LETTERS

Molecular architecture and assembly of the DDB1–CUL4A ubiquitin ligase machinery

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Protein ubiquitination is a common form of post-translational modification that regulates a broad spectrum of protein substrates in diverse cellular pathways[1]. Through a three-enzyme (E1–E2–E3) cascade, the attachment of ubiquitin to proteins is catalysed by the E3 ubiquitin ligase, which is best represented by the superfamily of the cullin-RING complexes[2,3]. Conserved from yeast to human, the DDB1–CUL4–ROC1 complex is a recently identified cullin-RING ubiquitin ligase, which regulates DNA repair[4–10], DNA replication[11–14] and transcription[15], and can also be subverted by pathogenic viruses to benefit viral infection[16]. Lacking a canonical SKP1-like cullin adaptor and a defined substrate recruitment module, how the DDB1–CUL4–ROC1 E3 apparatus is assembled for ubiquitinating various substrates remains unclear. Here we present crystallographic analyses of the virally hijacked form of the human DDB1–CUL4A–ROC1 machinery, which show that DDB1 uses one β-propeller domain for cullin scaffold binding and a variably attached separate double-β-propeller fold for substrate presentation. Through tandem-affinity purification of human DDB1 and CUL4A complexes followed by mass spectrometry analysis, we then identify a novel family of WD40-repeat proteins, which directly bind to the double-propeller fold of DDB1 and serve as the substrate-recruiting module of the E3. Together, our structural and proteomic results reveal the structural mechanisms and molecular logic underlying the assembly and versatility of a new family of cullin-RING E3 complexes.

Distinct from all known cullin adaptors, DDB1 is a large multi-domain protein featuring an independent β-propeller (BP) domain (BPB) flexibly connected to a clam-shaped double-propeller fold (BPA–BPC)[17]. To reveal how DDB1 incorporates into the CUL4A–ROC1 complex and mediates substrate recruitment, we determined the 3.1 Å crystal structure of a DDB1–CUL4A–ROC1 complex bound to the V protein of simian virus 5 (SV5-V), previously known as the VDC complex (Table 1). By docking the STAT proteins to DDB1, the V protein of the paramyxovirus is able to accelerate the ubiquitination and degradation of STAT1, thereby blocking the antiviral response of the host[16]. With all the components in their full-length forms, the 240 kDa quaternary protein complex represents a functionally complete form of the E3 apparatus.

The DDB1–CUL4A–ROC1–SV5-V complex has an overall elongated shape that is about 170 Å long and 100 Å wide, resembling SCF (SKP1–cullin–F-box-protein)[18] (Fig. 1). As a close paralogue of CUL1, CUL4A adopts a CUL1-like structure with an arc-shaped

Table 1 | X-ray refinement statistics

Resolution (Å)	3.1
R_{work}; R_{free} (%)	25.1; 31.6
Number of atoms	
Protein	16,937
Ligand/ion	5
Water	0
B-factors	
Protein	75.9
Ligand/ion	93.5
Water	NA
Root-mean-squared deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.57

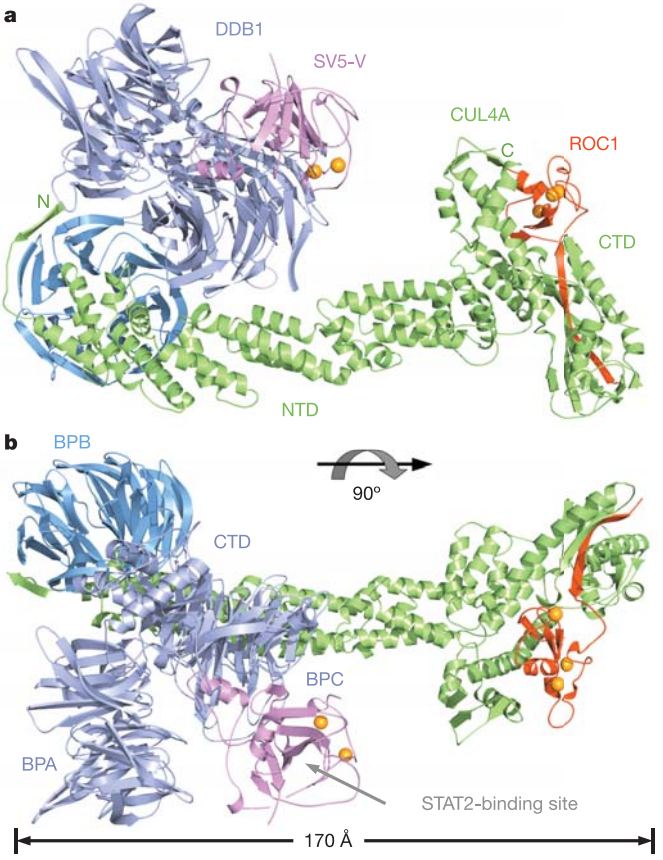

Figure 1 | Crystal structure of the DDB1–CUL4A–ROC1 ubiquitin ligase complex hijacked by the V protein of simian virus 5. a, **b**, Two orthogonal views of the complex structure are shown in ribbon diagram. DDB1, CUL4A, ROC1 and SV5-V are coloured in blue, green, red and magenta, respectively. The BPB domain of DDB1 is coloured in cyan. The zinc atoms in ROC1 and SV5-V are represented as orange spheres. A previously identified STAT2-binding site[17] of the viral protein, as well as the four domains of DDB1, are indicated.

