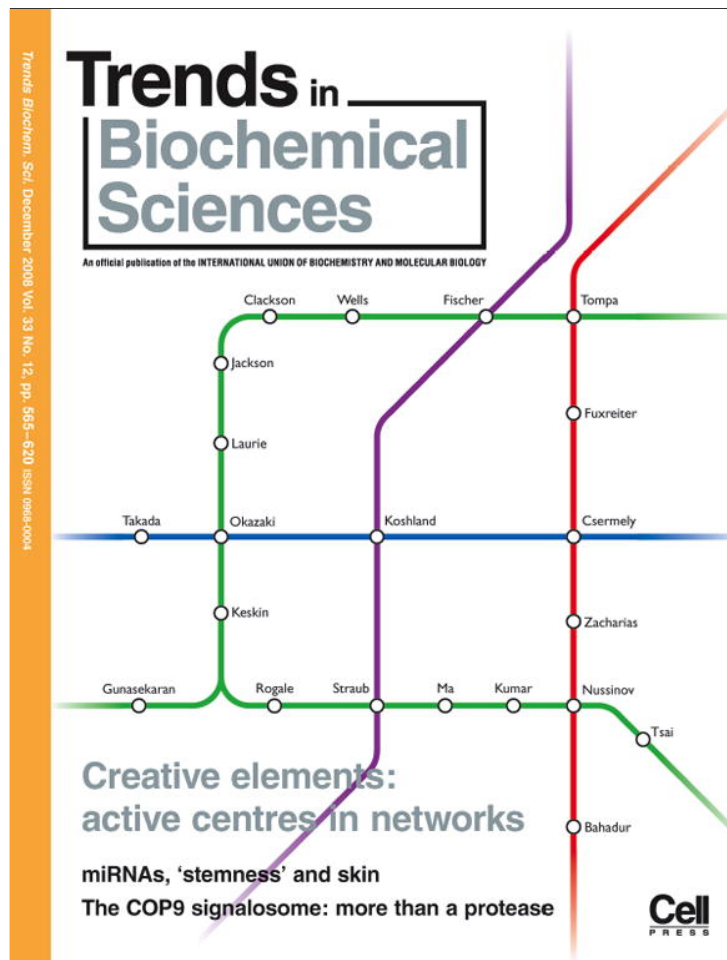


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# The COP9 signalosome: more than a protease

Ning Wei<sup>1\*</sup>, Giovanna Serino<sup>2\*</sup> and Xing-Wang Deng<sup>1</sup>

<sup>1</sup> Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06511, USA

<sup>2</sup> Dipartimento di Genetica e Biologia Molecolare, Università La Sapienza, Piazzale Aldo Moro 5, 00185 Roma, Italy

The COP9 signalosome (CSN) is a conserved protein complex that functions in the ubiquitin–proteasome pathway. After two decades of research, we now know that the CSN is a multi-subunit protease that regulates the activity of cullin–RING ligase (CRL) families of ubiquitin E3 complexes. The CSN is rapidly emerging as a key player in the DNA-damage response, cell-cycle control and gene expression. The independent functions of CSN5 (also known as JAB1) add to the complexity of the CSN machinery. Here, we provide an updated view of the structure, functions and regulation of this protein complex.

## The COP9 signalosome: a brief historical remark

The COP9 signalosome (CSN) is a conserved protein complex, typically consisting of eight subunits designated CSN1–CSN8 (Table 1). The CSN was originally identified in plants, based on a group of mutants exhibiting constitutive photomorphogenesis, pigmented seed-coats and premature death [1]. Later, inactivation of CSN genes was shown to cause profound detriments in many other organisms (Table 2). The discovery that the CSN contains Nedd8 (see Glossary) isopeptidase activity [2] immediately linked this complex to the cullin–RING family of ubiquitin E3 ligases (CRLs) and also shed light on the regulation of CRLs. In the past five years, deneddylation has been validated as a hallmark activity of the CSN. Additionally, substantial progress has been made in defining the specific role of the CSN in various aspects of cellular and physiological processes, using tools such as conditional knockout, subunit-specific knockdowns and new genetic model organisms. These studies also raise new questions about the function(s) and mechanisms of the CSN. Here, we provide an update on CSN structural analyses and discuss the latest research progress on cellular functions of the CSN complex and on CSN5.

## Structural features and architecture

An archetypical CSN comprises eight subunits: six subunits with a PCI (also known as PINT) domain and two subunits with an MPN domain [3] (Table 1). Exceptions to this otherwise general paradigm can be found in yeast. The fission yeast *Schizosaccharomyces pombe* possesses a smaller version of the CSN, consisting only of six subunits rather than eight [4], whereas a more distant CSN-like complex has been described in the budding yeast *Sacchar-*

*omyces cerevisiae* [3]. The PCI and MPN domains are also found in components of the ‘lid’ subcomplex of the 26S proteasome and in the eukaryotic translation initiation complex eIF3. The ‘lid’ and the CSN both have a 6 PCI:2 MPN stoichiometry and their corresponding subunits

## Glossary

**9–1–1 complex:** Rad9–Rad1–Hus1 complex. A heterotrimeric protein complex that forms a ‘doughnut-shaped’ sliding clamp around DNA after damage and/or replication fork stalling.

**CAND1:** cullin-associated and neddylation-dissociated 1. This protein specifically associates with deneddylated cullins to sequester them in an unassembled and inactive state. It was previously named TBP-interacting protein 120A (TIP120A).

**CRLs:** cullin–RING ligases, a superfamily of ubiquitin ligases. It typically consists of a cullin family member as a molecular scaffold and of a small RING protein, RBX1 (also named ROC1 or HRT1), which forms the core complex. An active CRL is formed when the cullin–RBX1 core is assembled with a substrate-binding module that recruits substrates. There are at least six different cullin proteins in humans, each assembling with a unique substrate-binding module.

**DEN1 (or NEDP1):** deneddylase 1 or Nedd8-specific protease 1. A dual function cysteinyl protease capable of processing the Nedd8 C terminus and of deconjugating Nedd8 chains on CUL1.

**eIF3:** eukaryotic translation initiation factor 3. It is the largest and most complex translation initiation factor, composed of 13 subunits in human. It stimulates assembly of the eIF2–GTP–Met-tRNA ternary complex and the formation of the 43S pre-initiation complex. It also facilitates mRNA recruitment to the 43S complex and mRNA scanning for AUG recognition.

