Correspondence

Evidence for a physical association of the COP9 signalosome, the proteasome, and specific SCF E3 ligases *in vivo*

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The COP9 signalosome (CSN) was initially discovered during the genetic analysis of light control of *Arabidopsis* seedling development and later shown to be a protein complex that is conserved among diverged organisms [1]. The accumulation of ubiquitin conjugated proteins and the failure to degrade specific proteins, such as HY5 and IAA6, in *csn* mutants [2–4] supports a role for CSN in the ubiquitin–proteasome pathway. In addition, CSN was found to be associated with SCF ubiquitin E3 ligases [2,5–7] and to promote the de-conjugation of NEDD8/RUB from the cullins [8]. Interestingly, both the lid subcomplex of the 26S proteasome and CSN contain eight subunits that display a remarkable subunit-to-subunit similarity [1]. Here we report evidence to support the existence of dynamic conglomerates consisting of the 26S proteasome, CSN and specific SCF E3 ligases. These substrate or E3-specific conglomerates could perform the entire substrate ubiquitination and proteasome-mediated degradation in a single location within the cell, thus conferring synergy to the specificity and the efficiency of the process.

Gel filtration size-fractionation was first used to examine the molecular association of CSN with known ubiquitin–proteasome pathway components. As shown in Figure 1, a small fraction of CSN (about 5–10%) co-fractionated with the 26S proteasome and SCF E3 ligase components [6] in higher molecular mass fractions, when protein phosphatase inhibitors and ATP were included in the buffer. It is possible that this interaction is labile and dynamic, therefore, our fractionation assay can, at any given moment, only detect a portion of CSN that is engaged with the proteasome and the E3 ligases. Consistent with this notion, a 30-minute *in vivo* cross-linking treatment of the tissue with 1% formaldehyde [9] before homogenization and fractionation leads to a significant increase (up to 40%) in the portion of CSN in higher molecular mass fractions (see supplementary Figure 1). This high molecular mass CSN co-fractionates with all tested subunits of the 26S proteasome and with the core subunits of SCF type ubiquitin E3 ligases. Thus, it is plausible that this cross-linking treatment probably helps to fix the transient and labile CSN conglomerates.

To determine whether the co-fractionating proteasome, CSN and SCF E3 ligases are physically associated, proteins were collected from the high molecular mass gel filtration fractions of extract without cross-linking treatment (fractions 2 to 4, Figure 1). These proteins were subjected to immunoprecipitation with antibodies against representative subunits of CSN, the 26S proteasome, and SCF E3 ligases. Antibodies against CSN6 co-immunoprecipitated all the tested subunits of the 26S proteasome and the SCF ubiquitin E3 ligases in addition to the CSN, whereas the pre-immune serum did not (Supplementary Figure 2). Antibodies against Rtp5, a base subunit of the 26S proteasome, also co-immunoprecipitated the subunits of CSN and SCF ubiquitin E3 ligases.

To confirm that multiple SCF E3 ligases can independently form large conglomerates with CSN and the proteasome, we immunoprecipitated tagged versions of three distinct *Arabidopsis* F-box proteins that are known to form SCF complexes, which functionally associate with CSN [4–6]. As shown in Figure 2, all tested subunits of SCF complexes, CSN and the proteasome can be co-immunoprecipitated with the F-box proteins. This confirms that multiple SCF E3 ligases are capable of forming conglomerates with CSN and the proteasome. Together with the co-fractionation

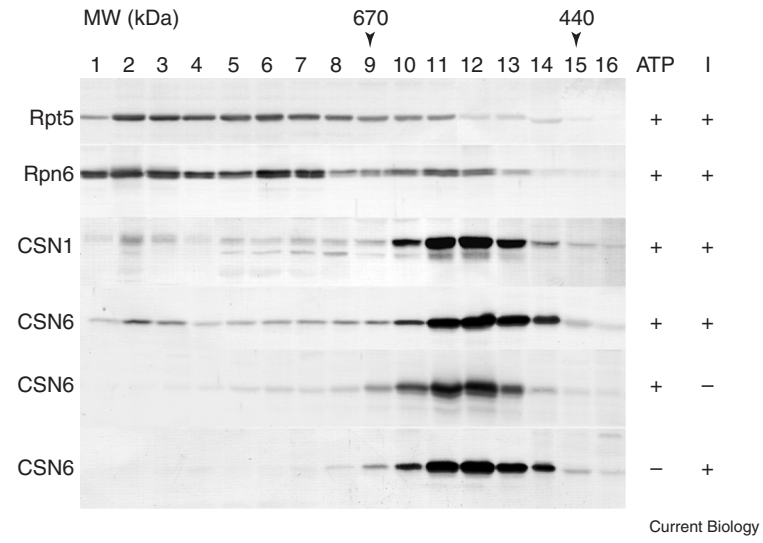

Figure 1. Co-fractionation of CSN with the 26S proteasome.
CSN and the 26S proteasome subunits co-fractionate in Superose 6 H/R gel filtration column with PBSM buffer (see web supplemental materials). The total protein was extracted from the top 1-millimeter layer of cauliflower head using the same buffer plus protease inhibitors. The proteins were detected with the antibodies indicated on a western blot following separation by SDS-PAGE. The – or + signs represent the presence or absence of 4 mM ATP or phosphatase inhibitors (I; 3mM NaF, 3mM $NaVO_3$, 50 mM glycerol phosphate, and 2mM sigma 104) in the buffer. Rpt5 and Rpn6 represent base and lid subunits of proteasome, and CSN1 and CSN6 represent CSN subunits.

