Cullin-Ring E3 Ligases (CRLs) regulate a multitude of cellular pathways through specific substrate receptors. The COP9 signalosome (CSN) deactivates CRLs by removing NEDD8 from activated Cullins. Here we present structures of the neddylated and deneddylated CSN-CRL2 complexes by combining single-particle cryo-electron microscopy (cryo-EM) with chemical cross-linking mass spectrometry (XL-MS). These structures suggest a conserved mechanism of CSN activation, consisting of conformational clamping of the CRL2 substrate by CSN2/CSN4, release of the catalytic CSN5/CSN6 heterodimer and finally activation of the CSN5 deneddylation machinery. Using hydrogen-deuterium exchange (HDX)-MS we show that CRL2 activates CSN5/CSN6 in a neddylation-independent manner. The presence of NEDD8 is required to activate the CSN5 active site. Overall, by synergising cryo-EM with MS, we identify sensory regions of the CSN that mediate its stepwise activation and provide a framework for understanding the regulatory mechanism of other Cullin family members.
Cullin-RING Ligases (CRLs) are modular, multi-subunit complexes that constitute a major class of ubiquitin E3 ligases. CRLs coordinate the ubiquitination of substrates as either a signal for degradation via the 26S proteasome, or to alter the function of the target protein. The CRL2 E3 ligase consists of a Cullin 2 (CUL2) scaffold in association with a catalytic RING-box protein (RBX1), with the substrate adaptors Elongin B (ELOB) and C (ELOC) at its N-terminal. When associated with the von Hippel–Lindau (VHL) tumour suppressor substrate receptor, the CRL2 complex is the primary regulator of the Hypoxia Inducible Factor 1α (HIF-1α) transcription factor. Mutations in the interface between VHL, ELOB and ELOC can deactivate CRL2 leading to an accumulation of HIF-1α, which can in turn drive tumorigenesis through the over-activation of oncogenes. Moreover, CRL2 has recently been identified as a potential target for small molecular inhibitors and PROTACs—a new class of cancer drugs that promote degradation of tumorigenic gene products. These fascinating systems have been described in detail by a number of excellent reviews.

Activation of CRL2, in common with other members of the CRL family, involves a cascade of E1, E2 and E3 enzymes, which conjugate the ubiquitin-like protein NEDD8 (N8) to residue K689 on the CUL2 scaffold. In its activated state, CRL2–N8 (the ~ stylisation denotes a covalent interaction) recruits the ubiquitin-conjugated E2 enzyme via the RING domain of RBX1. Ubiquitination now takes place, covalently adding ubiquitin-conjugated E2 enzyme via the RING domain of RBX1. Ubiquitination now takes place, covalently adding ubiquitin to the substrate molecule docked at the CRL2 N-terminal. The activity of CRL2 is negatively regulated by the 331 kDa Constitutive Photomorphogenesis 9 Signalosome (CSN) complex, frequently referred to as the COP9 signalosome complex.

The CSN was originally identified as consisting of eight subunits (designated as CSN1–8 by decreasing molecular weights of 57–22 kDa), and is organised in a spayed hand architecture, which has high sequence and structural homology to the proteasome lid. CSN1, 2, 3, 4, 7 and 8 are structurally homologous to each other and together contribute to the fingers of the spayed hand which arise from extended N-terminal α-helical repeats. Each CSN1-8 subunit includes an extended C-terminal helix which associates together forming a C-terminal helical bundle. CSN5 and 6 are also closely related structurally and form a globular heterodimer located on the palm of the hand. CSN5 is responsible for the deneddylase activity of the CSN. A ninth subunit, CSNAP, has recently been identified, and is thought to play a role in stabilising the CSN complex.

Electron microscopy (EM) based structural analysis has provided important insights into the mechanism of CRL1 regulation by CSN. CRL4A and CRL3 have also been observed to form such complexes. However, despite intense interest, structural information of the CSN bound to CRLs remains limited to CRL12,16,19, CRL4A, and a low-resolution map of a dimeric CSN–CRL3–N8 complex. While the exact role of CSN–CRL complexes remains unclear, it has been hypothesised that these complexes may function to regulate the cellular level of ubiquitin ligase activity of CRLs once they have been deneddylated, effectively sequestering E3 ligases from the intracellular environment.

Surprisingly, the CSN can also form complexes with each of the Cullin 1–5 family members even without NEDD8. Free CRLs such as CRL1 have been reported to readily bind and inhibit the CSN, albeit at relatively lower affinity than the deneddylated CRL. While the exact role of CSN–CRL complexes remains unclear, it has been hypothesised that these complexes may function to regulate the cellular level of ubiquitin ligase activity of CRLs once they have been deneddylated, effectively sequestering E3 ligases from the intracellular environment.

Results

Cryo-EM structures of the CSN–CRL2–N8 complex. To study the molecular interactions between deneddylated CRL2 (CRL2–N8) and the CSN, we performed single-particle cryo-EM to resolve a structure of the assembled CSN–CRL2–N8 (referred to as the holocomplex) (Supplementary Figs. 1 and 2). The H138A mutation in the catalytic site of CSN5 subunit makes it possible to assemble CSN–CRL2–N8 complexes in which NEDD8 remains covalently attached over the time scale of the experiment. To justify the use of the H138A point mutation, we performed a band-shift assay comparing the deneddylation activity of the CSNWT and CSNWT H138A mutant enzymes (Supplementary Fig. 3). As expected, CSNWT rapidly cleaves NEDD8 from CRL2–N8 within seconds of incubation, while little to no deneddylation activity is seen from the CSNWT H138A complex (Supplementary Fig. 3). This mutant form of CSN was used throughout the work described below, unless otherwise specified.

Using 3D classification, we were able to generate maps of three different structures: (a) a holocomplex map at 8.2 Å, (b) a map of the complex with little or no density for VHL at 8.0 Å, and (c) a map of the complex with little or no density for CSN5/CSN6/ VHL at 6.5 Å (Supplementary Figs. 2, 4, and 5). The two partial complexes likely arise from compositional heterogeneity in the original samples from which the structural analysis has succeeded in isolating subpopulations. To verify the existence of subcomplexes, we subjected the CSN–CRL2–N8 to native MS (Supplementary Fig. 6, Supplementary Data 1). In line with subpopulation observations from our cryo-EM, we identified subcomplexes of the CSN–CRL2–N8 missing the CSN5, VHL, ELOB or ELOC subunits. These observations support the notion that similar levels of heterogeneity observed in the cryo-EM analysis of other CSN–CRL complexes, CSN–CRL1–N8 and CSN–CRL4A–N8, is also likely to arise from variable subunit composition and may be ubiquitous to all CSN–CRL complexes.