**GGR:** global genome repair. A nucleotide-excision repair pathway that scans the entire genome for helix-distorting DNA damage.

**HEAT:** huntington, elongation factor 3, PR65/A, TOR. A tandemly repeated, 37–47 amino acid long module occurring in several cytoplasmic proteins. Arrays of HEAT repeats consist of 3–36 units forming a rod-like helical structure and seem to function as protein–protein interaction surfaces.

**JAMM:** JAB1/MPN domain metalloenzyme (or MPN+). It possesses a His-Xaa-His-Xaa<sub>10</sub>-Asp motif (in which Xaa represents any residue) accompanied by an upstream conserved Glu residue. The activity requires a zinc metal ion. The JAMM motif is found in the CSN5 subunit of the CSN, the RPN11 subunit of the proteasome and the C6.1A protein.

**MPN:** MPR1–PAD1–N-terminal domain. This domain is defined by sequence homology found in subunits of the proteasome, eIF3 and the CSN. It is usually located at the N-terminal half of a protein.

**Nedd8 (or Rub1):** Nedd8 (neural precursor cell-expressed developmentally downregulated-8) is used in metazoans, whereas RUB1 (related to ubiquitin 1) is used in yeast and plants. It is an ubiquitin-like protein that can be covalently conjugated to a cullin protein through a process termed neddylation. The enzymatic steps of neddylation are similar to ubiquitylation, but are catalyzed by Nedd8-specific set of E1, E2 and E3 enzymes. Recently, other neddylation targets, apart from cullins, have been reported.

**PCI (or PINT):** proteasome–COP9 signalosome–initiation factor 3 domain (or proteasome subunits, Int-6, Nip-1 and TRIP-15) domain. Like MPN, this domain is also defined by sequence homology from subunits of the proteasome, eIF3 and the CSN. It is usually located in the C-terminal region of a protein.

**SCF:** SKP1–cullin–F-box complex. It is a type of CRL ubiquitin ligase that contains a SKP1 adaptor protein, CUL1–RBX1 and an F-box-containing substrate receptor, which confers substrate specificity.

**TCR:** transcription coupled repair. A nucleotide-excision repair pathway that preferentially removes lesions from the coding strands of genes actively transcribed by RNA polymerase II.

**TPR:** tetratricopeptide repeat domain. A 34 amino acid sequence repeat, clusters of which fold into a helical structure and mediate protein–protein interactions.

Corresponding author: Deng, X.-W. (xingwang.deng@yale.edu)

\* These authors contributed equally to the article.

**Table 1. CSN composition based on mammalian CSN**

Subunit	Domain	Remarks	Refs
CSN1	PCI		[3]
CSN2	PCI	Two isoforms exist: the shorter form, known as Alien or Trip15, encompasses the N-terminal 300 amino acid residues	[26]
CSN3	PCI		[3]
CSN4	PCI		[3]
CSN5	JAMM or MNP+	Encoded by two highly homologous genes in <i>A. thaliana</i> : <i>CSN5a</i> and <i>CSN5b</i>	[20,77]
CSN6	MPN	Encoded by two highly homologous genes in <i>A. thaliana</i> : <i>CSN6a</i> and <i>CSN6b</i> . Not found in <i>S. pombe</i> CSN	[4,7,24]
CSN7a	PCI	Encoded by a single gene in most organisms. The <i>C. elegans</i> CSN7 is known as CIF-1 [1]. <i>S. pombe</i> CSN7b is an eIF3 subunit.	[6,7]
CSN7b	PCI		
CSN8	PCI	Not found in <i>C. elegans</i> or <i>S. pombe</i> CSN	[4,6,7]

share substantial sequence homology. In parallel to their structural similarities, both the 'lid' and the CSN exhibit metalloisopeptidase activities, which cleave ubiquitin conjugates and ubiquitin-like protein conjugates, respectively [2]. eIF3 has a more complex subunit composition, but a functional connection between the CSN and eIF3 has been uncovered in *Arabidopsis thaliana* [5]. Most interestingly, the CSN and eIF3 share a subunit in *Caenorhabditis elegans*, CIF-1 (CSN-eukaryotic initiation factor), which exhibits sequence homology to and functional characteristics of both CSN7 and eIF3m [6]. It is important to note that the previously named *S. pombe* Csn6 and Csn7b are, in fact, bona fide subunits of eIF3, rather than of the CSN [7].

Recent structural and bioinformatics analyses predict that the PCI and MPN domains might have additional roles. The PCI domain is now believed to consist of two subdomains: a TPR-like or HEAT domain, which is usually involved in protein–protein interactions and a 'winged-helix-like' domain [8,9]. This latter domain is usually found in DNA- and/or RNA-binding proteins, thus, pointing towards an interesting hypothesis that the CSN might

be able to bind nucleic acids [9]. Neither subdomain possesses catalytic activity. Because both subdomains are implicated in interactions with other PCI-containing proteins, the PCI domain probably functions as a scaffold for the complex and for other binding partners [3,8].

Although both CSN5 (also known as Jun activation domain-binding protein 1 [JAB1]) and CSN6 contain an MPN domain, only the CSN5 MPN domain contains an embedded JAMM (JAB1 MPN domain metalloenzyme) motif (also termed an MPN+ motif), which functions as the catalytic center for CSN isopeptidase activity (see later). The structural distinction between the two domains can be inferred from their respective crystal structures. Crystal structures have been obtained for the JAMM domain of an *Archaeoglobus fulgidus* JAMM-containing protein, AF2198 or AfJAMM [10,11] and for the MPN domains of human Mov34, a proteasome subunit and Prp8, a spliceosome component [12–14]. Although the JAMM motif can coordinate a Zn atom, which is a necessary co-factor for enzymatic activity, the MPN domain lacks metal-binding capability. The MPN domain might, therefore, represent a pseudoenzyme domain that has been converted into a protein–protein interaction platform. This hypothesis is strongly supported by experimental data [15–17]. In addition, the Prp8 MPN domain can bind ubiquitin with an affinity similar to many known ubiquitin-binding domains [12]. This finding raises the possibility that the CSN might bind ubiquitin or ubiquitin-like proteins.

