The CRL4\textsuperscript{DCAF1} cullin-RING ubiquitin ligase is activated following a switch in oligomerization state

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Abstract

The cullin-4-based RING-type (CRL4) family of E3 ubiquitin ligases functions together with dedicated substrate receptors. Out of the ~29 CRL4 substrate receptors reported, the DDB1- and CUL4-associated factor 1 (DCAF1) is essential for cellular survival and growth, and its deregulation has been implicated in tumorigenesis. We carried out biochemical and structural studies to examine the structure and mechanism of the CRL4\textsuperscript{DCAF1} ligase. In the 8.4 Å cryo-EM map of CRL4\textsuperscript{DCAF1}, four CUL4-RBX1-DDB1-DCAF1 protomers are organized into two dimeric sub-assemblies. This arrangement, the WD40 domain of DCAF1 mediates binding with the cullin C-terminal domain (CTD) and the RBX1 subunit of a neighboring CRL4\textsuperscript{DCAF1} protomer. This renders RBX1, the catalytic subunit of the ligase, inaccessible to the E2 ubiquitin-conjugating enzymes. Upon CRL4\textsuperscript{DCAF1} activation by neddylation, the interaction between the cullin CTD and the neighboring DCAF1 protomer is broken, and the complex assumes an active dimeric conformation. Accordingly, a tetramerization-deficient CRL4\textsuperscript{DCAF1} mutant has higher ubiquitin ligase activity compared to the wild-type. This study identifies a novel mechanism by which unnedylated and substrate-free CUL4 ligases can be maintained in an inactive state.

Keywords CRL4/DCAF1; E3 ligases; Oligomerization; Ubiquitin; VprBP
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Introduction

DCAF1 is an essential substrate receptor for the cullin 4 RING ubiquitin E3 ligase family. DCAF1 is implicated in fundamental cellular processes, ranging from DNA replication (McCall et al., 2008), cell cycle progression (McCall et al., 2008; Guo et al., 2016), transcription (Wang et al., 2017), to zygotic development and reproduction (Yu et al., 2013). CRL4\textsuperscript{DCAF1} (CUL4-DDB1-RBX1-DCAF1) activity requires the direct interaction between the DCAF1 substrate receptor and various reported substrates such as the replication factor MCM10 (Kaur et al., 2012), p53 (Hrecka et al., 2007; Guo et al., 2016), FoxM1 (Wang et al., 2017), TET methylcytosine dioxygenases (Yu et al., 2013), and protein phosphatase 2A (Yu et al., 2015). DCAF1 is conserved among metazoans and is ubiquitously expressed in different tissues (Zhang et al., 2001). Loss of DCAF1 in mice leads to early embryonic lethality (McCall et al., 2008). DCAF1 ablation is often associated with defects in cell cycle, cell division, and cell survival (Schaiba et al., 2019). A comprehensive high-throughput RNAi screening study in ~400 different cell lines found that loss of DCAF1 is pan-lethal (McDonald et al., 2017), with DCAF1 knock-down induced lethality being the highest among all reported CUL4 DCAF substrate receptors in the study (Fig EV1A). CRL4\textsuperscript{DCAF1} is also the second most abundant CRL4 complex, accounting for ~12% of all assembled CRL4 complexes, rendering DCAF1 a critical substrate receptor for the CRL4 system (Reichermeier et al., 2020).

The CRL4\textsuperscript{DCAF1} E3 ligase is frequently hijacked by viruses such as the human immunodeficiency virus type 1 and 2 (HIV-1, HIV-2) and their viral accessory proteins VPR and VPX (Romani & Cohen, 2012). Efficient replication of HIV in macrophages requires the interaction of VPR/VPX with DCAF1 (Kyei et al., 2015), which redirects the CRL4\textsuperscript{DCAF1} ubiquitin ligase activity toward degradation of host proteins such as UNG2 (Wu et al., 2016) and SAMHD1 (Schwefel et al., 2014). Viral-induced degradation of host proteins via
CRL4<sub>DCAF1</sub> was shown to enhance HIV infection by facilitating viral replication and propagation (Baldauf et al., 2017).

Several lines of evidence further implicate DCAF1 in cancer progression. Hrecka et al. (2007) reported that DCAF1 depletion stabilizes the expression of p53 and several of its target genes. Follow-up studies linked the role of DCAF1 to regulating p53 transcriptional activity in different cell types (Kim et al., 2012; Guo et al., 2016; Wang et al., 2016a). Recent work has also found that MERLIN, a protein that is commonly mutated in several glial cancers, mediates its tumor suppression activities via inactivation of CRL4<sub>DCAF1</sub> (Li et al., 2010, 2014).

