtRBX1 in the yeast two-hybrid interaction assay (*28*). (**E**) COP9 signalosome loss-of-function mutants accumulate preferentially RUB1-modified AtCUL1. Immunoblot with seedling protein extracts: wild-type, wt; CSN5 transgenic seedlings (antisense line J1L1 #20), csn5 tg; COP9 signalosome null-mutant, *cop9-1*; COP1 mutant, *cop1-6* (*31*). All techniques are as described in (*28*).

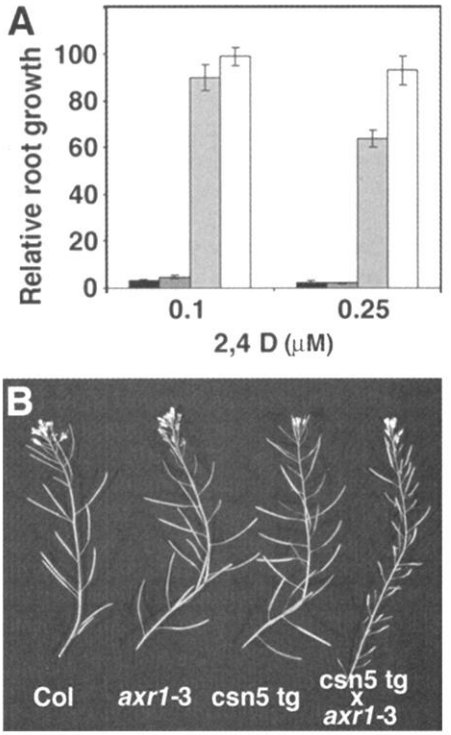

Fig. 4. Genetic interaction between *AXR1* and the COP9 signalosome (*31*). A weak CSN5 transgenic line (cosuppression line X1#7, no seedling phenotype but apical dominance phenotype) was crossed to the *axr1-3* mutant. (**A**) Root growth inhibition was reduced in the *axr1-3*/csn5 tg plants compared to the parent lines. Wild-type, black bars; csn5 tg, dark gray bars; *axr1-3*, light gray bars; *axr1-3*/csn5 tg, white bars. (**B**) The *axr1-3*/csn5 tg plants have reduced fertility. Mature inflorescences of 7-week-old wild-type (Col), *axr1-3*, csn5 tg, and *axr1-3*/csn5 tg plants are shown.

ic interaction between *axr1-3* and the CSN5 transgene cosuppression. Interestingly, studies centered around the ubiquitin-like modification SMT3 have revealed a parallel observation that yeast mutants deficient in SMT3 conjugation have similar phenotypes to mutants with defects in SMT3 deconjugation (*23*).

The conjugation of RUB1 to cullins promotes ubiquitin-chain formation (*24*), and it could be that RUB1 conjugation and deconjugation cycles are important for this process. Next to a possible biochemical role, the RUB1 modification could also be essential for the physical interaction between the COP9 signalosome and SCF^{TIR1} and thereby regulate the nucleo-cytoplasmic distribution of SCF^{TIR1}. This hypothesis is based on the finding that the COP9 signalosome is required for the light-dependent nucleo-cytoplasmic distribution of the putative E3 ubiquitin ligase COP1 (*25*).

We demonstrate a specific interaction between the COP9 signalosome and SCF^{TIR1}, and show that the COP9 signalosome is required for protein degradation in the context of auxin response. However, in analogy to other eukaryotes, it can be assumed that AtCUL1 and AtRBX1, the SCF^{TIR1} subunits that directly interact with the COP9 signalosome, are core components of multiple SCF-type E3 ubiquitin ligases that differ in their F-box domain subunit (*26*). Thus, the *Arabidopsis* COP9 signalosome may also interact with other yet-to-be-identified SCF-type E3 ubiquitin ligases. Indeed, we observed several phenotypes in the CSN5 transgenic plants, such as the loss of apical dominance and the change in leaf morphology, that cannot be solely explained by a loss of SCF^{TIR1} function (Fig. 1, C through E). Moreover, non–SCF-type E3 ubiquitin ligases like the aforementioned putative E3 ubiquitin ligase COP1 may also interact with the COP9 signalosome (*3*). Thus, the function of many different E3 ubiquitin ligases could be impaired in *cop/det/fus* mutants lacking the COP9 signalosome, and the combination of the resulting defects could account for the severe and pleiotropic phenotype of these mutants.