### The CSN holocomplex and subcomplexes

Most CSN subunits (except for CSN5; see later) are more stable *in vivo* as part of the holocomplex compared with their monomeric form. Studies with null mutants from *Aspergillus nidulans*, plants and vertebrate animals indicate that the loss of one subunit leads to loss of the entire complex [16,18]. As a result, *A. thaliana* null mutants for each of the CSN subunits exhibit nearly identical seedling lethal phenotypes and each germline CSN gene knockout in mouse causes early embryonic lethality (Table 2). The identical phenotype of different CSN null mutants presumably reflects the loss of the

**Table 2. Summary of physiological phenotypes in CSN subunit null mutants**

Organism	CSN genes <sup>a</sup>	Phenotypes	Refs
<i>S. cerevisiae</i>	<i>Csn5</i> (or <i>Rri1</i> ), <i>Csn9</i> , <i>Rri2</i> (or <i>csn10</i> ), <i>Pci8</i> (or <i>Csn11</i> ), <i>Csi1</i>	Viable, no major growth defects. Mild phenotype in pheromone response	[3]
<i>S. pombe</i>	<i>Csn1</i> , <i>Csn2</i> <i>Csn3</i> , <i>Csn4</i> , <i>Csn5</i>	Viable. Slow S phase. Hypersensitivity to $\gamma$ and UV radiation Viable. No growth defects	[4,58] [19,78]
<i>Dictyostelium discoideum</i>	<i>Csn2</i> , <i>Csn5</i>	Lethal. Necessary for cell proliferation	[17]
<i>N. crassa</i>	<i>Csn2</i>	Viable. Retarded growth; reduced aerial hyphae and conidia. Defect in the circadian clock.	[56]
<i>A. nidulans</i>	<i>CsnA</i> (or <i>Csn1</i> ), <i>CsnB</i> (or <i>Csn2</i> ), <i>CsnD</i> (or <i>Csn4</i> ), <i>CsnE</i> (or <i>Csn5</i> ), <i>CsnG</i> (or <i>Csn7</i> ) <i>CsnD</i> (or <i>Csn4</i> ), <i>CsnE</i> (or <i>Csn5</i> )	Viable in the asexual part of life cycle. Defective sexual development. Blocked fruit body formation DNA-damage response phenotype	[16,57] [79]
<i>A. thaliana</i>	All null CSN genes	Seedling lethal. Altered light response	[3,18,24]
<i>Drosophila</i>	<i>Csn4</i> , <i>Csn5</i> , <i>Csn8</i> <i>Csn4</i> , <i>Csn8</i> <i>Csn5</i> , <i>Csn8</i>	Lethal at larval stage. Defective oogenesis, hypersensitive to DNA damage Molting defects Melanotic capsules	[21,80,81] [21,81] [21,81]
<i>Mus musculus</i>	<i>Csn2</i> , <i>Csn3</i> , <i>Csn8</i> <i>Csn5</i>	Early embryonic lethal Early embryonic lethal. Massive apoptosis	[3,53,54,82] [48]

<sup>a</sup>Genes defined as CSN homologues gene in *S. cerevisiae*: *Pci8*, proteasome-COP9 signalosome-eIF3 protein 8; *Csi1*, COP9 signalosome interacting protein 1.

functional complex. A notable exception has been reported in fission yeast, in which CSN1 and CSN2 protein levels seem stable when other CSN subunits are deleted [19].

In addition to the eight-subunit holocomplex, various small complexes that are formed by a subset of CSN subunits, or by CSN5 and other proteins, have been detected by native-gel electrophoresis or by gel filtration chromatography [19–23]. Small CSN subcomplexes are poorly defined because the number of subcomplexes and their compositions remain unclear. Given that the subcomplexes are present in substantially lower abundance compared with the holocomplex, and that they can be detected more easily in mutants that lack an intact holocomplex [19,24], it can be argued that their formation is a consequence of holocomplex destabilization. However, the CSN5-containing small complex can change its cellular abundance according to cell-cycle stages and in response to cell density and cellular transformation state [22,23]. Thus, at least some of the small complexes are biologically active.

Apart from CSN5, CSN2 also has multifaceted features. Two isoforms are expressed from the mammalian *CSN2* gene: the full length CSN2 subunit of 443 amino acids and a shorter protein called Alien that corresponds to the N-terminal 300 amino acid residues of CSN2 [25,26]. The Alien form of CSN2 is believed to function as a co-repressor for a subset of nuclear receptors and to influence nucleosome assembly [25]. However, it is unclear how the expression of CSN2 isoforms is regulated and whether Alien can associate with the CSN.

### The CSN as a cullin deneddylase

The best characterized biochemical activity that can be ascribed directly to the CSN is the isopeptidase activity that removes a Nedd8 (or Rub1) modification from the cullin subunit of CRL ubiquitin ligase complexes [2,27,28] (Box 1, Figure I). Nedd8 conjugation and removal, termed neddylation and deneddylation, respectively, represent an important mechanism by which CRL activity is regulated. For comprehensive reviews on this largest class of ubiquitin E3 ligases, see Refs [29,30].

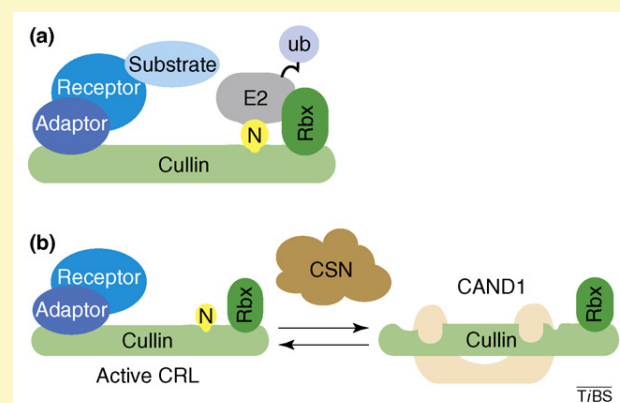
CSN-mediated cleavage of the Nedd8 moiety is catalyzed by the metalloisopeptidase activity centered within the CSN5 JAMM motif [2]. Point mutations that specifically inactivate the catalytic activity without disrupting complex assembly fail to rescue  $\Delta csn5$ -induced lethality in *Drosophila melanogaster* [2], cause seedling lethality in *A. thaliana* [24] and block fruit body formation in *A. nidulans* [16], thus, underlining the significance of JAMM-dependent activity to CSN functions. Moreover, the JAMM-defective point mutants can cause a dominant-negative phenotype when expressed in the presence of wild-type CSN5 [11,20]. Although the JAMM motif is contained within CSN5, it is important to note that cullin deneddylation activity is the property of an intact CSN complex rather than of CSN5 itself [2]. In fact, whereas CSN5 alone is inactive, the depletion or reduction in the level of each CSN subunit reported so far can elevate the cullin neddylation level owing to decreased deneddylation activity [3,24]. Neutralizing the CSN complex with an anti-CSN2 antibody is also sufficient to block deneddylation [31].