Despite the growing interest in studying DCAF1 physiological functions, the molecular determinants of DCAF1 regulation are currently poorly understood. CRL systems share common modes of regulation including activation by the ubiquitin-like protein NEDD8 (neural precursor cell expressed, developmentally downregulated 8) (neddylation) (Duda et al., 2005), inactivation by the multisubunit deneddylyase COP9 signalosome (CSN) (Lingaraju et al., 2014), and substrate receptor (SR) exchange mediated by CAND1 (Cullin-associated Nedd8-dissociated protein) (Pierce et al., 2013). Besides the standard CRL regulatory mechanisms, DCAF1 has been suggested to be governed by additional modes of activity control. For instance, MERLIN mediates direct binding to DCAF1 and has been linked to CRL4<sub>DCAF1</sub> ligase inhibition by as an as yet undefined mechanism (Li et al., 2010). Moreover, DCAF1 is known to dimerize in vivo and in vitro, and dimerization is proposed to enhance CRL4<sub>DCAF1</sub> ubiquitination activity in vitro (Ahn et al., 2011). The SECExplorer workflow fractionates native protein complexes by size-exclusion chromatography (SEC) followed by mass spectrometry to identify proteins in each fraction, along with an apparent molecular weight for the eluting complexes (Heusel et al., 2019). The size-exclusion profile in SECExplorer finds DCAF1 eluting with large native protein complexes of more than 1 MDa.

In the current study, we have used protein biochemistry and cryo-electron microscopy (cryo-EM) to characterize the CRL4<sub>DCAF1</sub> complex in vitro. We obtained an 8.4 A cryo-EM map of CRL4<sub>DCAF1</sub>, which finds the complex in a tetrameric arrangement. In the tetrameric conformation the DCAF1 WD40 domain mediates the interaction with the cullin C-terminal domain (CTD) and RBX1 of a neighboring CRL4<sub>DCAF1</sub> protomer. This architecture renders the RING domain of RBX1 inaccessible for the E2 ubiquitin-conjugating enzyme. Upon activation of CRL4<sub>DCAF1</sub> by neddylation, the interaction between the cullin CTD and DCAF1 is disrupted and the complex becomes dimeric and active. This dimeric state is also induced when the viral proteins VPR-UNG2 are bound to CRL4<sub>DCAF1</sub>, while the presence of MERLIN is compatible with the tetrameric autoinhibited state of the ligase. These results suggest a novel mechanism by which the activity of isolated CRL4<sub>DCAF1</sub> ligase is regulated by oligomerization.

Results

CRL4<sub>DCAF1</sub> exists in a dimer/tetramer equilibrium

The 1507 amino acid human DCAF1 receptor consists of a HLH (helix-loop-helix) and a WD40 domain at the C-terminus, which together mediate DDB1 binding (Fig 1A), and in turn bridges DCAF1 to the cullin scaffold (Fig EV1B). A highly acidic tail spans the very C-terminus of DCAF1, which is reported to mediate MERLIN and p53 binding (Mori et al., 2014; Wang et al., 2016a). The large N-terminal domain of DCAF1 is predicted to assume an Armadillo-like fold (Zimmermann et al., 2018). A Lis1 homology (LisH) motif spans residues 846–878 at the N-terminus and has been implicated in DCAF1 dimerization (Ahn et al., 2011).

To investigate the oligomeric state of CRL4<sub>DCAF1</sub>, we first purified the wild-type CRL4<sub>DCAF1</sub> complex and performed a neddylation reaction to generate N8-CRL4<sub>DCAF1</sub> complex (Fig 1B). This was followed by multi-angle light scattering coupled with size-exclusion chromatography (SEC-MALS) on the neddylated (N8-CRL4<sub>DCAF1</sub>) and unneddylated (CRL4<sub>DCAF1</sub>) complexes. While the SEC-MALS analysis of the neddylated state of CRL4<sub>DCAF1</sub> gives a molecular weight (MW) consistent with a dimeric complex (observed MW 780 kDa, calculated MW 810 kDa), the unneddylated form of CRL4<sub>DCAF1</sub> has an estimated MW equivalent to a tetramer (observed MW 1,561 kDa, calculated MW 1,582 kDa) (Fig 1C). The polydispersity values (M<sub>w</sub>/M<sub>n</sub>) are 1.002 and 1.000, respectively, in line with these complexes being present as single, mono-disperse species (Fig 1C). These data suggest that CRL4<sub>DCAF1</sub> switches its oligomeric form as a function of neddylation state. SEC-MALS analysis on an N-terminally truncated mutant of CRL4<sub>DCAF1</sub>(<sup>ANTD</sup> 987–1,507), lacking the LisH motif, finds a MW of 289 kDa, which corresponds to a monomeric complex with a MW of 287 kDa (Fig EV1C and D), linking the LisH motif in DCAF1 to dimerization (Ahn et al., 2011). SEC-MALS analysis on the isolated DDB1-DCAF1 (FL) shows this subcomplex elutes as a dimer (observed MW 563 kDa, calculated MW 592 kDa) (Fig EV1C and D), suggesting a role of the cullin-RBX1 scaffold in tetramer formation.