Next, we fitted into each map, the highest resolution crystallographic structure of the CSN (PDB 4D10) and a homology
model of the CRL2 (including the VHL-ELOB-ELOC) using molecular dynamics flexible fitting (MDF2)\(^\text{25}\) (Table 1; “Methods” section). In the model of the holocomplex (8.2 Å), the main interactions occur between the C-terminal end of CUL2 and the extended N-terminal helical repeats of CSN2 and CSN4 (Fig. 1a, b). Compared with their conformation in the highest resolution apo-CSN crystal structure\(^\text{16,19,20}\) (PDBs 4D10, 4D18, and 4WSN; Supplementary Movie 1). We additionally reviewed the conformations of CSN2 and CSN4 in all nine available intact CRL2 structures, the exact position of NEDD8 and the CUL2 structure (including the VHL-ELOB-ELOC) using the bis(sulfosuccinimidyl)suberate linker length (10 Å) and an extra 10 Å to allow for domain-level flexibility (“Methods” section). We imposed a cross-link distance threshold of 35 Å which takes into account the length of two lysine side chains (15 Å), the BS3 cross-linker length (10 Å) and an extra 10 Å to allow for domain-level flexibility (“Methods” section). We compared each of the apo-CSN structures of the apo-CSN (PDBs 4D10, 4D18 and 4WSN; Supplementary Data 2, Supplementary Fig. 9a, b). To generate a model of the CSN–CRL2–N8, we performed cross-link guided modelling which allows the placement of the WHB, NEDD8 and VHL subunits using identified cross-links from XL-MS (“Methods” section). We additionally reviewed the conformations of CSN2 and CSN4 in all nine available intact structures of the apo-CSN (PDBs 4D10, 4D18, 4WSN; Supplementary Fig. 7). We compared each of the apo-CSN structures with our holocomplexes and with other fitted models of the CSN in complex with CRL1–N8 and CRL4A–N8. Significant structural variation in CSN2 and CSN4 is observed within these apo-CSN structures, but in all cases, their conformations were substantially different to any found in the holocomplexes. In each of our CSN–CRL2 structures, the clamping motion of CSN2 and CSN4 is a swinging rotation about hinges located close to the CSN2 and CSN4 winged helix domains. For CSN2 this is coupled with an additional rotation about the axis of the superhelix formed from its N-terminal helical repeats. In the case of CSN4 the movement is coupled to the detachment of CSN4 from the Ins-2 loop of CSN6 by ~30 Å and leads to an ~12 Å shift in CSN5 (Fig. 1c, f). Only minor conformational changes were found in the CSN1, CSN3, CSN7B or CSN8 subunits. In the CRL2–N8 moiety, a number of relatively small rearrangements of CUL2, RBX1, ELOB, ELOC and VHL subunits were observed compared to its crystal structure\(^\text{26}\) (Supplementary Fig. 8a, b). In our EM maps and the other published CSN–CRL structures\(^\text{15,19,20}\), the exact position of NEDD8 and the CUL2 Winged-Helix B (WHB) domain were difficult to determine. To address this limitation, we carried out XL-MS experiments on the CSN–CRL2–N8 complex using the bis(sulfosuccinimidyl)suberate (BS3) cross-linker which targets lysine side chains (“Methods” section). We identified a total of 24 inter- and 60 intra-protein cross-links (Supplementary Data 2, Supplementary Fig. 9a, b). To generate a model of the CSN–CRL2–N8, we performed cross-link guided modelling which allows the placement of the WHB, NEDD8 and VHL subunits using identified cross-links from XL-MS (“Methods” section). We imposed a cross-link distance threshold of 35 Å which takes into account the length of two lysine side chains (15 Å), the BS3 cross-linker length (10 Å) and an extra 10 Å to allow for domain-level flexibility (“Methods” section). Our model of the CSN–CRL2–N8 satisfies all cross-link distances (Supplementary Fig. 9c). Three cross-links between CUL2-WHB (CUL2K382-WHBK720, CUL2K382-WHBK677 and CUL2K433, WHBK677) were used for the positioning of the WHB domain (Supplementary Fig. 9d, red text). A further two cross-links between CUL2 and NEDD8 (CUL2K382-N8K33 and CUL2K433, N8K69) allowed the positioning of NEDD8 near CSN5 (Fig. 1d, Supplementary Fig. 9d, green text). In this conformation, the isopeptide bond of NEDD8 is juxtaposed to the CSN5 active site. For the isopeptide bond of NEDD8 to reach the CSN5 active site, the WHB domain must be extended from its crystallographic conformation towards the CSN5 by 19 Å (Supplementary Fig. 9e).

### Table 1 Cryo-EM data collection, refinement and validation statistics

| Structure | CSN-CRL2-N8 (holocomplex) (EMB-4739) (PDB 6R7F) | CSN-CRL2-VHL-N8 (EMB-4744) (PDB 6R7N) | CSN-CRL2-VHL-N8 (CSN5/CSN6 refined) (EMB-4742) (PDB 6R71) | CSN-CRL2-N8 (VHL refined) (EMB-4736) (PDB 6R6H) | CSN-CRL2 (EMB-4741) (PDB 6R7H) |
|-----------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Data collection and processing | | | | | |
| Magnification | 47,170 | 47,170 | 47,170 | 47,170 | 47,755 |
| Voltage (kV) | 300 | 300 | 300 | 300 | 300 |
| Pixel size (Å) | 1.060 | 1.060 | 1.060 | 1.060 | 1.047 |
| Map resolution (Å) | 1.8-3.0 | 1.8-3.0 | 1.8-3.0 | 1.8-3.0 | 1.8-3.0 |
| Initial number of particles | 316,921 | 316,921 | 316,921 | 316,921 | 308,936 |
| Final number of particles | 20,055 | 22,471 | 24,552 | 24,049 | 17,191 |
| FSC threshold | C1 | C1 | C1 | C1 | C1 |
| Map resolution range (Å) | 6.7-22.7 | 5.9-8.0 | 5.9-11.5 | 6.3-17.4 | 5.6-16.8 |
| Map resolution range (Å) | 6.7-22.7 | 5.9-8.0 | 5.9-11.5 | 6.3-17.4 | 5.6-16.8 |

Structure of the deneddylated CSN–CRL2 complex. Having determined the structure of the CSN–CRL2–N8 complex, we next sought to detail any conformational differences in the deneddylated CSN–CRL2. The affinity of CSN for non-neddylated CRLs is significantly lower than for the neddylated forms, limiting the yield of the desired product\(^\text{19}\). To stabilise the formation of a complex between CSN and CRL2, we employed GraFix\(^\text{27}\) (“Methods”) prior to cryo-EM. Moreover, native MS confirmed
the formation of CSN–CRL2 complex (Supplementary Fig. 10, Supplementary Data 1). We next resolved a cryo-EM map of the CSN–CRL2 to 8.8 Å resolution (Supplementary Figs. 11, 12). Although the resolution of the CSN–CRL2 map is similar to that for CSN–CRL2–N8 holocomplex, we only observed partial density for VHL and CSN4. Next using the same procedure as for the neddylated CSN–CRL2–N8 complex, we fitted CSN and CRL2 subunits into the density map of the CSN–CRL2 complex ("Methods" section). We then utilised cross-link guided modeling to establish the position of the WHB which lacked clear density, similar to the neddylated holocomplex (Supplementary Fig. 13; Supplementary Data 3; "Methods" section).

To determine whether the lack of density for the CSN4 may be due to flexibility of the CSN4 N-terminal domain, we carried out XL-MS for the apo-CSN complex (Supplementary Fig. 14; Supplementary Data 4). A total of 18 cross-links were identified involving CSN4: 12 of which were intra-CSN4 cross-links, 4 between CSN2-CSN4 and 2 between CSN6–CSN4. We measured the distances of these cross-links on the structure of the apo-CSN (PDB 4WSN, 4D10 and 4D18) and CSN–CRL2–N8 (f) CSN5/CSN6 (PDB 4D10) upon binding of CRL2–N8 (holo). Subunits of the CSN–CRL2–N8 were compared with the apo-CSN crystal structure (PDB 4D10) following structural alignment. The structure of the CRL2–N8 has been hidden for clarity.
CRL2-bound structures, even in the absence of the CRL2 substrate.