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Promotion of NEDD8-CUL1 Conjugate Cleavage by COP9 Signalosome

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SCF ubiquitin ligases control various processes by marking regulatory proteins for ubiquitin-dependent proteolysis. To illuminate how SCF complexes are regulated, we sought proteins that interact with the human SCF component CUL1. The COP9 signalosome (CSN), a suppressor of plant photomorphogenesis, associated with multiple cullins and promoted cleavage of the ubiquitin-like protein NEDD8 from *Schizosaccharomyces pombe* CUL1 in vivo and in vitro. Multiple NEDD8-modified proteins uniquely accumulated in CSN-deficient *S. pombe* cells. We propose that the broad spectrum of activities previously attributed to CSN subunits—including repression of photomorphogenesis, activation of JUN, and activation of p27 nuclear export—underscores the importance of dynamic cycles of NEDD8 attachment and removal in biological regulation.

SCF ubiquitin ligases consist of at least four subunits: CUL1 and HRT1 (also known as ROC1 or RBX1), which harbor a core ubiquitin ligase activity; a variable F-box protein that serves as a substrate receptor; and SKP1, which links the two modules together (*1*). SCF activity is stimulated in vitro by attachment of the ubiquitin-like protein NEDD8 (that is, "neddylation") to CUL1, but the physiological role and dynamics of this modification are poorly understood (*2–5*). It is not known whether there are other forms of SCF regulation at play in vivo, or whether there are additional SCF subunits yet to be discovered.

To address these questions, we expressed Myc_9-tagged forms of two human proteins, the F-box protein hSKP2 and COOH-terminally truncated hCUL1 (Δ692-752; hereafter called CUL1ΔC), from retroviral vectors in NIH 3T3 cells. SCF complexes were purified from these cells on anti-Myc beads (*6*) and evaluated by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining (Fig. 1A). Bands corresponding to specific interacting proteins were excised and analyzed by matrix-assisted laser desorption/ionization mass spectrometry and nanoelectrospray tandem mass spectrometry (*7–9*).

The SKP2 (*6*) and CUL1ΔC eluates contained a rich harvest of associated proteins. Analysis of CUL1ΔC eluates (Fig. 1A) yielded SKP1, HRT1, and a number of F-box proteins, as expected. Unexpectedly, all eight subunits (CSN1 to CSN8) of the COP9 signalosome (CSN) (*10*) were also found associated with CUL1ΔC. The ~500-kD CSN was originally discovered in *Arabidopsis thaliana* as a suppressor of photomorphogenesis (*11*). CSN subunits have significant sequence homologies to components of both the lid subcomplex of the 26*S* proteasome and the translation initiation complex eIF3 (*12–14*), but the biochemical function of CSN remains elusive.

To address the specificity of CSN-CUL1ΔC interaction, we tested other SCF subunits and other cullins to see whether they also bound CSN. Flag-tagged CSN1 was transfected into HeLa cells, purified on anti-Flag beads, and analyzed by SDS-PAGE and Western blotting (Fig. 1B). CSN8 associated with Flag-CSN1, as did HRT1, SKP1, and SKP2. Although CSN complex was purified in association with CUL1ΔC, Flag-CSN1 also bound full-length endogenous CUL1. Moreover, a direct comparison revealed that CUL1ΔC and CUL1 bound CSN1 with equal efficiency (*6*). CUL2 and CUL3 were retrieved in Flag-CSN1 eluates (Fig. 1B), and preliminary data suggest that CUL4 and CUL5 also bound CSN (*15*). Because CSN binds all cullins, it might be a global signal integrator that radiates its output onto various associated ubiquitin ligases to modulate their activity.

To further define the SCF-CSN interaction, we performed a comprehensive two-hybrid analysis of SCF and CSN subunits (*6*). Whereas SKP1 did not interact with any CSN subunit, CUL1 interacted strongly with CSN2 and weakly with CSN6. Conversely, HRT1 interacted weakly with the NH_2-terminal domain of CSN1 and moderately with CSN6. Thus, COP9 signalosome interacts with the conserved catalytic core of SCF primarily via the CSN2 and CSN6 subunits.

To probe the functional relation between CSN and SCF, we turned to *Schizosaccharomyces pombe*; *S. pombe* contains a high molecular weight CSN-like complex, and the fission yeast CSN1 homolog Caa1 (*16*) is required for proper S phase progression (*17*). First, we sought to confirm that the fission yeast CUL1 homolog Pcu1 interacts with *S. pombe* CSN. Strains whose *pcu1*$^+$ and *caa1*$^+$ chromosomal loci were modified to encode proteins tagged with multimerized Myc epitopes were transformed with a plasmid

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