The RBX1 interaction with DCAF1 drives tetramer formation

To gain molecular insights into neddylation-dependent changes in the oligomeric state of CRL4<sub>DCAF1</sub>, we pursued structural analysis by single particle cryo-EM. In an effort to increase the stability of CRL4<sub>DCAF1</sub> protein sample for freezing cryo-EM grids, we had to cross-link the sample using a gradient fixation protocol (GraFix) (Stark, 2010). This enabled us to obtain an 8.4 A-resolution cryo-EM map of the unneddylated CRL4<sub>DCAF1</sub> complex (Fig EV2A–C). In the cryo-EM map, CRL4<sub>DCAF1</sub> assemblies into a tetrameric arrangement (Fig 2A and B). Intrinsic flexibility between the protomers of this assembly likely prevented us from obtaining higher-resolution structural insight. The cryo-EM map was interpreted by rigid body and restrained flexible fitting of crystallographic models of CUL4-DDB1-RBX1 (PDB 2HYE), and DDB1-DCAF1 (WD40) (PDB 5JK7), which allowed defining the overall architecture of the assembly.

The CRL4<sub>DCAF1</sub> map indicates a potential intermolecular interaction between the DCAF1 (WD40) of one molecule, with the cullin C-terminal domain (CTD) and RBX1 of a neighboring protomer (Fig 3A). An elementary dimer formed by two CRL4<sub>DCAF1</sub> molecules is also evident in the map and is consistent with the interaction of two neighboring LisH domains located proximal to the WD40 domain (Fig EV3A). The tetrameric CRL4<sub>DCAF1</sub> assembly in our structure is thus comprised of two CRL4<sub>DCAF1</sub> dimers. The structural data are consistent with the results obtained by SEC-MALS, illustrating that CRL4<sub>DCAF1</sub> is tetrameric, and that tetramerization is mediated by the interactions of the cullin CTD-RBX1 and DCAF1 WD40 domain.
domain at one dimerization interface, and the DCAF1 N-terminal LisH motifs at the other.

**Modeling of the DCAF1 Armadillo (ARM) and LisH domains**

The putative LisH density proximal to the WD40 domain showed helical features and strongly suggested the presence of a two-fold symmetry axis in line with previously observed LisH domain homodimers (PDB 1UUJ, PDB 1VYH) (Kim *et al.*, 2004; Tarricone *et al.*, 2004). The LisH domain fold is characterized by a two-helix bundle (helices 1 and 2) with a third helix (helix 3) crossing the helical bundle (e.g. PDB 1UUJ, PDB 6IWV). Upon homodimerization, the two-helix bundles form a four-helix bundle in which helices 1 and 2 align in an anti-parallel and slightly diagonal manner, respectively. In several structures, helices 3 of the homodimeric assembly additionally align in a diagonal manner.
perpendicular to the four-helix bundle (PDB 1UJJ, PDB 6IWV). The DCAF1 LisH domain structure was predicted by comparative modeling with high confidence (confidence score of 0.67, Song et al., 2013). To obtain a model for the homodimeric complex, the monomeric consensus model from comparative modeling (aa 846–883) was superposed on the dimeric LisH domains from PDB 6IWV. The homodimer interface was independently validated by docking two separated LisH monomer models against each other, imposing twofold symmetry constraints (see Materials and Methods). In these simulations, the LisH dimer interface previously observed in homologous dimeric LisH crystal structures gave the best docking scores (Fig EV3B). The dimeric homology model (obtained from superposition on a template structure) was then docked into the putative LisH density (Fig EV3A and C). The density supports an anti-parallel alignment of helices 2, while the putative density for helices 1 is fragmented. We observed only uninterpretable density at the expected location for helices 3 and refrained from modeling this helix given the limited local resolution.

A significant portion of uninterpreted density located between DDB1 BPA and DDB1 BPB of different protomers and close to the putative LisH density showed features indicative of several α-helical bundles. These features would be in agreement with an armadillo fold predicted for the segment N-terminal to the DCAF1 LisH domain (ARM, 1–817). To obtain a model for this part, we employed the deep learning-based structure prediction pipeline AlphaFold that has been shown to yield highly accurate predictions.

**Figure 2. The CRL4<sup>DCAF1</sup> assembles a tetrameric complex.**

A, B Different views of CRL4<sup>DCAF1</sup> cryo-EM map (8.4 Å) with fitted crystal structures of CUL4A (gray), RBX1 (red) (PDB 2HYE), DDB1 BPA (light blue), BPB (cyan), and BPC (dark blue), DCAF1 WD40 domain (yellow) (PDB 5jk7), LisH domain (orange), and ARM domain (brown) in (A) surface representation, and (B) cartoon representation.
Figure 3.
The DCAF1 WD40 domain is essential for tetramer formation.

A CRL4\textsuperscript{DCAF1} cryo-EM map (8.4 Å) with fitted model (left), and a close-up view of the CRL4\textsuperscript{DCAF1} map shows the interaction between DCAF1 (WD40) in yellow and RBX1 in red. The loop in DCAF1 (WD40) that contains the mutated arginine (R1247) is colored in violet. Circled numbers indicate the respective blades in the WD40 β-propeller. The RBX1 segment potentially contacting the V35A loop in DCAF1 is indicated by flanking residue numbers. Conservation plot in the region around the residue R1247 in DCAF1 is shown to the lower right.

B SEC-MALS analysis of CRL4\textsuperscript{DCAF1} and CRL4\textsuperscript{DCAF1} (R1247A) mutant. The chromatogram displays Rayleigh ratio curves of CRL4\textsuperscript{DCAF1} WT (blue) and mutant CRL4\textsuperscript{DCAF1} (R1247A) (red) together with the molar mass (MDa) of the main peaks calculated by MALS. The table summarizes the SEC-MALS observed molecular weights in the main peaks, the calculated molecular weight, polydispersity values, and oligomeric states of the tested complexes.