### Box 1. Regulation of CRLs by deneddylation and deubiquitylation

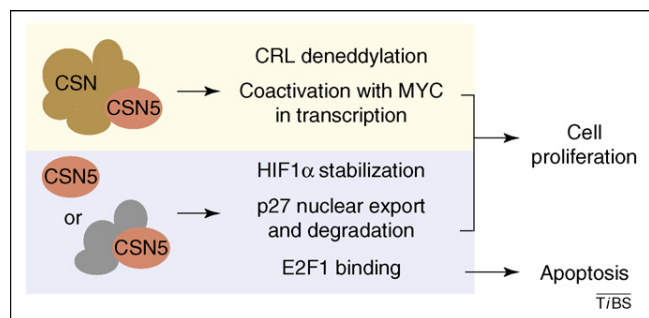
CRL complexes undergo an activation cycle that involves transient assembly and disassembly between the core complex, cullin–Rbx1 and the substrate-binding modules consisting of an adaptor and substrate receptors [29,30] (Figure Ia). This dynamic cycle makes the CRL core available to many different substrate-binding modules. In the case of SCF (or CRL1) complexes, the CUL1–Rbx1 core has the ability to recruit, via the Skp1 adaptor, ~70 F-box protein substrate receptors in humans, 300 in *C.elegans* and 700 in *A. thaliana*, each targeting a unique subset of cellular substrates [28,29]. Thus, the cycle enables the dissociation of 'old' ligases and the formation of new ligases to adapt to the changing needs of the cell. The CRL activation cycle is facilitated by a neddylation–deneddylation cycle. Neddylation is believed to activate CRLs, whereas deneddylation favors CRL disassembly and association of the core with CAND1 (Figure Ib). As a cullin deneddylase, the CSN has a crucial role, along with CAND1 and other components of the neddylation pathway, in maintaining dynamic cycles of CRLs [3,29] (Figure Ib).

CSN-mediated deneddylation also serves as a protective mechanism to prevent CRL components from self-destruction [33–36]. Neddylated CRLs are highly active and prone to auto-ubiquitylation of their own components if not deneddylated by the CSN in a timely fashion [24,29,30,87]. In the absence of the CSN, cullins are hyperneddylated and some of the CRL components, including certain cullins and a subset of F-box proteins, tend to be unstable [33–37] (Table 3). As a result, the turnover of the corresponding substrates is affected in CSN deficient cells (Table 3). Interestingly, some F-box proteins are more sensitive to the neddylation status than others; the reasons for these differences remain unknown.

The CSN also associates with a de-ubiquitylase (DUB) enzyme, Ubp12p in fission yeast or Usp15 in human, which contributes to the control of spurious CRL auto-ubiquitylation [78,83,88]. Consistent with this finding, a JAMM-domain defective CSN complex can convert polyubiquitylated Cul4A to monoubiquitylated Cul4A *in vitro*, probably via an associated DUB enzyme [69]. Evidence also indicates that CSN-associated Usp15 helps to maintain the stability of the CRL core subunit, Rbx1 [88] and to regulate the deubiquitylation of a CRL substrate, I $\kappa$ B- $\alpha$  [89]. Nonetheless, a JAMM-domain mutation, which does not affect the interaction with Ubp12p (or Usp15), still causes F-box protein destabilization [34], indicating that, in most instances, CSN-dependent cullin deneddylation represents the main activity that protects CRL subunits from degradation.



**Figure I.** The CRL family of ubiquitin ligases and its regulation by the CSN. (a) The CRL superfamily of ubiquitin ligases is characterized by an enzymatic core that contains a cullin family member (light green) and RBX1 (dark green), a RING protein. Specific substrates (light blue) are recruited to the core by a substrate-binding module consisting of an adaptor protein (or domains; blue) and various substrate receptors (turquoise). (b) Cullin neddylation enables the assembly of an active CRL, whereas deneddylation by CSN (light brown) facilitates the dissociation of the substrate-binding module and the association of CAND1 (beige) with the core complex. Color code: E2, gray; ubiquitin (Ub), sky blue; Nedd8 (N), yellow.



**Figure 1.** Different CSN5-containing complexes and their specific functions. The CSN holocomplex (light brown) exhibits CRL deneddylation activity and collaborates with MYC in transcriptional activation of ‘wound signature’ genes. By contrast, monomeric CSN5 (light red) or a CSN5-containing small complex (grey) are responsible for HIF1- $\alpha$  stabilization, p27 nuclear export and degradation and E2F1-mediated apoptosis. Combining CSN5 specific functions with that of the CSN holocomplex identify CSN5 as being most closely linked to cell proliferation and apoptosis pathways.

In addition to the CSN, deneddylase 1 (DEN1; also known as NEDP1) has been identified as a Nedd8 protease [32,33]. The roles of DEN1 and CSN seem to be quite different. DEN1 seems to function predominantly as a Nedd8 C-terminal hydrolase, whereas overwhelming biochemical and genetic evidence indicates that the CSN is the key factor that determines the deneddylation rate of cullins [16,27,28,32,33].

#### CSN5: a multifunctional subunit in multiple complexes

Among the eight CSN subunits, CSN5 is unique; not only does it harbor the catalytic center of the CSN isopeptidase activity, it can also stably exist independently of the CSN *in vivo* [3]. Reducing cellular CSN5 levels depletes monomeric CSN5 much more dramatically than the CSN holocomplex [22,23,34–36]. Similarly, transient CSN5 overexpression in cultured cells usually causes an increase in CSN5 monomers with little effect on CSN holocomplex levels [37]. Although early reports did not attempt to distinguish between these two forms of CSN5, several recent studies have explicitly addressed this issue [23,34,37–39]. As a result, it is now clear that CSN5 actively participates in important biological functions, both as part of the CSN holocomplex and independently of the CSN (Figure 1). This behavior of CSN5 raises the interesting possibility that the CSN could function, in part, as a ‘depot’ for CSN5 that, upon release, would acquire new functions.