To evaluate any local changes across the CSN–CRL2–N8 in the absence of NEDD8, we aligned the cryo-EM models of neddyalted and deneddylated holocomplexes using the C-terminal helical bundle as a reference point (Supplementary Fig. 15a, b). We systematically compared the conformations of each subunit (Supplementary Fig. 15c–j, Supplementary Movie 1). Compared with its structure in the CSN–CRL2–N8, the N-terminal helices of CSN2 are shifted by ~21 Å towards the CUL2 C-terminal domain (Fig. 2b). This change in CSN2 in the absence of NEDD8, leads to a structural difference in CUL2 which rotates upwards towards the rest of the CSN by 20 Å (Fig. 2c). The position adopted by CUL2 in the deneddylated holocomplex, places ELOB closer to CSN1, forming a CSN1–ELOB interface (Fig. 2d). An interface between CSN1–ELOB can also be seen in the partial structures of CSN–CRL2–N8 missing VHL and CSN5/CSN6 (Supplementary Fig. 2, Supplementary Fig. 16). The dissociation of VHL and CSN5/CSN6 causes the N-terminus of CUL2 to shift downwards, away from the CSN (Supplementary Fig. 15b, Supplementary Fig. 16). The plasticity of the CSN results in changes in the C-terminus of CUL2, which is clamped between CSN2 and CSN4, in the CSNWT condition, indicating that an interface exists between CSN1–ELOB (Supplementary Fig. 22). It is noted that since the CSNWT–CRL2 sample used for HDX were not treated with GraFix (as they were for cryo-EM), it is unlikely that the CSN1–ELOB interface is caused by glutaraldehyde cross-linking. In the deneddylated Δ(CSN–CRL2–N8 - CSN) condition, ELOB (17–25) was not found due to the lack of proteomic coverage. However, CSN1 (143–156) is stabilised, suggesting that the CSN1–ELOB interface is present in the deneddylated holocomplex.

To assess the affinity between CSN4 and CRL2, we performed PLIMSTEX (protein–ligand interactions by mass spectrometry, titration and HDX) experiments (“Methods”). In these experiments, we performed HDX-MS of CSN or CSNWT in the presence of increasing concentrations of CRL2 or CRL2–N8, to derive dissociation constants (Kd) between CSN4 and CRL2/ CRL2–N8. PLIMSTEX differs from differential HDX-MS in several ways. First, no differential comparison is performed from PLIMSTEX. Second, PLIMSTEX experiments utilise a single deuteration time for all samples. PLIMSTEX requires a sufficiently long deuteration time to observe differences between holo and apo states, but short enough to prevent over-deuteration of interfaces as the complex naturally dissociates and reassociates. Finally, PLIMSTEX is a titration experiment which observes deuteration changes as a function of ligand concentration, while differential HDX-MS utilises a 1:1 molar ratio of our CSN and CRL2 substrates.

We tested the affinity of CSN4 for CRL2 or CRL2–N8 in CSN–CRL2–N8, CSN–CRL2 and CSNWT–CRL2 complexes. Our PLIMSTEX experiments identified three regions of CSN4 which exhibit a dramatic decrease in deuteration uptake when exposed to the CRL2 substrate. These regions correspond to CSN4 α-helices which are in contact with CRL2–N8 in our cryo-EM fitted model of the CSN–CRL2–N8 (Supplementary Fig. 23). The Kd measurements for the different regions of CSN4 in CSNWT–CRL2 (11.3–34.5 nM), CSN–CRL2 (138.1–218.8 nM) and CSN–CRL2–N8 (118.9–389.0 nM) are each in the low nanomolar region, suggesting limited differences in local affinity brought about by changes such as the neddylation status of CRL2. These Kd values for the CSN–CRL2 system all fall within a similar overall range to the published global Kd to the CSN–CRL1 system19 (1.6–310 nM; Supplementary Table 1), indicating a crucial role for CSN4 in stabilising CSN–CRL2. In addition, HDX-MS based Kd measurements also allowed us to localise individual regions of CSN4 responsible for interacting with CRL2 at the peptide level. (Supplementary Fig. 23).
Remodelling of the CSN5 active site in the presence of NEDD8.

We next considered the release mechanism of the CSN5/CSN6 subunits of both the neddylated and deneddylation holocomplexes. In both Δ(CSN−CRL2~N8 - CSN) and Δ(CSNWT−CRL2 - CSNWT) experiments, the Ins-2 loop of CSN6 was destabilised, correlating with the release of CSN6 from its interface with CSN4 and in line with the allosteric activation mechanism of CSN by CRL4A~N820 (Fig. 4a, b). An interesting difference between the neddylated and deneddylation complexes is that the CSN6 α4 helix is destabilised only in the absence of NEDD8 (Fig. 4b i). Similarly, the CSN5 α7 helix is also destabilised in both neddylated and deneddylation conditions (Fig. 4a, b ii). The CSN6 α4 and CSN5 α7 helices are topologically knotted in the CSN5/CSN6 heterodimer and tether the globular domains of CSN5/CSN6 to the C-terminal helical bundle13. These observations suggest that structural changes are required in the helical knot to bring about release of the CSN5/CSN6 globular domains from their apo conformation.

Another finding is that we identified destabilisation in the Ins-2 loop of CSN5 (Fig. 4a, b ii). The Ins-2 loop of CSN5 has a lesser understood role in CSN activation. In isolated CSN5, the Ins-2 loop is highly disordered22 (Supplementary Fig. 24a), while it folds into a helical-loop structure when incorporated into the CSN13 (Supplementary Fig. 24b). Accompanying the changes in the CSN5 Ins-2 loop, in both comparative HDX-MS experiments, we detected destabilisation of the α5 helix area which surrounds the CSN5 active site (Fig. 4a, b ii). The changes in both the CSN5 Ins-2 and α5 helix indicate a major conformational remodelling in the area adjacent to the CSN5 active site, which can be triggered through the binding of both CRL2 or CRL2~N8 to the CSN in a NEDD8-independent manner. It is only in the presence of NEDD8, that the CSN5 active site is further destabilised suggesting that in a final activation step, NEDD8 induces conformational changes in the active site itself (Fig. 4a–d). To eliminate the possibility that the observed changes in the CSN5 active site are due to the H138A point mutation, we compared the deuterium uptake profiles of CSN5 from apo-CSNWT and CSN5H138A constructs (Supplementary Fig. 25). Calculating the deuterium uptake differences between peptides of the CSN5WT and CSN5H138A and visualising this through a Woods plot, identified no significant uptake differences (Supplementary Fig. 25). With this considered, the deprotection observed in the CSN5 active site of the CSN−CRL2~N8 complex can be seen to result from the binding of CRL2~N8 and not the H138A mutation of the CSN5 active site.

Discussion

Here we have combined EM and MS analyses to provide insights into the mediation of CRL2 by the CSN. We have described the molecular structures of CSN−CRL2~N8 and its deneddylation CSN−CRL2 counterpart. Furthermore, we combined cryo-EM maps with comparative HDX-MS to expand on the stepwise activation mechanism of the CSN, involving a conformational network of both NEDD8-independent and dependent stages. We suggest that the steps which lead to deneddylation are mostly NEDD8-independent, except for the remodelling of the CSN5 active site which requires NEDD8 to encounter the CSN5 active site.