C Autoubiquitination of DCAF1 by wild-type CRL4\textsuperscript{DCAF1} (FL) (top) or mutant CRL4\textsuperscript{DCAF1} (FL) (R1247A) (bottom) observed after incubation with UBA1, UbcH5a, Ubiquitin (WT), at 30°C for 0 to 15 min as indicated (n = 3).

D Ubiquitination of UNG2 by wild-type CRL4\textsuperscript{DCAF1}-VPR (FL) (top) or mutant CRL4\textsuperscript{DCAF1}-VPR (FL) (R1247A) (bottom) observed after incubation with UBA1, UbcH5a, Ubiquitin (WT), at 30°C for 0 to 15 min as indicated (n = 3).

Source data are available online for this figure.

Figure 3. The DCAF1 WD40 domain is essential for tetramer formation.

The subnanometer CRL4\textsuperscript{DCAF1} cryo-EM map allowed fitting of the DCAF1 WD40 and RBX1 domains into the four copies with reasonable confidence. Within the RBX1/DCAF1 region, we identified a highly conserved loop at the surface of the WD40 propeller that approaches RBX1, namely, loop 1246\textsuperscript{VRS}1249 (Fig 3A). We introduced an alanine mutation at the conserved arginine residue 1247 within this loop, and analyzed the mutated CRL4\textsuperscript{DCAF1} complex by SEC-MALS. SEC-MALS analysis showed that this single mutation R1247A is sufficient to disassemble the tetrameric complex into a fully dimeric one (Fig 3B). These data support the model interpretation where the interaction between DCAF1 and the RBX1 RING domain is a key driver in tetramerization, and further confirms the assignment of the two elementary CRL4\textsuperscript{DCAF1} dimers that together form the tetramer.

The cryo-EM map finds the RBX1 RING domain shielded by the WD40 domain of DCAF1, rendering it inaccessible for an Ub-loaded E2 (Fig 3A). To test whether the tetrameric CRL4\textsuperscript{DCAF1} assembly represents an inactive autoinhibited state, we carried out auto-ubiquitination assays using the wild-type and R1247A mutant of CRL4\textsuperscript{DCAF1} (FL). The wild-type tetrameric CRL4\textsuperscript{DCAF1} complex shows a prominent reduction in DCAF1 auto-ubiquitination as compared to the dimeric mutant CRL4\textsuperscript{DCAF1} (R1247A) (Fig 3C). Reduced catalytic activity of the tetrameric CRL4\textsuperscript{DCAF1} compared to the dimeric mutant was also observed toward the viral substrate VPR-UNG2 (Fig 3D). On the other hand, preventing tetramer formation by incorporating the mutant DCAF1 (R1247A) into the complex overcomes auto-inhibition and gives rise to a more active E3 ligase (Fig 3C and D). Taken together, these data suggest that the tetrameric CRL4\textsuperscript{DCAF1} ligase represents a conformation with a significantly reduced catalytic activity.

Tetrameric CRL4\textsuperscript{DCAF1} counteracts neddylation and de neddylation

In our MALS studies, the neddylated CRL4\textsuperscript{DCAF1} complex predominately existed in a dimeric state (Fig 1C). To test whether the tetramer allows for direct access of the neddylation machinery, we used the tetrameric CRL4\textsuperscript{DCAF1} cryo-EM map and modeled the position of the putative RBX1-UBC12 complex (PDB 4PSO) (Fig 4A). The presence of UBC12, the NEDD8 E2, resulted in substantial steric clashes with the neighboring DCAF1 molecule (Fig 4A), suggesting that the tetrameric confirmation of CRL4\textsuperscript{DCAF1} is inhibitory to neddylation without structural rearrangements. To test this hypothesis, we established a neddylation assay, where we directly compared the amount of neddylated CRL4\textsuperscript{DCAF1} (WT) and CRL4\textsuperscript{DCAF1} (R1247A) dimeric mutant. While CRL4\textsuperscript{DCAF1} (R1247A) is efficiently and fully neddylated by UBC12 within 1 min (Fig 4B, left panel), the tetrameric CRL4\textsuperscript{DCAF1} is substantially more resistant to neddylation, as evident by longer incubation times, of up to 180 min,
Figure 4. The tetrameric conformation renders CRL4<sup>DCAF1</sup> refractory toward regulation by neddylation and deneddylation.

A CRL4<sup>DCAF1</sup> cryo-EM map and a close-up view of fitted UBC12-RBX1 structure (PDB 4P50) that shows steric clashes with DCAF1 (WD40)<sub>1</sub> of one CRL4<sup>DCAF1</sup> molecule (yellow) (PDB 5JK7) and RBX1<sub>2</sub> of the second CRL4<sup>DCAF1</sup> molecule (red), UBC12 is shown in orange (PDB 4P50), DDB1 BPA (light blue), BPB (cyan), and BPC (dark blue), CUL4A in gray. Molecules belonging to different protomers are indicated by superscripts (1 or 2).