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helical amino-terminal domain (NTD) and a globular carboxy-terminal domain (CTD). Lacking the SKP1/BTB fold, DDB1 devotes its entire BPB domain for CUL4A binding, anchoring at the tip of the NTD of cullin. The BPA–BPC double-propeller fold of DDB1, however, is positioned in the complex facing towards the E2-binding subunit of the E3 ligase, ROC1. As a result, the viral protein—which sits at the opening of the large pocket of the DDB1 double-propeller fold—is about 45 Å away from the RING finger subunit. By comparing this overall architecture of the virally hijacked DDB1–CUL4A–ROC1 complex with SCF[18], we conclude that the cullin-docking function and the substrate-presenting function of DDB1 are separately performed by the BPB domain and the BPA–BPC double-propeller fold, respectively.

The complex formation between DDB1 and CUL4A is predominantly mediated through two separate interfaces, both involving the BPB domain of DDB1 (Fig. 2a). At one interface, the top surface of the propeller fold of DDB1-BPB is recognized by the H2 and H5 helices of CUL4A, which are unexpectedly the same ones used by CUL1 to bind SKP1[18] (compare Fig. 2b, c). At the other interface, CUL4A further embraces the BPB domain of DDB1 using an N-terminal extension sequence, which is unique among all cullin family members[17]. Running across two blades of DDB1-BPB, this CUL4A N-terminal sequence wraps around the propeller of DDB1 at the peripheral side via a hydrogen-bond network and hydrophobic packing (Fig. 2a, d). The equal importance of these two separate interfaces in DDB1–CUL4A binding is reflected in the strict conservation of most of the interacting residues among CUL4 and DDB1 orthologues (Supplementary Fig. 1). Furthermore, mutations of the DDB1 or CUL4A residues involved in either interface can significantly weaken the complex formation[13,17]. Overall, the bipartite binding mode between DDB1 and CUL4A not only strongly supports a specific role of DDB1 in the CUL4A-based E3 machinery, but also demonstrates a surprising variation in cullin–adaptor interactions.

The crystal structures of DDB1 alone and in complex with SV5-V have previously revealed two distinct structural states of DDB1[17], which show dramatic differences in the relative orientation between the substrate-presenting BPA–BPC double-propeller fold and the cullin-binding BPB domain. In the quaternary-complex crystal, DDB1 adopts yet a new domain arrangement. Intriguingly, superposition analysis of the three structures indicates that all three domain configurations of DDB1 are in fact geometrically allowed in the context of the full DDB1–CUL4A–ROC1 complex (Fig. 2e). The BPA–BPC double-propeller folds of DDB1 in the resulting three models all similarly point towards the E2-binding RING subunit, even though the orientations of the fold itself are correlated by large degrees of rotation (compare Figs 1b and 2e). Because the BPA–BPC double-propeller fold of DDB1 has a very limited number of contacts with the cullin scaffold (Supplementary Fig. 2), we speculate that on binding to different cellular factors, DDB1 might be able to undergo conformational changes, which can modulate the ubiquitin ligase activity of the E3 machinery.

Although a multidomain structure might allow DDB1 to directly recruit substrates to the E3 platform, ubiquitination of several known DDB1–CUL4A substrates has been shown to require additional cellular factors[7,19]. Mimicked by the viral V protein, endogenous substrate-specific receptors might be used by DDB1 to expand its substrate repertoire. To further clarify the substrate targeting mechanisms of the DDB1–CUL4A E3 machinery, we used a proteomic approach optimized for isolating CUL4A and DDB1 protein complexes from mammalian cells by tandem-affinity purification[20]. In these analyses, more than 30 proteins were consistently identified by mass spectrometry, giving rise to a protein-interaction network shown in Fig. 3a.

Notably, besides several known cullin regulators and DDB1-binding proteins, numerous novel protein factors were identified in both the CUL4A and DDB1 complexes. In particular, a group of 16 proteins, including DDB2 and CSA, clearly stands out by sharing a predicted WD40 domain. The consistent presence and abundance of these WD40 proteins in the complexes strongly suggest that they represent bona fide members of the human DDB1–CUL4A E3 machinery (Supplementary Tables 2 and 3). It is also of note that reciprocal tandem-affinity pull-down analyses with DDB2 and two randomly selected WD40 proteins (H326 and WDTC1) confirmed that they are individually associated with the DDB1–CUL4A complex (Fig. 3a). Because many of these WD40 proteins do not have any known function other than DDB1–CUL4A binding, we propose to name them the DCAF (DDB1–CUL4A-associated factor) proteins (Supplementary Table 2).