Our map of the deneddylation CSN−CRL2 holocomplex represents a complex in which the CSN is still associated with its CRL2 reaction product. Resolving this structure has provided several important details into how activation of the CSN is achieved. Our comparison of the neddylated and deneddylation holocomplex structures indicated that the CSN2 contacts the CRL2 C-terminal domain in a slightly different conformation to when the CRL2 is modified with NEDD8. Between both neddylated and deneddylation conformations, we suggest that the clamping by CSN2 involves destabilisation of the CSN2 helical modules 6–9 which function possibly as a hinge that allows the
CSN2 to bend upwards towards the CRL2. The plasticity of these N-terminal helices presumably permits the binding of deneddylated and alternative Cullin isoforms. HDX-MS further indicates that RBX1 and CSN4 form an interface, which is more prominent in the absence of NEDD8, as shown through stabilisation of the two interfaces (Supplementary Fig. 21b). Overall, the conformational variations seen in the CSN2 N-terminal helical modules (Fig. 2b), the bend of CRL2 (Fig. 2c) and the HDX differences in CSN4/RBX1 (Supplementary Fig. 21b) may therefore be attributable to the seemingly promiscuous affinity that allows CSN2 to bind to each of the different Cullins regardless of neddylation.

We uncovered structural and dynamical aspects of both the neddylated and deneddylated CSN–CRL2 complexes. Beginning our interpretation from valuable studies of the CSN–CRL1~N8 and CSN–CRL4A~N8 systems, we propose several major conformational switches of the CSN which must be activated by the CRL2 substrate to bring about deneddylation. While some of these steps are conserved ubiquitously among other CSN–CRL complexes (e.g. CSN2/CSN4 clamping in CSN–CRL1~N8 and CSN–CRL4A~N8), our study suggests additional steps for the CRL2-bound CSN complex (Fig. 5). In the first activation step, the CSN and CRL2~N8 associate through major conformational changes in CSN2 and CSN4, which clamp onto the CRL2 (Fig. 5a, b). Our data suggest that the conformational change in CSN4 breaks its interface with CSN6 through the CSN6 Ins-2 loop and with the eventual release of the CSN5/CSN6 heterodimer (Fig. 5c). Removal of the CSN6 Ins-2 loop has been shown in CSN–CRL1~N8 to disrupt the CSN4–CSN6 interface, leading to sustained enzymatic activity of the CSN13. In future studies, targeted deletion of the CSN6 Ins-2 loop can be performed alongside differential HDX-MS for the CSN–CRL2 system, to further probe the differences in active site remodelling of CSN5 as a result of disrupting the CSN4–CSN6 interface.

The release of CSN5/CSN6 appears consistent with the destabilised knotted helices of CSN6 that our HDX has identified. It is plausible that these two helices function as the mechanical hinges which allow the CSN5/CSN6 to be released from their auto-inhibited conformations but remain tethered to the rest of the CSN. Although the resolution presented by CSN5 in our cryo-EM structures prevents us from making molecular level observations, our HDX data can provide local detail for the CSN5 active site. The release of CSN5/CSN6 is accompanied by HDX changes in

Fig. 3 Effect of NEDD8 on the CSN4/CSN6 interface. a Investigating the effect of CRL2~N8 and CRL2 binding on CSN and CSNWT, respectively. The two experiments involve deuterating the complexes for 0.25, 5 and 30 min time points, a quench step to halt the deuteration, and digestion to the peptide level. Peptides are then identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and a database search. b Effect of CRL2~N8 binding on the CSN. c Relative deuterium uptake over 30 min for the CSN6 Ins-2 peptide (T170SVIDIING177). Error bars indicate the deuterium uptake standard deviation for triplicate measurements (N = 3). d Effect of CSN on the C-terminal domain of CRL2~N8. Colour scheme follows that of b. Source Data are provided as a Source Data file.
areas surrounding the CSN5 active site, including the CSN5 Ins-2 loop. Up to here, the changes experienced by the CSN can be brought about in a NEDD8-independent manner. In the next stage, the presence of NEDD8 acts as a selectivity filter which results in remodelling of the CSN5 active site itself (Fig. 5d). These changes presumably expose the CSN5 JAMM ligands of the metalloprotease site and allow subsequent deneddylation to occur (Fig. 5e). Finally, deneddylation ensues with the cleavage of NEDD8 from CRL2 and the dissociation of the complex (Fig. 5f).

The fact that CSN can then reassociate with its CRL2 reaction product following dissociation, as shown by our study and structure of the CSN–CRL2, suggests that the non-neddylated complex may possess an alternative role to deneddylation. By associating with non-neddylated CRLs, the CSN sterically blocks access of both ubiquitination E2 enzymes and substrates to the CRLs. Further studies will be required to fully understand the deneddylation-independent roles of the CSN. Interestingly, a comparison of our non-neddylated CSN–CRL2 with the apo-CSN and the CSN–CRL2 complexes with NEDD8 may provide insights into the structural basis of these alternative roles.
and neddylyated CSN–CRL4–N8 and CSN–CRL2–N8 complexes, highlighted the dramatic differences in the conformation of CSN6. In the absence of NEDD8, the CSN5/CSN6 are released much further than any other observed conformation of CSN6. We hypothesise that this difference may arise from the lack of steric hinderance usually presented by NEDD8, allowing CSN5/CSN6 to rotate and meet the Cullin scaffold much closer than in the neddylyated structure.

In our study we have made interpretations of the CSN2 and CSN4 conformational clamping through comparing our cryo-EM holocomplexes with crystal structures of the apo-CSN. From reviewing the conformations of all nine currently available independent copies of the apo-CSN molecule it is apparent that CSN2 and CSN4 appear to exist in a range of open conformations that are quite distinct from the closed conformations that we observed in CSN–CRL2 complexes (Supplementary Fig. 8), and the similarly closed conformations observed in CSN complexes with CRL1–N8 and CRL4A–N8. However, each of the apo-CSN crystal structures is characterised by crystal contacts involving CSN2 and CSN4 which in principle could have biased their conformations.

Therefore, we used XL-MS to further monitor the conformation of CSN4 within the solution structure of apo-CSN and found that both open and closed conformations of apo-CSN are required to satisfy a number of unique cross-links (Supplementary Fig. 14). Since our study used lysine cross-links, our distance measurements do not allow for a high level of scrutiny in our modelling due to the uncertainty of lysine side chain rotamers in our models. Nonetheless, given that the violated cross-link distances are well above 40–50 Å for one model and close to 35 Å for the other (Supplementary Fig. 14), we can assume that both open and closed conformations are simultaneously represented within the cross-link ensemble. Thus it seems likely that crystal formation has stabilised CSN conformations already present in solution. Hence, while we cannot completely rule out the possibility that the crystallographically defined open conformations of apo-CSN are atypical, it seems likely that the solution structure of the apo-CSN corresponds to an ensemble containing a range of open structures including those seen crystallographically as well as some closed or nearly closed structures. In comparison, CSN2 and CSN4 in the cryo-EM structures of CSN–CRL2 complexes adopt consistently closed conformations sandwiching the C-terminal end of CUL2 with rather little apparent conformational variation. It is likely here that conformational variation is limited by interactions formed with CUL2. Thus it would appear that the interaction between CSN and CRL2 involves the transition of CSN from an ensemble of conformers with substantial variation in the distance between CSN2 and CSN4 to a bound complex in which CSN2 and CSN4 clamp onto the C-terminal region of CUL2 which is characterised by greatly reduced conformational variation.