CSN5, more than any other CSN subunit, can bind numerous important cellular regulators, as summarized in past reviews [3,40]. Depending on the specific target, CSN5 exerts different effects on their stabilities (Table 3). On the one hand, CSN5 stabilizes several of its binding partners, including hypoxia-inducible factor 1  $\alpha$  subunit (HIF1- $\alpha$ ), c-Jun and transformed mouse 3T3 cell double minute 2 (Mdm2) [36,41,42]. On the other hand, CSN5 promotes the degradation of other binding proteins including p27, p53, the 9–1–1 (Rad9–Rad1–Hus1) DNA repair complex, estrogen receptor  $\alpha$  (ER- $\alpha$ ), misfolded cystic fibrosis transmembrane conductance regulator (CFTR) and West Nile virus capsid protein (WNVCP) [37,43–47]. Interestingly, CSN5-induced degradation tends to be pre-

ceded by nuclear export of its binding targets (e.g. p27, p53 and 9–1–1 complex), often by a CRM1-dependent (leptomycin-B sensitive) pathway [44,45,47]. In these cases, blockage of the nuclear export can prevent CSN5-induced degradation of the targets. Notably, CSN5 or a CSN5-associated small complex, rather than the CSN holocomplex, drives p27 nuclear export and degradation [22,23]. Likewise, CSN5-dependent degradation of misfolded CFTR is independent of the JAMM-mediated CSN holocomplex isopeptidase activity [37]. Unfortunately, the precise molecular mechanism(s) underlying CSN5-mediated nuclear export and degradation have not been revealed.

Of note, two cellular functions stand out as being more closely linked to CSN5 than to any other CSN subunits: apoptosis and cell proliferation (Figure 1). CSN5 associates tightly with cellular apoptosis pathways. *Csn5* knockout mice [48,49] exhibit massive apoptosis that is more pronounced and severe than in mouse knockouts of *Csn2*, *Csn3* or *Csn8*. In *Csn5*-deleted thymocytes, apoptosis correlates with increased expression of the pro-apoptotic protein BCL2-associated X protein (Bax) and decreased expression of anti-apoptotic B-cell CLL lymphoma 2 (Bcl-2) family proteins, including Bcl-xL [49]. The lack of Bcl-xL is largely responsible for apoptosis because the phenotype can be efficiently rescued by forced Bcl-xL expression [49]. In addition, CSN5 specifically interacts with transcription factor E2F1 and enhances E2F1-dependent apoptosis by a mechanism that is independent of deneddylation activity [39].

A role for CSN5 in promoting cell proliferation was initially uncovered based on its interaction with p27, a cyclin-dependent kinase (CDK) inhibitor, in cultured cells [47]. Further research, including clinical-based and high-throughput analyses using disease samples and various tumor cell lines, has provided compelling support that CSN5 promotes proliferation. More so than for other CSN subunits, *CSN5* amplification is associated with malignant transformation and many types of human tumors (e.g. breast, pancreatic, prostate, ovarian, certain lung cancers, lymphoma, oral squamous cell carcinomas, hepatocellular carcinoma and melanomas) [38,40]. Of note, high CSN5 expression often correlates with reduced p27 levels and poor outcome in cancer cases. Consistent with its role as an activator of cell proliferation, cells with lower CSN5 levels proliferate poorly [23,35,48].

Mechanistically, CSN5 induces growth and oncogenic transformation through at least two different pathways. First, CSN5 functions as a co-factor in myelocytomatosis oncogene (MYC)-mediated transcriptional activation of the 512 genes belonging to the so-called ‘wound signature’ that promotes cell proliferation, invasion and angiogenesis [38]. These genes are believed to be reactivated in breast cancer metastasis. Importantly, the transcriptional co-activation of CSN5 specifically requires JAMM-dependent deneddylase activity and the entire CSN complex [50]. Further research showed that CSN5 facilitates SCF<sup>Skp2</sup>-mediated ubiquitylation of MYC through the Skp2 F-box protein [38]. In line with this finding, knockdown of CSN5 and CSN4 in cultured cells can significantly reduce the rate of cellular proliferation; this proliferation defect can be fully rescued by forced expression of Skp2 [35].