B SDS–PAGE showing the neddylation reaction time course of CRL4<sup>DCAF1</sup> (FL) WT and CRL4<sup>DCAF1</sup> (FL) (R<sub>1247A</sub>) mutant in the presence of 1 μM UBC12 for 0 to 180 min as indicated. Left panel with t = 0 to 10 min, and right panel with t = 5 to 180 min (n = 3).

C Different views of CRL4<sup>DCAF1</sup>-CSN cryo-EM map with fitted crystal structures of CUL4A (gray) (PDB 2HYE), RBX1 (red), DDB1 BPA (light blue), BPB (cyan), BPC (dark blue), DCAF1 WD40 (yellow), DCAF1 NTD (yellow-green), and CSN (PDB 4D10) (wheat).

Source data are available online for this figure.
required to fully neddylate the CRL4\textsubscript{DCAF1} (WT) complex (Fig 4B, right panel). These data illustrate the inhibitory effect of RBX1 sequestration by DCAF1 (WD40) in the tetrameric CRL4\textsubscript{DCAF1} conformation. Within the dimer to tetramer equilibrium, it is the dimeric form of CRL4\textsubscript{DCAF1} that likely is preferentially neddylated.

The impaired neddylation of tetrameric CRL4\textsubscript{DCAF1} led us to investigate deneddylation, the opposing regulatory mechanism for CRLs catalyzed by the COP9 signalosome (CSN). Fitting the crystal structure of CSN (PDB 4D10) into the tetrameric CRL4\textsubscript{DCAF1} cryo-EM map suggests that tetramerization and CSN binding are mutually exclusive. This is evident in the steric clashes between CSN and the CRL4\textsubscript{DCAF1} tetramer (Fig EV4A). As previously reported, neddylated CRLs (N8-CRLs) bind CSN with very high affinity (Cavadini et al., 2016; Mosadeghi et al., 2016). The unnededlated CRLs, on the other hand, bind CSN with significantly lower affinities (Cavadini et al., 2016). Pull-down assays using an active site mutant of CSN (CSN5 E76A) and CRL4\textsubscript{DCAF1} (FL) or a CRL4\textsubscript{DCAF1} mutants, which lacks the N-terminus (973–1,507), shows that N8-CRL4\textsubscript{DCAF1} (FL) and N8-CRL4\textsubscript{DCAF1} mutants stoichiometric complexes with CSN (Fig EV4B, lanes 1 and 4). While the unnededlated CRL4\textsubscript{DCAF1} form stoichiometric complexes with CSN (Fig EV4B, lanes 2 and 3). Consistent with these findings, a 20 Å cryo-EM map of the N8-CRL4\textsubscript{DCAF1}-CSN complex shows that the complex adopts a dimeric architecture when bound to CSN, revealing the central LisH-mediated elemental dimer (Figs 4C and EV4C–E). Taken together, tetramerization of CRL4\textsubscript{DCAF1} is a conformational state of the ligase that interferes with CSN binding and activation.

Viral substrate binding to CRL4\textsubscript{DCAF1} favors the active, dimeric state of the ligase

During its maturation cycle, HIV-1 hijacks the CRL4\textsubscript{DCAF1} ligase via its VPR accessory protein, which binds the WD40 domain of DCAF1 and recruits host UNG2 for ubiquitination and subsequent degradation (Ahn et al., 2010; Wu et al., 2016). We set out to examine this viral-induced DCAF1 substrate for its ability to break the tetrameric CRL4\textsubscript{DCAF1} complex up into dimers. Generated CRL4\textsubscript{DCAF1} substrate, as well as the mechanistic characterization of this essential ubiquitin ligase. We identify a tetrameric CRL4\textsubscript{DCAF1} state that counteracts neddylation (Fig 4B) and auto-ubiquitination (Fig 3C) and by these measures likely represents the inhibited state of the E3 ubiquitin ligase. Substrate binding to CRL4\textsubscript{DCAF1} favors a dimeric, active conformation, which is also the preferred substrate of CSN, further underlining that this is the active conformation of the ligase. Structurally, the mechanism of DCAF1 inhibition by RBX1 sequestration is reminiscent of what has been previously observed for CSN2/RBX1 interactions in CSN structures (Emberley et al., 2012; Cavadini et al., 2016), as well as the arrangement seen in the GLOMULIN-CRL1-RBX1 complex (Duda et al., 2012). To our knowledge, this is the first report of a CRL with an inbuilt auto-inhibitory mechanism.