To further explore the nature of such clamped complexes, we additionally performed PLIMSTEX experiments which provided localised affinity values between CSN4 and CUL2 for each combination of the CSN–CRL2. From a functional perspective, our PLIMSTEX experiments also determined that the H138A mutation of CSN5 leads to measurable changes in Kd between CSN4 and CRL2. It is also important to note that the Kd values presented are not representative of the global Kd between CSN and CRL2 complexes but are only local affinities between the CSN4 and CRL2 subunits. PLIMSTEX experiments require that the labelling time of the experiment is carefully selected based on preliminary experiments. While powerful, a concern is that if the labelling time is too long, protein interfaces may become over-deuterated, leading to an underestimated deuterium uptake decrease and a higher Kd value. Likewise, some interfaces may appear invisible if the labelling time is not sufficiently long to allow deuteration to occur.

In both neddylyated and non-neddylyated CSN–CRL2 complexes, we identified an interface between CSN1 and ELOB. So far, CSN1 has been shown to interact with Skp2 and Fbw7 in
CRL1–N816 and DDB1 in CRL4A–N8 holocomplexes20. Mutations of the CSN1–DDB1 interface did not affect binding to the CSN nor perturb deneddylation activity20. In the absence of Cullin 1 or RBX1, CRL1 substrate receptors do not associate with the CSN116. Although no CSN activator role has been assigned to CSN1–substrate receptor interactions, their presence increases the interface between CSN and their CRL substrates16 and may stabilise these interactions.

Furthermore, there may be additional roles for the CSN as suggested by the compositional plasticity seen in our CSN–CRL2–N8 classes. In our map of the CSN–CRL2–N8, the Cullin arm was observed to shift downwards away from CSN3 in maps deficient of the VHL substrate receptor (Supplementary Fig. 26), consistent with the coupling of VHL binding to a conformational change in the rest of the CRL2 portion of the protein. This type of behaviour may allow the CRL2 to adapt to individual substrates and substrate receptors which vary in size and geometry as has been suggested with the CRL4A system16. In addition, it may reflect changes associated with remodelling of the CRL2 by the dissociation of the VHL and the binding of alternative substrate receptors. In future work we will determine whether the CSN can mediate substrate receptor dissociation.

Overall, our study has provided greater detail into the role of CRL2 and NEDD8 in regulating the activation mechanism of CSN. We propose that the series of mechanistic responses of the CSN that lead up to deneddylation, can be triggered even by the CRL2 reaction product in a NEDD8-independent manner. The presence of NEDD8 on the activated CRL2 substrate would then trigger the remodelling within the catalytic site of the CSN5 subunit during the final stage of CSN activation. We envision that this type of mechanism would have implications for the entire family of CRL proteins and their regulatory relationship with the CSN. Our study therefore provides a template not only for assisting investigations of other CRL-based systems but also for bringing together data from different structural biology techniques that will otherwise be reported independently.

Methods

Preparation and expression of bacmids. WT and catalytically reduced CSN5H138A bacmids were a kind gift from Radoslav Enchev (The Francis Crick Institute, London)19. pCDNA3–myc-CUL2 was a gift from Yue Xiong (Addgene plasmid #19892)25. HA-VHL wt-pBabe-puro was a gift from William Kaelin (Addgene plasmid #242)19. RBX1, ELOB and ELOC were cloned from cDNA from the Mammalian Gene Collection purchased from Dharmaco2n. To improve protein yield, an N-terminally truncated (Δ53) natural isoform of VHL was also produced for use with MS. Both isoforms of VHL were sluced into a pET-52b (+) vector (Novagen) to add an N-terminal Step-Tag II. Genes were assembled into pACBac using I-Ceu/I/BstXI restriction sites via the MultiBac system17. RBX1 and CUL2 were assembled into one vector and ELOB, Strep-II-VHL(AN) and ELOC into a second vector. Correct assembly was confirmed by sequencing of entire genes. DH10EmBacY cells were transformed with each assembly and blue/white selection was performed on 1-agar plates containing 50 μg ml−1 kanamycin, 7 μg ml−1 gentamycin, 10 μg ml−1 tetracyclin, 100 μg ml−1 Blue-Gal (Thermo Scientific) and 40 mg ml−1 IPTG. DH10MultiBac bacmid DNA was isolated from white colonies and Recombinant bacmid vectors were generated in SfiI-selected Sf21 cells (a clonal isolate from Sf21, Life Technologies #11496015) using standard amplification procedures.

Expression and purification of recombinant CRL2. High Five Cells (BTI-TN-5B-1, from embryonic tissue of the cabbage looper, Trichoplusia ni, Life Technologies #885502) were co-infected with bacmids containing RBX1/CUL2 and ELOB/Strep-II-VHL(AN)/ELOC and incubated at 27 °C and 130 r.p.m. for 72 h. Cells were harvested by centrifugation at 250 × g for 10 min at 4 °C before storage at −80 °C. Freeze-thawed pellets were resuspended in 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM DTT containing complete EDTA-free protease inhibitor tablets (Roche) and Benzonase (Sigma-Aldrich). Cells were lysed by sonication and clarified by centrifugation at 25,000 × g for 1 h (Beckman JA-20 rotor). Supernatant was bound to a 3 × 5 ml Strept Hap HP columns (GE Healthcare) in tandem, equilibrated with 50 mM Tris pH 7.5, 150 mM NaCl and 2 mM DTT. Protein was eluted by the addition of 2.5 mM desthiobiotin. The eluted peak fractions were concentrated to 2 ml and loaded onto a Superdex 200 16/600 (GE Healthcare) size exclusion column equilibrated with 50 mM HEPES pH 7.5, 130 mM NaCl and 1 mM TCEP. All CRL2 and CRL2–N8 samples used throughout were in 50 mM HEPES pH 7.5, 150 mM NaCl and 1 mM TCEP.

In vitro neddylation of CRL2. APPBP1-Uba3, UbcH12 and Nedd8-His were purchased from (Enzo Life Sciences). The neddylation reaction was carried out for 10 min at 37 °C with 8 μM CRL2, 350 nM APPBP1-Uba3, 1.8 μM UbcH12 and 50 μM b-mercaptoethanol in a reaction buffer containing 50 mM HEPES pH 7.5 and 100 mM NaCl, supplemented with 1.25 mM ATP and 10 mM MgCl2. The reaction was quenched with 15 mM DTT and ice prior to loading onto a 1 ml Strept Hap HR column (GE Healthcare). CRL2–N8 was eluted with reaction buffer supplemented with 2.5 mM desthiobiotin and neddylation was confirmed by SDS-PAGE.

Expression and purification of recombinant CSN. High Five Cells were co-infected with bacmids gifted by Radoslav Enchev (The Francis Crick Institute, London) and protein was expressed as described for CRL2, with an additional Ni-affinity step prior to gel filtration to exploit the His6-tag on the CSN5 subunit. For Ni-affinity using 2 × HisTrap HP columns (GE Healthcare) in tandem, the same buffer was used, but protein was eluted by a 0–300 mM Imidazol gradient across 45 ml. For size exclusion chromatography using a Superdex 200 16/600 (GE Healthcare), the column was equilibrated with 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP and 2% glycerol. All CSN and CSN5V7 samples used throughout were in 30 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT and 1% glycerol.