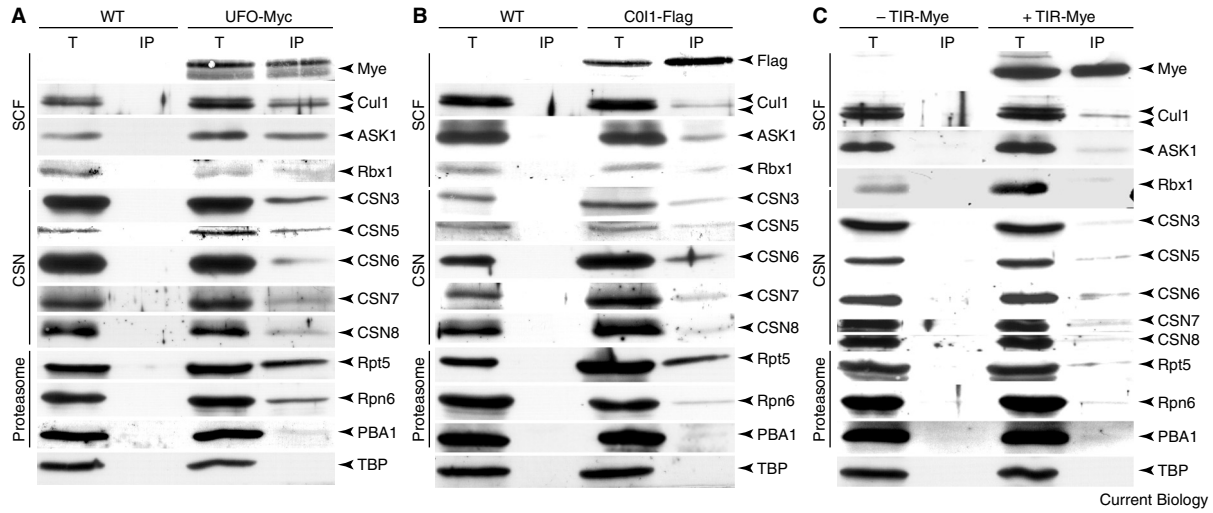

Figure 2. Multiple SCF E3 ligases are physically associated with CSN and proteasome *in vivo*.
Myc-tagged UFO protein (UFO-Myc) (A), Flag-tagged COI1 protein (COI1-Flag) (B), and Myc-tagged TIR1 (TIR1-Myc) (C) immunoprecipitate SCF subunits, CSN, and proteasome. The immunoprecipitates were run on SDS-PAGE, blotted, and subjected to hybridization by antibodies against different SCF, CSN, and proteasome subunits as shown. Anti-TBP (TATA-binding protein) antibody was used as an immunoprecipitation control. T indicates total protein control and IP indicates the immunoprecipitated protein with corresponding monoclonal antibodies against Flag or Myc tags. Flower extracts from wild-type (WT) and transgenic *Arabidopsis* were used for experiments in (A) and (B) and whole transgenic *Arabidopsis* seedling extracts from uninduced (-TIR1-Myc) and induced (+TIR1-Myc) samples were used for (C).

of all these three complexes, our co-immunoprecipitation studies support a physical association of the 26S proteasome, CSN and specific SCF E3 ligases in large conglomerates.

Based on this finding, we propose that CSN, the proteasome and individual SCF ubiquitin E3 ligases, possibly including an E2 enzyme, can form protein degradation conglomerates. These conglomerates would confer substrate specificity and ensure the completion of the entire ubiquitination and degradation process of a given substrate without releasing any intermediate, once the substrate is recruited into this conglomerate. At this point, we can not differentiate between a simple physical association of the complexes involved and a scenario in which at least a fraction of the CSN can act as an alternative lid subcomplex of a new type of 26S proteasome. The latter would suggest the presence of two distinct types of proteasome with alternative lids, which might provide a basis for the observed substrate specificity of CSN-mediated protein degradation [3]. However, future study will be needed to differentiate between specific interaction modes of the E3 ligase–CSN–proteasome conglomerates.

Supplemental Data

Supplemental data for this article are available at http://images.cellpress.com/supmat/supmatin.htm.

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