Several studies have suggested that the cullin CTD is flexible and may act as a regulatory element. The CTD of CUL4A and CUL4B are inherently flexible and can adopt both dimeric and tetrameric conformations, depending on the cell cycle stage and cellular conditions (Rathod et al., 2009; Li et al., 2010). Conversely, CUL1 has a more rigid CTD, which is crucial for its function as a scaffold in ubiquitin conjugation reactions (Bachinger et al., 2008; Fischer et al., 2011; Baek et al., 2020; Banchenko et al., 2021). These conformational changes triggered upon neddylation in turn may alter the interface between DCAF1 WD40 and RBX1. Unlike recent findings for the CUL1 systems (Baek et al., 2020), it is currently unclear how CUL4 ligases interact with ubiquitin loaded E2 enzymes. Given the extensive conformational rearrangements observed for CUL1 CTD, however, it is likely that a neddylated CRL4\textsubscript{DCAF1} in its E2 ubiquitin bound form would further impair tetramer formation in

Discussion

We herein provide the subnanometer map of the CRL4\textsubscript{DCAF1} E3 ubiquitin ligase, as well as the mechanistic characterization of this essential ubiquitin ligase. We identify a tetrameric CRL4\textsubscript{DCAF1} state that counteracts neddylation (Fig 4B) and auto-ubiquitination (Fig 3C) and by these measures likely represents the inhibited state of the E3 ubiquitin ligase. Substrate binding to CRL4\textsubscript{DCAF1} favors a dimeric, active conformation, which is also the preferred substrate of CSN, further underlining that this is the active conformation of the ligase. Structurally, the mechanism of DCAF1 inhibition by RBX1 sequestration is reminiscent of what has been previously observed for CSN2/RBX1 interactions in CSN structures (Emberley et al., 2012; Cavadini et al., 2016), as well as the arrangement seen in the GLOMULIN-CRL1-RBX1 complex (Duda et al., 2012). To our knowledge, this is the first report of a CRL with an inbuilt auto-inhibitory mechanism.
neous CRL4 activity and auto-ubiquitination in the absence of this inhibitory mechanism. We and others have observed spontaneous tetrameric complex and thereby convert it to a dimer. CUL4DCAF1 being in a dimer-tetramer equilibrium, we cannot exclude that Ub/Nedd8-loaded E2 complexes, or in fact also CSN, possess additional molecular mechanisms to open the CUL4DCAF1 tetrameric complex and thereby convert it to a dimer.

At this stage, we can only speculate as to the enzymatic role of CRL4DCAF1 substrate versus non-substrate binding proteins on the tetrameric configuration of CRL4DCAF1.

cells. Moreover, although our findings are consistent with CUL4DCAF1 being in a dimer-tetramer equilibrium, we cannot exclude that Ub/Nedd8-loaded E2 complexes, or in fact also CSN, possess additional molecular mechanisms to open the CUL4DCAF1 tetrameric complex and thereby convert it to a dimer.

At this stage, we can only speculate as to the enzymatic role of this inhibitory mechanism. We and others have observed spontaneous CRL4 activity and auto-ubiquitination in the absence of neddylation, in case of CRL4DDB2 (Scrima et al, 2008; Fischer et al, 2011; Cavadini et al, 2016), CRL4CRBN (Fischer et al, 2014; Petzold et al, 2016), and CRL4DCAF15 (Bussiere et al, 2020) using UBCH5a as ubiquitin-conjugating enzyme. This appears to be in contrast to the CUL1 system, which has been reported to be strictly dependent on neddylation for activity (Baek et al, 2020). Furthermore, studies with CSN inhibitors have reported CRL receptor instability (Schlierf et al, 2016) due to auto-ubiquitination of the receptor following neddylation.
persistent CRL activation. Although at lower flux, receptor auto-ubiquitination likely also occurs in cells with an active neddylation/deneddylase machinery. DCAF1 tetramerization may thus provide an additional layer of inhibition preventing futile DCAF1 receptor auto-ubiquitination and degradation cycles for substrate- or neddylation-free CRL4DCAF1. Given the sensitivity of cells toward DCAF1 loss, this may preserve the cellular CRL4DCAF1 pool. A somewhat different albeit non-mutually exclusive explanation is that the inactive form of CRL4DCAF1 serves to counteract spurious activation of the enzymes. This would assure that only a tightly bound substrate shifts the equilibrium to a dimeric, active form of the enzyme to then bestow CRL4DCAF1 neddylation and full activity.

We speculate that the CRL4DCAF1 dimer-tetramer transition is primarily regulated through neddylation and substrate binding. However, a possible regulatory mechanism involving post-translational modifications including phosphorylation can not be excluded at this point. We note that there are a number of residues in the CUL4 C-terminus expected to be phosphorylated (S642, Y744, and N751), based on the PhosphoSitePlus resource (Hornbeck et al., 2015). When phosphorylated, these residues have the potential to impact tetramerization in light of our structures and would allow activation.

The pronounced pan-letality of DCAF1 loss, poses an important role of DCAF1 for cell survival. As CUL1-based CRLs may intrinsically be more tightly regulated by neddylation than the CRL4 family, an additional auto-inhibitory circuitry, as observed for CRL4DCAF1 may thus be required for proper ligase function, targeting and regulation. Future experiments are required to assess the biological role of this inhibitory mechanism, and to examine whether other oligomeric CRLs use a similar mechanism.