Cryo-EM of CSN–CRL2–N8. The CSN–CRL2–N8 complex was formed by incubation between CRL2–N8 (1.1× molar excess) and CSN at room temperature for 90 min. The preparation (~0.5 MDa) was subjected to size exclusion chromatography using a Superose 6 Increase 3.2/300 column (GE Healthcare), equilibrated in 15 mM HEPES pH 7.5, 100 mM NaCl, 0.5 mM DTT and 1% glycerol to reduce the contribution of apo components. Fractions from the leading edge of the peak were exchanged into 15 mM HEPES pH 7.5 and 100 mM NaCl using PD SpinTrap G-25 columns (GE Healthcare) before initial assessment by negative stain EM. Cryo-grids were prepared using a Vitrobot (FEI). A cryo-EM dataset was collected beamline M02 from Quantifoil grids with an extra carbon layer with an extra carbon layer on top of perfect carbon layer (M02, Diamond Light Source, UK) on a Titan Krios 300 kV with Gatan K2 detector (M02), Å pix−1 = 1.06. Movies of 25 frames (dose = 1.85 e−Å−2) were motion corrected in RELION39 (2.0) using MOTIONCOR239 (01-30-2017) and subsequent CTF estimation of micrographs was performed using CTFFIND40 (4.1.5) (Supplementary Fig. 1). Auto-picking selected ~317,000 particles from ~310,000 micrographs. Particle were subjected to reference free 2D classification to assess data quality and to remove contaminants selected by auto-picking. This process reduced the particle number to ~250,000. Following particle selection through 2D classification, particles were divided into 15 3D classes. Three of these classes (~69,000 particles) were selected for further classification and processing, as described in Supplementary Fig. 2.

Cryo-EM of CSN–CRL2. The CSN–CRL2–N8 complex was formed by incubation between CRL2 (1.1× molar excess) and CSN at room temperature for 90 min. Samples were loaded onto a 5–50% glycerol Gradia27 gradient containing 0–0.2% glutaraldehyde and ultracentrifuged at 86,000 × g for 2 h at 4 °C. Gradients were manually fractionated and the resultant aliquots assessed by SDS-PAGE to determine the extent of cross-linking. Fractions were also assessed using negative stain EM in-house. In order to reduce the glycerol content of samples for cryo-EM, fractions containing the desired complex (as determined by negative stain EM) were pooled together and gel filtered into 15 mM HEPES pH 7.5, 100 mM NaCl and 0.5 mM DTT using a Superose 6 Increase 10/300 GL column (GE Healthcare). Fractions were again assessed by negative stain before preparing cryo-grids using a Vitrobot (FEI). A cryo-EM dataset was collected at the Electron Bio-Imaging Centre (eBIC—Diamond Light Source, UK) on a Titan Krios 300 kV with Gatan K2 detector (M02), with a sampling of 1.047 Å pixels−1. Movies of 85 frames (dose = 1.0 e−Å−2) were motion corrected in RELION39 (2.0) using MOTIONCOR239 (01-30-2017) and subsequent CTF estimation of micrographs was performed using CTFFIND40 (4.1.5). Auto-picking selected ~309,000 particles from ~680 micrographs. Particle were subjected to reference free 2D classification to assess data quality and to remove contaminants selected by auto-picking. This process reduced the particle number to ~208,000. Following particle selection through 2D classification, particles were divided into six 3D classes. Three of these classes (~69,000 particles) were selected for further classification and processing, as described in Supplementary Fig. 2.

Band-shift assays. In order to test the activity of the CSN and CSNWT, 3 μg of each complex was separately incubated with 3 μg of CRL1–N8 at 37 °C for 0, 15, 30, 45 and 60 s. Deneddylation was quenched through rapid denaturation by the
addition of NuPAGE lithium dodecyl sulfate sample buffer (Thermo Fisher) and placing on a heat block, pre-heated to 90 °C. Samples were analysed by SDS-PAGE. Denatured CRL2, CSNWT, and C4A were separated by a 10% gel by filling the gel band corresponding to CUL2, by comparing with CRL2–N8 and CRL2 controls.

Homology modelling of the CRL2. Homology modelling of the CRL2 was necessary due to a combination of missing domain structure for the CUL2 and the CSN. We then converted the structures into MDFF-compatible format. In the first stage, we performed structural alignment of the CRL2 (SN4W) and CRL2 (1LDI) structures in PyMOL (2.06). Using MODELLER49 (r16), a single model of the CRL2 was generated using the slow molecular refinement option of MODELLER. The model was evaluated for correctness, including the correct positioning of residues (such as the CUL2 K689 NEDD8-acceptor site) already present in the SN4W crystal structure. In the second stage, we aligned the CRL2 model with the VHL-ELOB-ELOC-CUL2 fragment (4WQO) to generate a template for homology modelling. The isoform of VHL (missing residues 1–53) was used for modelling and the entire VHL-ELOB-ELOC-CUL2 fragment was kept as a single model of the CRL2 (with VHL/ELOB/ELOC) generated using the slow molecular refinement option of MODELLER. The final model of the CRL2 shows the RMSD of 3.6 Å when compared with the initial crystal structure (SN4W) but includes a complete WHA domain and VHL subunit. The script used can be found in Supplementary Note 1.

Model fitting of EM maps. All models were fitted using CSN subunits sourced from the 4D10 crystal structure (chains A–H) and the CRL2 (VHL-ELOB-ELOC) structure generated and described in the Homology Modelling of the CRL2 section. We performed map fitting first by performing rigid body fitting of the CSN and CRL2 subunits to each map in Chimera52 (1.13.1rc) then using the MDFF53 (0.5) feature of NAMD for additional refinement of the rigid body fitting step. Elongated subunits such as CSN2, CSN4 and CUL2 were first dissected into smaller rigid bodies to permit better fitting into their densities. Following map fitting all of the CSN and CRL2 subunits, we then converted the structures into MDFF-compatible topology files using the protein structure file builder function of VMD54 (1.93). MDFF was performed in two stages: an initial energy minimisation step (scaling factor = 0.3 for 50,000 steps) which coerced each subunit into their map densities, and a second equilibration run (scalling factor = 10 for 200,000 steps) which applied molecular dynamics to produce structurally and energetically realistic structures. Secondary structure, cis-peptide and chirality characteristics of the initial models were calculated and enforced throughout each step to avoid a loss of internal structure for each subunit. For each of the CSN–CRL2–N8 and CSN–CRL2 structures, subunits/domains which lacked clear density were not included to avoid interference with the fitting of other subunits. These were the WHB domain (CUL2 residues 656–745) for all maps, NEDD8 in all NEDD8–including maps, and VHL in the CSN–CRL2–map. The cross-correlation coefficient which calculates the degree of overlap between the cryo-EM map and a simulated map of the same resolution from the atomic model, are reported for each model in Table 1 of the manuscript.

