

Supplementary Data

Subunit folds

The six PCI proteins, CSN1, CSN2, CSN3, CSN4, CSN7 and CSN8, share a common domain composition: an N-terminal array of tandem α -helical tetratricopeptide/-like repeats, a ~ 34 residue motif, followed by a PCI domain, which encompasses a WH subdomain, a linker, and one or two α -helices at the C-terminus (**Fig. 1d**). The helical repeat domains of the PCI proteins all adopt right-handed solenoidal folds, but their appearance varies substantially due to differences in their length, curvature and the loops that decorate the helical repeats (**Extended Data Fig. 3a-f**). The overall fold of CSN1 contrasts most markedly to the other PCI proteins. Unlike CSN2-4 and CSN7-8, which contain twisted linear arrays of helical repeats (**Extended Data Fig. 3b-f, j**), CSN1 has two repeat domains separated by a loop that form an antiparallel stack. This fold, which was first characterised in *A. thaliana* CSN1¹, gives CSN1 a fan-like appearance that contrasts to the elongated suprahelical folds assumed by the other PCI proteins (**Fig. 1a and Extended Data Fig. 3a, i**).

Arrangement of the PCI ring

The PCI ring is formed by the oligomerization of the WH subdomains in each of the PCI proteins. The WH interface has two recurrent features: *i*) a β -sheet-forming interaction between PCI units (**Extended Data Fig. 5a**) that involve two residues in opposing strands, within hydrogen-bonding distance, where one strand straddles a bulged residue in the neighbouring WH subdomain (**Extended Data Fig. 5b, c**); and *ii*) the side chain of a conserved Tyr/Phe residue in the turn prior to $\beta 1$ interacts with the helix-turn- $\beta 2$ unit of the adjacent PCI module (**Fig. 2d and Extended Data Fig. 5b, c, e**). The subunits capping the ends of ring, CSN7 and CSN8, appear incompatible with these interactions, presumably to discourage oligomerization of additional PCI proteins. CSN7 has a small residue, Val108, in place of the conserved Tyr/Phe residue on its solvent-exposed edge (**Extended Data Fig. 5e**). Similar lack of conservation is also found for CSN7 homologues (data not shown). CSN8, located at the end of the PCI ring, carries a helix-turn- $\beta 2$ motif reduced by six residues relative to the other PCI proteins (**Extended Data Fig. 5d**) and therefore presents a face that appears incompatible with the addition of further PCI proteins. These apparent capping features are conserved among CSN7 and CSN8 orthologues and also their paralogues in eIF3 and the 19S lid (data not shown). Phylogenetic analysis² shows CSN8 is absent in some eukaryotes, (notably, *Schizosaccharomyces pombe*³) and other organisms where neither CSN8 nor CSN3 are not found (*Saccharomyces cerevisiae*, for example⁴). This has a structural explanation: orthologues lacking CSN8 or the CSN3/CSN8 module likely have much the same core architecture as larger CSNs, supporting previous arguments that proteins are dispensable in some species and have been lost in evolution.

Effect of C-terminally truncated subunits on CSN integrity

We expressed the CSN holoenzyme in insect cells (**Methods**). The presence of all eight CSN complex members was required for stable formation of an active complex. The absence of individual subunits affected the integration of other subunits, most notably incorporation of CSN5 (**Extended Data Fig. 7c, d**). Using viruses encoding different CSN subunit