Materials and Methods

Cloning, protein expression, and purification

Wild-type (WT) and mutant constructs of full-length human DCAF1 (Q9Y4B6) were cloned into pLAF vector, which is derived from the pBacPAK8 system (Clontech). Cullin 4A (FL) (Q13619) and DDB1 (FL) (Q16531) were assembled into one vector (pBIG1a) using Gibson-based technique (biGBac) (Weissmann et al., 2016). Recombinant baculoviruses were prepared in Spodoptera frugiperda (sf9) cells using the Bac-to-Bac system (Life Technologies). Recombinant protein complexes were expressed in Trichoplusia ni High Five cells by co-infection of 35 ml of single baculoviruses per 2 l of High Five culture. DCAF1 wild-type and mutants were expressed with N-terminal Strept (II) tag, and Cullin 4A and DDB1 were expressed with N-terminal His6 tags. MERLIN A (1–346) was expressed with an N-terminal glutathione S-transferase (GST) tag. CRL4DCAF1-CSN complex was co-expressed using the CSN4-RBX1 fusion construct, as described in Cavadini et al. (2016).

Cells were harvested 36-48 h after infection and lysed by sonication in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8.0, 200 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), including 0.1% Triton X-100, 1x protease inhibitor cocktail (Roche Applied Science), and 1 mM phenylmethanesulfonyl fluoride (PMSF). This was followed by ultracentrifugation at 40,000 g for 45 min to separate the soluble fraction of the lysate (supernatant). The supernatant was loaded on Strept-Tactin (IBA Life Sciences) affinity chromatography in buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5 mM TCEP. The Strep (II) elution fractions were further purified via ion exchange chromatography (Poros HQ 50 µm, Life Technologies) and subjected to size-exclusion chromatography (Superose 6, GE Healthcare) in a buffer containing 50 mM HEPES pH 7.4, 200 mM NaCl, and 0.25 mM TCEP. Pure fractions were collected and concentrated using 10,000 MW cut-off centrifugal devices (PALL) and stored at −80°C.

APPBP1-UBA3, UBC12, and NEDD8 were expressed and purified as described before Duda et al. (2008), Huang et al. (2007), and Huang et al. (2008).

In vitro Ubiquitination of CRL4DCAF1

In vitro ubiquitination was performed by mixing wild-type or mutant CRL4DCAF1 at 2 µM with a reaction mixture containing E1 (UBA1, Boston Biochem) at 0.1 µM, E2 (UBCH5a, Boston Biochem) at 0.1 µM, wild-type Ubiquitin (Ubiquitin, Boston Biochem) at 5 µM as indicated. Reactions were carried out in 50 mM Tris pH 7.7, 200 mM NaCl, 10 mM MgCl2, 0.2 mM CaCl2, 3 mM ATP, 2 mM DTT, and 0.1 mg/ml BSA, and incubated for 0–15 min at 30°C. Reactions were then analyzed by Western blot using anti-ubiquitin (P4D1) primary antibody (Santa Cruz, 1:500) and Horseradish Peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:10,000). Blots were incubated with SuperSignalTM West Pico PLUS Chemiluminescent Substrate solution (Thermo Fisher) and scanned on Fusion FX7 imaging system (Witec AG).

Pull-down assay of CRL4DCAF1 and CSN

N8-CRL4DCAF1 (FL), N8-CRL4DCAF1(NTD) (987–1,507), CRL4DCAF1 (FL), and CRL4DCAF1(NTD) were loaded on 20 µl Strept-Tactin Macroprep beads (IBA life sciences), and incubated with untagged and inactive CSN (CSN5 E76A) at 1:1 molar ratio for 1 h at 4°C. Bound complexes were washed three times with 1 ml buffer containing 50 mM HEPES pH 7.4, 200 mM NaCl, and 0.25 mM TCEP. Beads were boiled in SDS loading dye.

Size-Exclusion Chromatography-Multi-Angle Light Scattering (SEC-MALS)

The molecular masses of CRL4DCAF1 WT and mutant complexes were investigated by SEC-MALS using an ÄKTA FPLC system (GE Healthcare) equipped with a Superose 6 10/300 size-exclusion column (GE Healthcare Life Sciences). 50 µl of the protein samples at a concentration of 2 mg/ml was loaded. The light scattering measurements in the SEC-MALS system used an Optilab T-rEX refractive index detector and a miniDYN TREOS 3 angle MALS detector (Wyatt Technology). The samples were loaded on the SEC-MALS system in a buffer containing 50 mM HEPES pH 7.4, 200 mM NaCl and 1 mM TCEP. The protein fractions at/near the void volume eluted as heterogeneous high molecular weight aggregates, and we therefore did not further investigate their behavior (Fig EV5A–C).

Cryo-electron microscopy and image processing

In order to increase the stability of the CRL4DCAF1 and CRL4DCAF1-CSN complexes, gradient fixation (GraFix) protocol was performed
The purified human NEDD8 mutant (Methionine 1 to Cysteine, M1C) was incubated with 8 mM DTT at 4°C for 1 h to completely reduce the protein sample. Buffer exchange was performed in non-reducing buffer conditions, 50 mM Tris pH 7.5 and 150 mM NaCl. PT22-maleimide (TTP Labtech) were dissolved in 100% DMSO and mixed with NEDD8 in 4:1 ratio. The labeling reaction took place at room temperature after 3-h incubation in a vacuum desiccator, and the reaction continued overnight at 4°C. Labeled NEDD8 was purified on a Superdex 200 16/60 column in 50 mM Tris pH 7.5, 150 mM NaCl, 0.25 mM TCEP, and 10% (v/v) glycerol. The sample was then concentrated and stored at −80°C.