**Table 3. A summary of proteins whose stability is affected by the complete or partial loss of the CSN<sup>a,b</sup>**

Name	Species	Function	Targeting E3	CSN deletion or knockdown	Refs
<b>Proteins that are stabilized in CSN deleted or knockdown cells</b>					
Spd1p	<i>Sp</i>	Ribonucleotide reductase inhibitor	Cul4–DDB1	$\Delta csn1$ and $\Delta csn2$	[4,70]
Rum1p	<i>Sp</i>	Cdk inhibitor	SCF <sup>Pop1p</sup>	$\Delta csn5$	[83]
FRQ (Frequency)	<i>Nc</i>	Circadian clock regulator	SCF <sup>FWD-1</sup>	<i>csn2</i> mutant	[56]
PSIAA6	<i>At</i>	Repressor of auxin-induced gene (from pea)	N/A	<i>csn5</i> hypomorphic mutants	[28]
HY5	<i>At</i>	Transcription factor	COP1	<i>csn4</i> null mutant	[18]
eIF3e (also called Int6)	<i>At</i>	eukaryotic translation initiation factor 3 (eIF3) subunit	N/A	<i>csn1</i> and <i>csn7</i> mutants	[5]
MEL-1 (also called katanin)	<i>Ce</i>	Microtubule-severing protein, required for meiosis	Cul3–MEL26	<i>csn1</i> and <i>csn3</i> RNAi	[6]
CDT1	<i>Dm</i>	Replication licensing factor	Cul4–DDB1 <sup>CDT2</sup> , SCF <sup>Skp2</sup>	<i>csn1</i> and <i>csn5</i> RNAi cells, <i>csn5</i> knockout double-positive thymocytes	[71]
Cyclin E	<i>Dm, Mm, Hs</i>	G1 cyclin	SCF <sup>Fbw7</sup>	<i>csn2</i> and <i>csn5</i> knockout embryos, <i>csn5</i> knockdown cells.	[34,48,66,80]
p53	<i>Mm, Hs</i>	Tumor suppressor, transcription factor	Mdm2, COP1, Pirh2, ARF–BP1	<i>csn2</i> and <i>csn5</i> knockout embryos, <i>csn5</i> knockout thymocytes	[48,49,53]
p27 <sup>kip1</sup>	<i>Mm, Hs</i>	Cyclin-dependent kinase (CDK) inhibitor	SCF <sup>Skp2</sup> , Cul4a–DDB1	<i>csn5</i> knockout embryos and MEFs, <i>csn5</i> knockdown cells, <i>csn1</i> knockdown cells	[34,35,38,48,51]
$\beta$ -catenin	<i>Hs</i>	Multifunctional cell growth regulator	SCF <sup>TRCP</sup>	<i>csn5</i> knockout thymocytes	[49]
MYC	<i>Hs</i>	Transcription factor	SCF <sup>Skp2</sup> , SCF <sup>Fbw7</sup>	<i>csn5</i> knockdown cells	[38]
9–1–1 complex	<i>Hs</i>	DNA-damage sensor and mediator	N/A	<i>csn5</i> knockdown cells	[44]
CFTR	<i>Hs</i>	(Misfolded) cystic fibrosis transmembrane conductance regulator	N/A	<i>csn5</i> knockdown cells	[37]
Smad7	<i>Hs</i>	Regulator of TGF- $\beta$ signaling	N/A	<i>csn5</i> knockdown cells	[84]
Topoisomerase II	<i>Hs</i>	Modulate DNA topological states	N/A	<i>csn5</i> knockdown cells, under glucose starvation	[85]
<b>Proteins that are destabilized in CSN deleted or knockdown cells</b>					
Pop1p	<i>Sp</i>	F-box WD-repeat substrate receptor for SCF <sup>Pop1p</sup>	SCF <sup>Pop1p</sup> auto-ubiquitylation	$\Delta csn5$	[83]
Btb3p	<i>Sp</i>	BTB–POZ substrate adaptor-receptor in Cul3–Btb3p	Cul3–Btb3p auto-ubiquitylation	$\Delta csn5$	[83]
FWD-1	<i>Nc</i>	F-box protein for SCF <sup>FWD-1</sup>	SCF <sup>FWD-1</sup> auto-ubiquitylation	<i>csn2</i> mutant	[56]
GLH-1	<i>Ce</i>	Germline RNA helicase 1	N/A	<i>csn5</i> RNAi cells	[86]
MEL26	<i>Ce</i>	BTB–POZ protein in Cul3–MEL26	Cul3–MEL26 auto-ubiquitylation	<i>csn1</i> RNAi	[6]
Rbf1, Rbf2	<i>Dm</i>	Homologues of the human Retinoblastoma repressor protein	N/A	<i>csn4</i> and <i>csn5</i> mutants	[66]
Cyclin F (also called Fbx1)	<i>Hs</i>	Degraded at G2–M transition; contains an F-box domain	N/A	<i>csn5</i> knockdown cells	[34]
Skp2	<i>Hs</i>	F-box protein for SCF <sup>Skp2</sup>	SCF <sup>Skp2</sup> auto-ubiquitylation	<i>csn5</i> knockdown cells	[34,35,38]
Fbw7 (also called hCdc4)	<i>Hs</i>	F-box protein for SCF <sup>Fbw7</sup>	SCF <sup>Fbw7</sup> auto-ubiquitylation	<i>csn5</i> knockdown cells	[34]
c-Jun	<i>Hs</i>	AP-1 transcription activator	Itchy, SCF <sup>Fbw7</sup> , Cul4A–DDB1 <sup>(DET1-COP1)</sup>	<i>csn1</i> and <i>csn5</i> knockdown cells	[36]
HIF1- $\alpha$	<i>Hs</i>	Hypoxia inducible factor, transcription factor	CRL2 <sup>VHL</sup>	<i>csn5</i> knockdown cells	[41]

<sup>a</sup>Abbreviations for experimental organism: *At*, Arabidopsis thaliana; *Sp*, Schizosaccharomyces pombe; *Nc*, Neurospora crassa; *Ce*, Caenorhabditis elegans.; *Dm*, Drosophila melanogaster; *Hs*, Homo sapiens; *Mm*, Mus musculus.

<sup>b</sup>Other abbreviations: BTB, broad complex–tramtrack–bric-a-brac domain protein Btb3; Fbw7, F-box and WD repeat domain-containing 7; FWD-1, F-box WD40-repeat-containing 1; HY5, long hypocotyl 5; Int6, integration site 6; MEL-1, meiosis defective 1; MEL26, maternal effect lethal 26; Pop1p, F-box/WD repeat protein Pop1; PSIAA6, Pisum sativum indole-3-acetic acid 6; POZ, pox virus and zinc finger; Skp2, S-phase kinase-associated 2; Smad7, SMAD family member 7; VHL, Von-Hippel-Lindau protein.

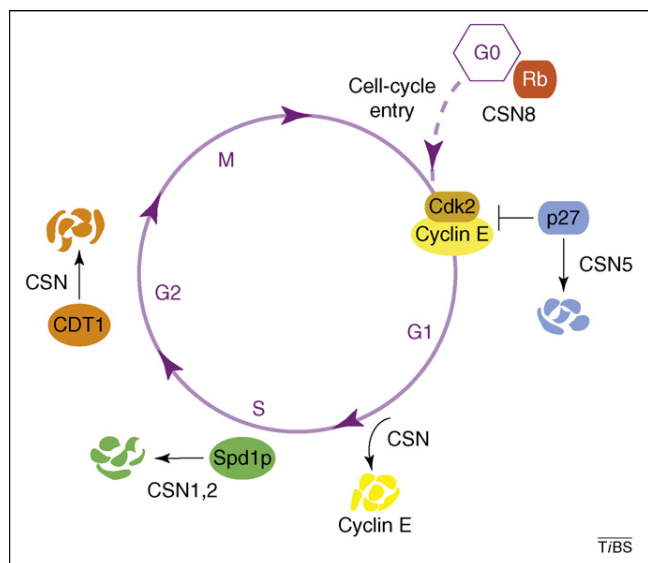
Second, in a manner independent of the CSN holocomplex, CSN5 (or a CSN5-containing small complex) promotes cell proliferation by inducing p27 degradation [22,23] and HIF1- $\alpha$  stabilization [41]. Because p27 can also be ubiquitylated by SCF<sup>Skp2</sup> and cullin 4 (CUL4)–DDB1 (damage-specific DNA-binding protein 1) [51], which are regulated by CSN, it is possible that p27 turnover is affected by CSN5 and the CSN holocomplex. In addition, CSN5 might promote cancer progression through p53 downregulation, in part, by modulating its phosphorylation state [42,52].