truncations, we tested which domains within a given subunit are required for CSN complex integrity. CSN1 (**Extended Data Fig. 6a**) and CSN4 (**Extended Data Fig. 6d**) are dependent on the presence of their C-terminal helix (CSN1 isoform-2 residues: 466-527; and CSN4: 364-406) for integration into CSN. The deletion of C-terminal helix of CSN3 (residues 364-423) impaired its ability to incorporate into the holoenzyme (**Extended Data Fig. 6c**). We note that the same CSN3 truncation appeared stably integrated into a CSN1-CSN2-CSN3-CSN8 subcomplex, which has been described previously⁵ (**Extended Data Fig. 6c**). Pull-down experiments using CSN7 truncations were not possible due to low expression levels of the mutant protein (data not shown). Others have shown the CSN7 PCI domain to be important for interaction with CSN4⁶ and its PCI domain alone is insufficient for complex incorporation⁷. For CSN2 (**Extended Data Fig. 6b**) and CSN8 (**Extended Data Fig. 6e**), deletion of their C-terminal helices (CSN2 residues: 417-443 and CSN8 residues: 159-209/166-209) had no effect on complex incorporation and integrity, suggesting that interactions outside the helical bundle drive their integration. Even though CSN2 incorporates into CSN without its C-terminal helix, it failed to do so when both its C-terminal helix and WH subdomain were removed (**Extended Data Fig. 6b**). This is mirrored *in vivo*, where in *Arabidopsis thaliana*, deletion of the CSN2 C-terminal helix and PCI domain resulted in the inability to accumulate CSN and seedling lethality². Stable integration of the PCI subunits (CSN1-4, 8 and presumably CSN7^{6,7}) appears to depend on the presence of the C-terminal helices, or C-terminal helices and WH subdomains. Pairwise interactions or other contacts outside the PCI ring/helical bundle do not appear to be sufficient for integration.

The C-terminal helices of the MPN domain-containing subunits, CSN5 and CSN6 are also crucial for complex integration. Three different C-terminal truncations of CSN5 failed to incorporate into CSN (**Extended Data Fig. 6f**) without affecting the integrity of the remainder of the complex (**Extended Data Figs 6f and 7c**). For mouse CSN, the CSN6 C-terminal helices (residues 171-324 and 221-324) have previously been shown to be essential for CSN6 incorporation and CSN complex formation⁴. We find that the CSN6 MPN domain deletion construct comprising residues 192-327, which retains only three C-terminal helices, was able to pull-down the intact CSN holoenzyme (CSN^{6ΔMPN}) (**Extended Data Fig. 6g**). These results imply that the basic interaction framework provided by the three C-terminal helices of CSN6 is crucial for CSN6 incorporation and complex stability. Taken together, these results highlight the major role the C-terminal helical bundle and PCI ring have in CSN structural integrity.

Supplementary Discussion

Architectural and mechanistic similarities between CSN and the 19S proteasome lid

The 26S proteasome consists of the 20S catalytic core and the 19S regulatory particle (RP) which is an assembly of the so called 19S lid and the 19S base. Proteins that are targeted to the proteasome are de-ubiquitinated by the 19S lid, unfolded by the 19S base and finally degraded in the 20S catalytic core. Several EM structures, most notably the near atomic resolution structure of the yeast 26S proteasome⁹, elucidate the molecular architecture of the proteasome. Furthermore, in mechanistic and structural studies, full deubiquitinating activity of the 19S lid is dependent on the presence of the 19S base and that binding of substrates to the lid induce significant structural changes⁸⁻¹⁰.

Despite low sequence identity between the individual components, the 19S lid shares significant structural similarity with CSN (**Extended Data Fig. 4b-f**), as suggested by previous EM structures of both CSN and the proteasome⁸⁻¹². The lid subunits RPN7, RPN6, RPN3, RPN5, RPN11, RPN8, RPN9 and RPN12, correspond to the CSN subunits CSN1, CSN2, CSN3, CSN4, CSN5, CSN6, CSN7 and CSN8, respectively (**Extended Data Fig. 4b-d**), whereas SEM1, a small protein of limited structure in the 19S lid, has no counterpart in CSN.