Model building and refinement

The two template structures with PDB IDs 2HYE and 5JK7 were iteratively re-refined against the crystallographic structure factors using Coot and Phenix (Angers et al., 2006; Emsley et al., 2010; Wu et al., 2016; Liebschner et al., 2019). The template models for CUL4A, RBX1, and DDB1 BPB were extracted from coordinates with PDB ID 2HYE (Table EV2), and the template models for DCAF1 and DDB1 BPA/BPC were extracted from coordinates with PDB ID 5JK7 and docked into the cryo-EM map using Chimera (Petersen et al., 2004). The structure was then refined using the Rosetta density-guided FastRelax protocol (torsional followed by Cartesian space) in combination with constraints to the starting model coordinates and symmetric scoring (Wang et al., 2016b). Before deposition, the model was truncated to poly-alanine and validation was performed using Phenoix and Molprobity (Chen et al., 2009). Figures were generated using ChimeraX (Petersen et al., 2020).

Labeling of NEDD8 with PET22-maleimide

The purified human NEDD8 mutant (Methionine 1 to Cysteine, M1C) was incubated with 8 mM DTT at 4°C for 1 h to completely reduce the protein sample. Buffer exchange was performed in non-reducing buffer conditions, 50 mM Tris pH 7.5 and 150 mM NaCl. PT22-maleimide (TTP Labtech) were dissolved in 100% DMSO and mixed with NEDD8 in 4:1 ratio. The labeling reaction took place at room temperature after 3-h incubation in a vacuum desiccator, and the reaction continued overnight at 4°C. Labeled NEDD8 was purified on a Superdex 200 16/60 column in 50 mM Tris pH 7.5, 150 mM NaCl, 0.25 mM TCEP, and 10% (v/v) glycerol. The sample was then concentrated and stored at −80°C.
Neddylation of Cullin 4 with wild-type or fluorophore-labeled NEDD8

*In vitro* neddylation was performed by mixing wild-type or mutant CRL4^DCAF1^ at 2 μM with a reaction mixture containing 0.15 μM APPBP1-UBA3, 6 μM wild-type NEDD8, and 1 μM UBC12. Reactions were carried out in 50 mM Tris-HCl pH 7.7, 200 mM NaCl, 2.5 mM MgCl₂, 5 mM DTT, and 1.25 mM ATP, and incubated at room temperature for 5–180 min as indicated.

The neddylation reaction with the fluorophore-labeled NEDD8 involves 0.3 μM APPBP1-UBA3, 1.2 μM UBC12, 4–8 μM CRL4^DCAF1^, 8–20 μM NEDD8, 2.5 mM MgCl₂, and 1.5 mM ATP. The reaction was carried out in HEPES pH 7.4, 200 mM NaCl, and 1 mM TCEP buffer at room temperature.

**Fluorescence polarization (FP)-based CSN activity assays**

NEDD8 was labeled with PT22-maleimide (TTP LabTech) and enzymatically conjugated to CRLs as described. 7- to 10-μl reaction mix contains 50 mM Tris-HCl pH 7.5, 200 mM NaCl, and 1 mM TCEP, 0.1% pluronic acid, and different concentrations of PT22-labeled CRL4^DCAF1^ (80 nM–1.5μM), and 15 nM CSN wild-type for CRL4^DCAF1^ and CRL4^DCAF1^, MERLIN (1–346), and 150 nM CSN wild-type for CRL4^DCAF1^, VPR and CRL4^DCAF1^, VPR-UNG2. Reactions took place at room temperature in 384-well plates in a BMG PheraStar plate reader (BMG LabTech). Initial rates were calculated by measuring the change in fluorescence polarization at 590 nm after excitation at 540 nm. Product formation was calculated as described in (Marks et al., 2005). Initial rate constants were calculated by plotting product formation (M) over time (s) using GraphPad Prism version 7.00 (GraphPad Software). $K_m$ and $K_{cat}$ were calculated by non-linear fitting of initial velocities $V_0$, in the Michaelis–Menten equation.

**Data availability**

The cryo-EM maps of the CRL4^DCAF1^ and CRL4^DCAF1^-CSN complexes are deposited in the Electron Microscopy database under the accession codes EMD-12964 (https://www.ebi.ac.uk/emdb/entry/EMD-12964) and EMD-12965 (https://www.ebi.ac.uk/emdb/entry/EMD-12965), respectively. The model coordinates for the CRL4^DCAF1^ structure are deposited in the Protein Data Bank under the accession code 7OKQ (https://www.rcsb.org/structure/7OKQ). Table EV1 summaries cryo-EM data collection, refinement, and validation statistics. Table EV2 includes the validation statistics for re-refined template models.

**Expanded View** for this article is available online.

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**Author contributions**

WIM prepared the specimens for EM data collection. WIM, SC, and ADS. collected EM data. WIM performed EM data processing, with help from SC and ADS. Model building and refinement was carried out by GK. WIM performed the activity assays with input from AP and WAR. WIM performed the biochemical assays. JR and KR contributed in data discussion. NHT, WIM, and AB wrote the manuscript with input from all authors.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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