Native mass spectrometry. All spectral data were collected using a SYNAPT G2–Si (Waters Corp., Manchester, UK) high-definition mass spectrometer and were ionised using a NanoLockSpray® dual electrospray inlet (Waters Corporation) run with positive polarity in sensitivity mode. Capillary voltages were pulled using a Flaming/Brown P-900 micropipette puller (Sutter Instrument) and coated with Au/Pd (80:20) using a sputter coater (Quorum Q150RS). The following mass spectrometer parameters of PLGS were set to: lock mass for charge 1 of 556.2771 Da e−, low energy threshold of 135.0 counts, elevated energy threshold of 30.0 counts and intensity threshold of 750 counts. Worklow parameters for mass tolerance were peptide and mass tolerance set to ± 2 mDa, minimum fragment ion matches per peptide set to 1, minimum fragment ion matches per protein set to 7, minimum peptide matches per protein set to 3, primary digest reagent set to non-specific, number of missed cleavages 0, false discovery rate of 0.01. The output peptides were filtered using DynaXm (3.0) using the following filtering parameters: minimum intensity of 2500, minimum and maximum peptide sequence length of 5 and 30, respectively, minimum MS/MS products of 3, minimum product mass per amino acid of 0.1, and a minimum peptide score of 5. In addition, all the spectra were visually examined and only those with high signal to noise ratios were used for HDX MS analysis. The amount of relative deuterium uptake for each peptide was determined using DynaXm (3.0) and are not corrected for back exchange. State (listing the deuterium uptake per-peptide, per-timepoint and per-experimental state) and difference (listing the difference in deuterium uptake between identical peptides of two states compared for each timepoint) were exported from DynaXm. These files were input to DeuterX55 (1.0.8) which formats the differential data into the Woods Plot format. Statistical filtering of peptides is then performed to identify those which exhibit significant uptake differences between the two states. DeuterX applies a blanket confidence interval (specifically, the 98% confidence interval given as 0 ± DU where DU is the Deuterium uptake threshold in Daltons) across all peptides of each timepoint. Non-significant peptides for each timepoint are then exported into a formatting script which is used to project the filtered data onto a 3D model of the protein (Supplementary Figs. 19 and 20).

PLIMSTEX for CSN–CRL2 complexes. In addition to performing traditional time-resolved HDX-MS experiments, we also performed PLIMSTEX (protein–ligand interactions by MS, titrations and HDX) measured on combinations of CSN/CSNWT–CRL2–N8–CRL2 to determine kinetic parameters. PLIMSTEX was used for three complexes: (1) CSN–CRL2–N8, (2) CSN–CRL2 and (3) CSNWT–CRL2 complexes. The final concentration of CSN or CSNWT was fixed at 250 nM. CRL2 or CRL2–N8 were titrated at either 1:0.1, 1:0.5, 1:1, 1:2 or 1:5 molar ratios of CSN:CRL2 (0–250 nM) for each of the three complexes consisted of five undeuterated references of CSN or CSNWT, three samples of deuterated CSN or CSNWT, followed by three each of the above molar ratios of CSN:CRL2. Datasets for CSN–CRL2–N8, CSN–CRL2 or CSNWT–CRL2 underwent labelling for either 15 sec or 2 min depending on which exposure time yielded better deuterium uptake differences as a function of [CRL2] or [CRL2–N8–CRL2] (250 nM) and the HDX-MS data acquisition parameters. HDX data was also acquired using a transfer collision energy ramp for high-energy acquisition of product ions. Leucine Enkephalin (LeuEnk-Sigma) was used as a lock mass for mass accuracy correction and the MS was calibrated with sodium iodide. The online Enzyme pepsim column was washed with pepsim wash (1.5 M Gu-HCl, 4% MeOH and 0.8% FA) recommended by the manufacturer and a blank run using the pepsim wash was performed between each sample to prevent significant peptide carry-over from the pepsim column. Optimised peptide identification and peptide coverage for all samples was performed from undeuterated controls (three–four replicates). All deuterium time points were performed in triplicate on different samples on distinct plates.

Sequence identification was made from MS² data from the undeuterated samples using the Waters ProteinLynx Global Server 2.51 (PLGS). Processing parameters of PLGS were set to: lock mass for charge 1 of 556.2771 Da e−, lock mass window of 0.4 Da, low energy threshold of 135.0 counts, elevated energy threshold of 30.0 counts and intensity threshold of 750 counts. Worklow parameters for mass tolerance were peptide and mass tolerance set to ± 2 mDa, minimum fragment ion matches per peptide set to 1, minimum fragment ion matches per protein set to 7, minimum peptide matches per protein set to 3, primary digest reagent set to non-specific, number of missed cleavages 0, false discovery rate of 0.01. The output peptides were filtered using DynaXm (3.0) using the following filtering parameters: minimum intensity of 2500, minimum and maximum peptide sequence length of 5 and 30, respectively, minimum MS/MS products of 3, minimum product mass per amino acid of 0.1, and a minimum peptide score of 5. In addition, all the spectra were visually examined and only those with high signal to noise ratios were used for HDX MS analysis. The amount of relative deuterium uptake for each peptide was determined using DynaXm (3.0) and are not corrected for back exchange. State (listing the deuterium uptake per-peptide, per-timepoint and per-experimental state) and difference (listing the difference in deuterium uptake between identical peptides of two states compared for each timepoint) were exported from DynaXm. These files were input to DeuterX55 (1.0.8) which formats the differential data into the Woods Plot format. Statistical filtering of peptides is then performed to identify those which exhibit significant uptake differences between the two states. DeuterX applies a blanket confidence interval (specifically, the 98% confidence interval given as 0 ± DU where DU is the Deuterium uptake threshold in Daltons) across all peptides of each timepoint. Non-significant peptides for each timepoint are then exported into a formatting script which is used to project the filtered data onto a 3D model of the protein (Supplementary Figs. 19 and 20).
digestion (CSNWT–CRL2, CSNWT and CSN–CRL2–N8) and (ii) digested in solution (CSNWT–CRL2) and generated peptides were pre-fractionated by gel filtration (CSNWT–CRL2). Gel electrophoresis was performed using the NuPAGE system according to manufacturer’s protocols. In-gel digestion was performed as described before. Digestion in solution was performed in the presence of RapiGest (Waters) according to manufacturer’s protocols. For gel filtration, peptides were dissolved in 30% acetonitrile (ACN), 0.1% trifluoroacetic acid and separated on a Superdex Peptide PC 3.2/30 column (GE Healthcare) at a flow rate of 50 μl min⁻¹.

**Mass spectrometry for XL-MS.** Peptides were dissolved in 2% ACN, 0.1% FA and separated by nano-flow liquid chromatography ( Dionex Ultimate 3000 RSLC, Thermo Scientific; mobile phase A: 0.1% (v/v) FA; mobile phase B: 80% (v/v) ACN, 0.08% (v/v) FA). Peptides were loaded onto a trap column (μ-Pre-column, C18, 100 μm i.d., particle size 3 μm; Thermo Scientific) and separated with a flow rate of 300 nL per minute. On an analytical C18 capillary column (Acclaim PepMap 100, C18, 75 μm i.d.; Particle Size 3 μm; Thermo Scientific), with a gradient of (v/v) mobile phase B over 66 min. Separated peptides were directly eluted into a Q Exactive Plus hybrid quadrupole-Orbitrap (CRL2 and CRL2–N8) or an Orbitrap Fusion Tribrid Mass Spectrometer (CSN–CRL2–N8) (Thermo Scientific).