### The CSN cellular functions

The CSN is involved in diverse cellular processes. Here, we focus on the aspects in which substantial progress has been made in advancing an understanding of the complex.

#### The CSN and the cell cycle

The CSN is crucially involved in cell-cycle progression and cell-cycle entry (Figure 2). Defects in cell proliferation are, arguably, the most documented phenotype of *csn* mutants from various organisms (Table 2). For example, the inner cell mass of blastocysts from the mouse *Csn2*, *Csn5* and



**Figure 2.** Multifaceted roles of the CSN in cell-cycle regulation. The diagram illustrates the effects of the CSN on specific cell-cycle regulators. CSN8 is required for cell-cycle entry from the G0 quiescent state by its role in gene expression and in the regulation of Rb proteins (red). CSN5 promotes p27 (light blue) and cyclin E (yellow) degradation and facilitates G1-phase progression. In fission yeast, CSN1 and CSN2 are required to degrade the S-phase inhibitory protein Spd1p (green). CSN1 and CSN5 are required for the degradation of the replication licensing factor CDT1 (orange oval) in S, G2 and M phase.

*Csn8* knockouts all fail to undergo sustained cell divisions in outgrowth assays [48,53,54]. Yet, several in-depth cell-cycle analyses showed that, although the CSN is an important cell-cycle regulator, it is not absolutely required for cell proliferation in continuously cycling cells. *Csn8*-deleted mouse embryonic fibroblasts (MEFs) and pre-activated T cells can undergo multiple cell-division cycles and proliferate [54]. Cell divisions also occur in *A. thaliana csn4* and *csn5* mutants, although with an abnormal dynamics [55]. Similarly, fission yeast and *Neurospora crassa* can proliferate, albeit sluggishly, in the absence of CSN1 and CSN2 [56]. Moreover, *A. nidulans* can complete the asexual life cycle in the absence of the CSN [57]. These findings indicate that CSN is not an essential housekeeping factor for general cell-cycle progression.

Nonetheless, the CSN obviously has important roles in cell-cycle regulation, because *csn* mutants display abnormal dynamics of cell division cycles. In fission yeast, *csn1* and *csn2* deletion mutants exhibit a delayed S-phase progression [58]; *Csn5* knockout mouse thymocytes and, to some extent, *Csn8* knockout peripheral T cells and MEFs exhibit a greater G2 population, indicating a delay in progressing from S phase to G2 and M phase [49,54]. Similarly, *A. thaliana csn4* and *csn5* mutants undergo a noticeable G2 arrest [55]. Collectively, these reports indicate that a conserved function(s) of the CSN is involved in facilitating the S–G2–M-phase progression.

The role of the CSN in the G1 phase is more controversial. In *Drosophila*, most CSN genes, except for CSN8, were identified as positive G1 regulators in a high throughput RNAi screen [59]. Indeed, the CSN5 small complex probably functions to promote G1 progression, because *Csn5*<sup>+/-</sup> MEFs, which contain a reduced level of the Csn5 small complex, enter into the S phase more slowly

than *Csn5*<sup>+/+</sup> cells after the release from serum starvation [48]. This phenotype was accompanied by elevated p27 levels and reduced CDK2- and CDK4-associated kinase activities [48]. However, there is no convincing evidence in mammalian cells indicating that the CSN holocomplex positively regulates G1, because complete knockout of *Csn8* or *Csn5* in mouse T lymphocytes or MEFs does not cause G1 arrest [49,54]. On the contrary, microinjection of the purified active CSN into synchronized mammalian cells can inhibit the G1–S-phase progression [31]. Moreover, CSN3 knockdown in human cells accelerates cell proliferation, indicating that CSN3 inhibits cell proliferation; however, in this case, no cell-cycle phase information was available [60]. Further experiments on how the CSN influences the G1 phase are required to clarify this issue.

In addition to its function in continuously cycling cells, new light has been shed on the crucial role of the CSN in cell-cycle entry from quiescence (G0). Although *Csn8*<sup>-/-</sup> MEFs can divide under normal growth conditions and can undergo quiescence after serum starvation, they are unable to re-enter the cell cycle upon serum re-addition [54]. Similarly, resting *Csn8*<sup>-/-</sup> T cells in peripheral lymphoid organs fail to initiate cell proliferation in response to antigenic or mitogenic stimulation [54]. Upon exiting the cell cycle, many growth-related genes are shut down. These genes must be activated for cells to re-enter the cell cycle. However, the *Csn8*<sup>-/-</sup> T cells are unable to induce the robust expression of the genes necessary for cell-cycle entry, including many G1 cyclins, CDKs and E2F1. Instead, *Cdkn1a*, which encodes the CDK inhibitor p21<sup>cip/waf</sup> is highly induced in the mutant. As a result, retinoblastoma (Rb) proteins cannot be properly phosphorylated and the gene activation program cannot be executed. Thus, the necessity of the CSN in cell-cycle entry is, in fact, a manifestation of its function in gene expression.

#### The CSN and gene expression

Starting with the isolation of the first *csn* mutant, *A. thaliana csn8* (also called *cop9*), aberrant expression of light responsive genes has been noticed [1]. Following the characterization of the CSN across different organisms in various developmental scenarios, it seems that abnormal gene expression is a common phenotype in *csn* mutants. For example, resting peripheral T cells express many growth-related genes at a very low background level. As part of the T-cell activation program, these genes are strongly induced upon antigen stimulation. The *Csn8*-knockout T cells display an aberrant gene-expression profile in two aspects [54]. First, the background expression of a subset of cell-cycle genes including *E2f1*, *Ccnd2* and *Ccen1*, which encode E2F1, cyclin D2 and cyclin E, respectively, are elevated in the absence of stimulation. Second, the same genes cannot be highly activated after induction. Consequently, these genes display a signal-independent constitutive expression pattern reminiscent of many light-induced genes in *A. thaliana csn* mutants, for example, constitutive expression of ribulose biphosphate carboxylase small subunit (*rbcs*) in the dark and lack of induction of chlorophyll a and/or b-binding protein 1 (*CAB1*) in the light [3,18]. Other examples of gene-expression defects

include decreased mRNA expression of *IκB-α* in *Csn5*-deleted thymocytes [49] and decreased F-box protein 4 (*Fbx4*) expression in *Csn5* knockdown cells [34]. *Drosophila csn* mutants also exhibit altered temporal gene expression patterns [61].