The structural similarities translate into functional similarities. As described in the main text, a helical bundle forms the principle organizational centre in the CSN holocomplex. The CSN5-CSN6 dimer contacts all other subunits and thus depends on their presence for stable integration. A helical bundle is also present in the 19S lid and, like for CSN, gauges the assembly of the complex¹³ (**Fig. 3 and Extended Data Figs 4f, 6a-g, 7c, d**). The conformational changes induced by substrate binding to the proteasome are similar of those observed for CSN following CRL binding and involve the RPN5 (CSN4) and RPN6 (CSN2) N-terminal solenoids, as well as the RPN11 (CSN5) and RPN8 (CSN6) MPN domain dimer (**Fig. 5 and Extended Data Fig. 4c, d**).

The structural similarities extend to the MPN dimers in 19S lid and the CSN. However, comparisons of the recently published structures of the isolated RPN11-RPN8 MPN dimer^{14,15} (**Extended Data Fig. 4e**) with the CSN5-CSN6 dimer imply significant functional differences. The RPN11 Ins-1 loop also assumes a non-productive conformation that requires remodelling prior to activity. In contrast to CSN5, however, RPN11 Ins-1 does not take part in the zinc coordination, because the residue spatially equivalent to Glu104 in human CSN5 is typically a Gly in RPN11 (**Extended Data Fig. 8f**). This less stringent stabilization of the RPN11 inactive state compared to CSN5 is reflected by the observation that high substrate concentrations can overcome autoinhibition of RPN11-RPN8¹⁴. In the context of the entire 26S proteasome, protease activity and specificity is regulated by additional structural elements such as the ATP-dependent 19S base^{8-10,14}. Such regulatory elements are absent from the CSN holocomplex and might explain the more stringent stabilisation of the CSN5 inactive state, which is a key determinant of substrate specificity.

Architectural similarities between CSN and eIF3

eIF3 in humans is a 13-subunit complex involved in the assembly of translation initiation factors on the ribosome¹⁶. The EM map of the eIF3 octameric core (composed of eIF3a, eIF3c, eIF3e, eIF3f, eIF3h, eIF3k, eIF3l and eIF3m) closely resembles that of the native 13-subunit eIF3 holocomplex (composed of the core and additional subunits eIF3b, eIF3d, eIF3g, eIF3i and eIF3j)¹⁷⁻²⁰. CSN subunits CSN1, CSN2, CSN3, CSN4, CSN5, CSN6, CSN7 and CSN8 correspond to eIF3 core subunits eIF3e, eIF3c, eIF3l, eIF3a, eIF3f, eIF3h, eIF3m and eIF3k, respectively (**Extended Data Fig. 4b, g, h**). The rigid body fit of CSN into the ~12 Å cryo-EM map of native, 13-subunit eIF3 in the 43S preinitiation complex²⁰ shows that the eIF3 density is well accounted for by the eight CSN subunits (**Extended Data Fig. 4h**). Additional eIF3 subunits appear to decorate the 8-subunit core. For example, unaccounted density near CSN5/eIF3f is estimated to correspond to a globular domain/protein of ~10 kD of unknown identity.

eIF3f, the CSN5 paralogue, is suggested to have deubiquitinase activity²¹. Although the overall MPN domain fold is presumably similar to CSN5 and Rpn11, sequence analysis and secondary structure predictions failed to identify a Zn²⁺-coordinating motif. When fitting the CSN crystallographic model into different eIF3 EM envelopes^{19,20}, unaccounted density is found consistently near the expected position of the eIF3m (CSN7) N-terminus (**Extended Data Fig. 4g, h**). As eIF3m possesses an N-terminal repeat region that is extended compared to CSN7, the additional density likely originates from the ~178 N-terminal residues predicted to form four additional helical repeats^{22,23} (**Extended Data Fig. 4g, h**).