Typical mass spectrometric conditions for the Q Exactive Plus were: spray voltage of 1.6–2.1 kV; capillary temperature of 250°C; normalised collision energy of 30%, activation Q of 0.25. The mass spectrometer was operated in data-dependent mode. Survey full scan MS spectra were acquired in the Orbitrap from 350–1600 or 2000 m/z with a resolution of 70,000 at an automatic gain control (AGC) target of 3 × 10⁶. The top 20 most intense ions were selected for Higher Energy Collisional Dissociation (HCD) MS/MS fragmentation in the Orbitrap (isolation window, 1.5 or 1.6 m/z). MS/MS spectra were acquired at a resolution of 17,500 at an AGC target of 1 × 10⁶ or 5 × 10⁶. Previously selected ions within previous 30 s were dynamically excluded for 30 s. Only ions with charge states 2− were selected. Singly charged ions as well as ions with unrecognised charge state were excluded. Internal calibration of the Orbitrap was performed using the lock mass option (lock mass: m/z 445.120025). Typical mass spectrometric conditions for the Orbitrap Fusion were: spray voltage of 2.5 kV; capillary temperature of 275°C; collision energy of 30% and activation Q of 0.25. The mass spectrometer was operated in data-dependent mode. Survey full scan MS spectra were acquired in the Orbitrap from 500–1700 or 2000 m/z with a resolution of 120,000 at an AGC target of 5 × 10⁶. The most intense ions were selected for high MS/MS fragmentation in the Orbitrap (3 s cycle time with a maximum injection time of 128 ms; isolation window, 1.6 m/z). MS/MS spectra were acquired at a resolution of 30,000 at an AGC target of 5 × 10⁶. Previously selected ions within previous 30 s were dynamically excluded for 30 s. Only ions with charge states 3−8 were selected. Singly and doubly charged ions as well as ions with unrecognised charge state were excluded. Internal calibration of the Orbitrap was performed using the lock mass option (lock mass: m/z 445.120025).

**Data analysis for XL-MS.** Raw files were converted into Mascot generic format (mgf) files using pXtract (https://pfnl.ict.ac.cn/software/pXtract/index.html). Mgf files were searched against a reduced database containing CSN and CRL2 proteins using pLink1.0 search engine. Search parameters were: instrument spectra, HCD; enzyme, trypsin; max missed cleavage sites, 3; variable modifications, oxidation (methionine) and carbamidomethylation (cysteine); cross-linker, BS3; min peptide length, 75; max peptide length, 350; mobile phase A: 0.1% (v/v) FA; mobile phase B: 80% (v/v) ACN, 0.1% triethylamine; flow rate of 50 µl min⁻¹. Peptides were dissolved in 2% ACN, 0.1% FA and separated by nano-flow liquid chromatography (Dionex UltiMate 3000 RSLC, Thermo Scientific; mobile phase A: 0.1% (v/v) FA; mobile phase B: 80% (v/v) ACN, 0.08% (v/v) FA). Peptides were loaded onto a trap column (μ-Pre-column, C18, 100 μm i.d., particle size 3 μm; Thermo Scientific) and separated with a flow rate of 300 nL per minute. On an analytical C18 capillary column (Acclaim PepMap 100, C18, 75 μm i.d.; Particle Size 3 μm; Thermo Scientific), with a gradient of (v/v) mobile phase B over 66 min. Separated peptides were directly eluted into a Q Exactive Plus hybrid quadrupole-Orbitrap (CRL2 and CRL2–N8) or an Orbitrap Fusion Tribrid Mass Spectrometer (CSN–CRL2–N8) (Thermo Scientific).

Typical mass spectrometric conditions for the Orbitrap Fusion were: spray voltage of 1.6–2.1 kV; capillary temperature of 250°C; normalised collision energy of 30%, activation Q of 0.25. The mass spectrometer was operated in data-dependent mode. Survey full scan MS spectra were acquired in the Orbitrap from 350–1600 or 2000 m/z with a resolution of 70,000 at an automatic gain control (AGC) target of 3 × 10⁶. The top 20 most intense ions were selected for Higher Energy Collisional Dissociation (HCD) MS/MS fragmentation in the Orbitrap (isolation window, 1.5 or 1.6 m/z). MS/MS spectra were acquired at a resolution of 17,500 at an AGC target of 1 × 10⁶ or 5 × 10⁶. Previously selected ions within previous 30 s were dynamically excluded for 30 s. Only ions with charge states 2− were selected. Singly charged ions as well as ions with unrecognised charge state were excluded. Internal calibration of the Orbitrap was performed using the lock mass option (lock mass: m/z 445.120025). Typical mass spectrometric conditions for the Orbitrap Fusion were: spray voltage of 2.5 kV; capillary temperature of 275°C; collision energy of 30% and activation Q of 0.25. The mass spectrometer was operated in data-dependent mode. Survey full scan MS spectra were acquired in the Orbitrap from 500–1700 or 2000 m/z with a resolution of 120,000 at an AGC target of 5 × 10⁶. The most intense ions were selected for high MS/MS fragmentation in the Orbitrap (3 s cycle time with a maximum injection time of 128 ms; isolation window, 1.6 m/z). MS/MS spectra were acquired at a resolution of 30,000 at an AGC target of 5 × 10⁶. Previously selected ions within previous 30 s were dynamically excluded for 30 s. Only ions with charge states 3−8 were selected. Singly and doubly charged ions as well as ions with unrecognised charge state were excluded. Internal calibration of the Orbitrap was performed using the lock mass option (lock mass: m/z 445.120025).
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Acknowledgements

We thank Radoslav Enchev (The Francis Crick Institute, London) for the COP9 baculovirus plasmids, and Lucile Vyletova and Ruth Knight (Institute of Cancer Research, London) for their help with baculovirus expression and insect cell maintenance. We are grateful to Chris Richardson (Institute of Cancer Research, London) for IT support and to Jane Sandall (Institute of Cancer Research, London) for laboratory support. We thank the staff at EBC (Diamond Light Source), particularly Dan Clare and Yurie Chaban, for their support during cryo-EM data collection. We thank Antoni Borysk (King’s College London) for helpful guidance with HDX-MS instrumentation. We thank Jurgen Caesen (Hasselt University, Belgium) for helpful interpretation of the HDX-MS data. We thank Guido Gross and Don L. Rempel for providing their PHIMSTEX data analysis software. The London Interdisciplinary Biosciences Consortium (LIDCs) BBSRC Doctoral Training Partnership (BM/B009531/1) supports A.M.C.L. S.V.F., E.P.M. and F.R. are funded by Cancer Research UK (C12209/A16749). C.S. acknowledges funding from the Federal Ministry for Education and Research (BMBF, ZIK programme, 03Z222HN22), the European Regional Development Funds (FEPE, Z5/2016/04/78115) and the MLU Halle-Wittenberg. C.M. and A.P. are funded by the Wellcome Trust (109854/Z/15/Z) and a King’s Health Partners R&D Challenge Fund through the MRC.

Author contributions

E.P.M. and A.P. conceived and designed the research. A.M.C.L and N.B.C performed their support during cryo-EM data collection. We thank Antoni Borysk (King’s College London) for laboratory support. We thank Jurgen Caesen (Hasselt University, Belgium) for their contribution to the peer review of this work. Peer reviewer reports are available.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11772-y.

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