As discussed earlier, the CSN can function in conjunction with MYC to modulate the transcription of many of MYC target genes [50]. The finding that *Ccnd2* and *E2f1*, which are both MYC target genes, are de-regulated in *Csn8* knockout T cells [54] provides further genetic evidence in support of this notion. Previous work showed that co-transcriptional ubiquitylation of MYC by SCF<sup>skp2</sup> enhances MYC transcriptional activity [62,63]. More recently, the *A. thaliana* unusual floral organs (UFO) F-box component of SCF<sup>UFO</sup> was shown to act as a co-activator with transcription factor Leafy (LFY) in the activation of floral identity genes [64]. As a regulator of CRL E3s, it is logical to deduce that the CSN regulates gene expression by modulating the ubiquitylation of the transcription factor substrates. Indeed, the functions of both mammalian SCF<sup>skp2</sup> and plant SCF<sup>UFO</sup> require CSN [34,35,38,65]. It has been postulated that the coordinate actions of MYC, the CSN and SCF<sup>skp2</sup> generate a continuous supply of a short-lived ubiquitylated MYC protein that is highly active in transactivation [38].

Recently, the CSN was found to be recruited to its target gene promoters. Chromatin immunoprecipitation (ChIP) experiments show that CSN4 co-occupies the *Drosophila PCNA* gene promoter in association with Rbf1 and Rbf2, the fly homologs of the Rb [66]. Likewise, CSN1 can be recruited to the promoter regions of the *Ccnd2*, *Cdk4* and *Cdkn1a* genes in mouse T lymphocytes [54]; CSN2 and/or Alien can be recruited to the *E2f1* promoter in U2OS osteosarcoma cells [67].

Furthermore, strong evidence indicates that the CSN is present in close vicinity to transcription complexes. *Drosophila* Rbf2 can pull-down the entire CSN along with many components of histone acetyltransferase (HAT) complexes in stoichiometric amounts [66]. In agreement with this finding, CSN1 interacts with U2 small nuclear ribonucleoprotein-associated protein 130 (SAP130), a component of a mammalian transcription complex named STAGA (SPT3-TAF9-GCN5 acetyltransferase complex) or TFTC (TBP-free TAF complex) [68]. SAP130 preferentially associates with neddylated and, therefore, active CRLs in a manner that is regulated by CAND1 and CSN1 [68]. In addition, the CSN2 Alien isoform binds the nucleosome assembly protein NAP1 and functions as a co-repressor, in part, by modulating nucleosome formation [25]. Together, these reports point to a tantalizing possibility that the CSN might have direct roles in gene expression regulation.

#### The CSN and DNA repair

The role of the CSN in DNA repair and DNA-damage responses has been reinforced by several recent studies (Table 2). The CSN often participates in DNA repair pathways through the CUL4–DDB1 family of CRLs, which are key players in DNA repair and genome stability maintenance [69,70]. The first report connecting the CSN to DNA repair came from studies on fission yeast *csn1* and *csn2*

mutants. These mutants were hypersensitive to DNA-damaging radiation and exhibited a delay in S-phase progression [58]. This phenotype was later attributed to a defect in Spd1p degradation [4]. Spd1p anchors the small subunit of the ribonucleotide reductase (RNR) enzyme to the nucleus, thereby maintaining it in an inactive state when the cellular demand for deoxynucleotide synthesis is low. In S phase or in response to DNA damage, Spd1p is degraded to enable the formation of active RNR enzymes in the cytosol. Spd1p degradation requires the Cul4–DDB1 ubiquitin ligase and CSN1 and CSN2, which together define a DNA-damage-response pathway that is important for genome integrity in fission yeast [4,70].

Another target protein that links the CSN to DNA repair is CDT1, an important DNA replication licensing factor and a ubiquitylation substrate of SCF<sup>skp2</sup> and/or CUL4–DDB1<sup>CDT2</sup>. The CSN is required in CUL4–DDB1<sup>CDT2</sup>-mediated CDT1 ubiquitylation and degradation during S–G2 phase and after DNA damage, thus, uncovering a novel CSN-dependent checkpoint [71]. In addition, the CSN is required for global genome repair (GGR) and TCR [69].

#### How is the CSN regulated?

As a potent protease that can act on all neddylated cullins, cellular CSN activity must be tightly regulated. The CSN can selectively deneddylate specific CRLs through differential protein interactions. For example, UV irradiation can trigger the rapid release of the CSN from the Cul4–DDB1<sup>DDB2</sup> complex, leading to its transient hyperneddylation [69]. Simultaneously, UV irradiation induces the association of the CSN with the chromatin-bound Cul4–DDB1<sup>CSA</sup> complex which, interestingly, is recruited to the hyperphosphorylated form (IIo) of the RNA polymerase II large subunit [69]. The molecular basis for this differential association remains to be elucidated. In addition, evidence indicates that substrate loading onto an assembled CRL might inhibit CSN deneddylation activity, thereby triggering transient CRL activation and substrate ubiquitylation [72]. Again, it is unclear whether substrate loading triggers a transient release of the CSN from the CRL or impedes CSN enzymatic activity.

Presumably, CSN functions could be regulated by differential gene expression or post-translational modifications. Because CSN subunits can be phosphorylated [52], this modification could potentially affect CSN activity and/or the dynamic equilibrium between the CSN holocomplex and small complexes. In addition, CSN6 can be cleaved by caspases during apoptosis, although it is unclear how CSN6 processing might affect CSN functions [73,74].

#### Concluding remarks

The progress in delineating the specific roles of the CSN during various cellular processes also raises outstanding questions concerning CSN functions and mechanisms. Recent CSN knockout studies show that, as expected, a CSN deficiency causes cullin hyperneddylation. Perplexingly, several CRL substrates (e.g. p27, Pcd4 and IκB-α) can still undergo signal-dependent degradation normally despite the loss of the CSN and its deneddylation activity [49,68,75]. The disagreement between CRL neddylation

and the outcome of substrate turnover indicates that a gap exists in our current model on the function of neddylation and/or deneddylation and, thus, the functional implication of CSN-mediated deneddylation. In addition, a deletion of the CSN1 N-terminal domain can cause seedling lethality in *A. thaliana*, even though this mutant retains deneddylation activity [76]. This observation raises questions concerning whether the CSN has important functions aside from its isopeptidase activity (or additional functions after Nedd8 cleavage). Elucidation of these questions will undoubtedly advance an understanding of the functions and the mechanisms of the CSN.

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