The CSN4 N-terminal helical solenoid does not fit in the EM maps of eIF3 (**Extended Data Fig. 4g, h**). Introducing the conformational change observed for CSN4 in the presence of a CRL substrate (**Fig. 5a-c** and **Extended Data Fig. 9a**), however, provided a good fit allowing eIF3a placement in the maps (**Extended Data Fig. 4g, h**). The structure of the isolated PCI domain of eIF3a²⁴ mirrors the conformational state of CSN4 in CSN, not that in the isolated eIF3 complex or the eIF3 part of the 43S complex (data not shown). These data suggest that eIF3a may change conformation following incorporation into the eIF3 complex.

Autoinhibition of apo-CSN5

Apo-CSN5 is inactive when assayed in isolation and is unable to cleave neddylated cullins or NEDD8-AMC conjugates²⁵. In the apo-CSN5 crystal structure²⁵, helices $\alpha 10$ and $\alpha 11$ (**Extended Data Fig. 6h**) drape over the active site of a neighbouring molecule, blocking access. Because these helices make extensive crystal contacts in the apo-CSN5 structure and the crystallized construct lacks the C-terminal region that they interact with when in CSN, this conformation is likely to be non-physiological. The apo-CSN5 Ins-1 loop, while not coordinating the Zn²⁺ ion as is observed for the holoenzyme, positions Ins-1 Arg106 within salt-bridging distance of Asp151, a Zn²⁺-coordinating residue (**Extended Data Fig. 8a**). Arg106, in turn, partially obstructs substrate access (**Extended Data Fig. 8b, c**). The Ins-1 loop in the apo-CSN5 structure also makes lattice contacts. Biochemical experiments do, however, support a role for Arg106 in apo-CSN5 regulation, as Arg106 mutations restored limited catalytic activity allowing isolated CSN5 to cleave NEDD8-AMC²⁵. This contrasts to the CSN5 Glu104Ala mutant, which broadens the specificity of the holoenzyme allowing deneddylation of non-CRL substrates, while maintaining activity towards neddylated CRLs. The Ins-1 loop thus appears to play dual roles: (*i*) serving in CRL-induced allosteric activation via CSN5 Glu104 within the holoenzyme; and (*ii*) by CSN5 Arg106 preventing the isolated CSN5 subunit from becoming active in the absence of the remainder of CSN, ensuring specificity towards neddylated CRLs.

Restriction of Zn²⁺ access is commonly used in metalloprotease inhibition

The CSN5 Glu104-mediated mechanism of CSN holoenzyme inhibition bears some mechanistic resemblance to zymogen-mediated inhibition of zinc metalloproteases. A protein side chain that coordinates the catalytic Zn²⁺ ion and prevents the catalytic water molecule from binding is found in zymogens of the metzincin class of zinc metalloproteases²⁶. In these enzymes, a ~15-80 residue N-terminal pro-region harbouring the inhibitory residue is removed in the maturation process of the protease. In the zymogen of astacin²⁷, for example, the pro-region residue Asp21 directly coordinates the Zn²⁺ ion and together with the bulky

pro-peptide blocks substrate access (**Extended Data Fig. 8d, e**). In matrix metalloproteases (MMPs), such as fibroblast collagenase/MMP1, stromelysin-1/MMP3 and gelatinase-A/B, the S- γ atom of a conserved cysteine residue within the pro-region acts as an inhibitory Zn²⁺ ligand. A similar mechanism is found in zymogens of the adamalysin family (ADAM: a disintegrin and metalloproteinase)²⁸. Protease inhibition also can occur *in trans*; mature MMPs are subject to inhibition by tissue inhibitors of metalloproteinases (TIMPs) proteins²⁹, which utilize an N-terminal cysteine residue to chelate the catalytic zinc, thereby inhibiting the enzyme^{30,31}.

Although no general zymogen-like state has been described for the MPN family of deubiquitination/deneddylating enzymes, we now find that CSN, through its CSN5 Glu104 residue is also able to limit access to the catalytic Zn²⁺ ion. This inhibition is only overcome in the presence of a neddylated CRL substrate, explaining the selectivity of the enzyme.

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