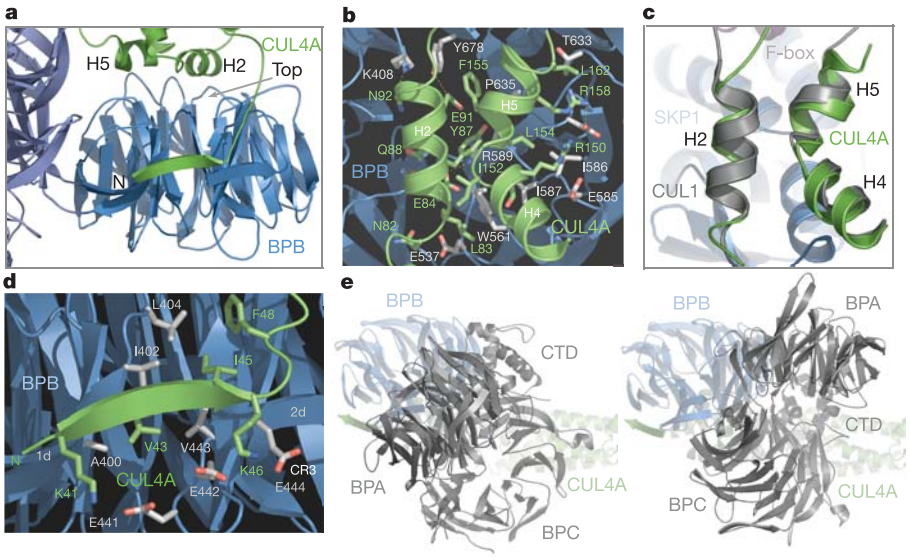

Figure 2 | Binding interfaces and structural flexibility of DDB1 on CUL4A. **a**, An overall view of the structural elements mediating the binding of the DDB1 BPB domain (cyan) with the CUL4A NTD (green). **b**, The interface between the top surface of DDB1-BPB (cyan) and the H2–H5 helices of CUL4A (green) viewed from above with participating side chains shown as sticks (grey in DDB1 and green in CUL4A). **c**, Superposition of the H2–H5 helices of CUL4A (green) and CUL1 (grey) with CUL1-bound SKP1 (blue) shown in the background. The view is the same as that shown in panel **b**. CUL4A-bound DDB1 is not shown for clarity. **d**, The interface between the CUL4A N-terminal extension sequence (green) and the peripheral side of the DDB1 BPB domain (blue). **e**, Superposition analyses of DDB1 alone (right) and DDB1–SV5-V complex (left) structures in the context of the full DDB1–CUL4A–ROC1 complex. The models are docked through the BPB domain and viewed from the same angles as shown in Fig. 1b.

As DDB2 and CSA have already been demonstrated necessary for DDB1–CUL4A-mediated ubiquitination of certain substrates, we speculated that the DCAF proteins we have identified might represent a novel family of proteins serving as the substrate-recruiting module for the E3 machinery. Using co-affinity purification experiments, we first confirmed our mass spectrometry results by showing the *in vivo* interactions of DDB1 with a panel of randomly selected DCAF proteins (Supplementary Fig. 3). Given that both DDB2 and CSA have been reported to directly bind DDB1, we next examined the direct interactions between DDB1 and two DCAF proteins. As shown in Fig. 3b, bacterially produced and purified glutathione *S*-transferase (GST)-tagged WDR23 (DCAF11) and H326 (DCAF8) were indeed able to interact with the purified recombinant DDB1 protein in an *in vitro* GST pull-down experiment.

If the DCAF proteins represent a family of substrate receptors for DDB1–CUL4A, then they might share a common DDB1-docking motif, analogous to the substrate receptors of other cullin E3 complexes[3]. As no recognizable common sequence other than the WD40 repeats was found among the DCAF proteins, we postulated that this motif might consist of amino acids exposed on the surface of their WD40 domains. Structure-based sequence analysis of the DCAF proteins revealed the presence of two conserved DxR motifs within the WD40 domain of almost all members (Fig. 3c). These two motifs are separately located at the end of strand c of two consecutive blades of the WD40 fold, and we propose to name these two motifs the 'double DxR box'. On the basis of several known WD40 domain structures[21,22], the conserved Asp and Arg residues in the double DxR box are predicted to be solvent-exposed, lining at the bottom surface of the WD40 propeller fold (Supplementary Fig. 4).

Although other unique and hidden features of the DCAF proteins might contribute to their specific interactions with DDB1, several lines of evidence suggest that the double DxR box is one of the main determinants for their binding to the CUL4A adaptor. First, a naturally occurring mutation (R273H) found in a subset of xeroderma pigmentosum group E (XPE) patients has been mapped to an arginine within the double DxR box motif present in DDB2. As previously reported[23] and confirmed by us (Fig. 3d, lane 2), this mutation abolishes the binding of DDB2 to DDB1. Second, our mutagenesis analysis of two representative DCAF proteins, WDR23 (DCAF11) and H326 (DCAF8), shows that both arginine residues in the double DxR box contribute to DDB1 binding. As shown in Fig. 3d, although single mutation of either of the two arginine residues in the double DxR box of the two DCAF proteins markedly weakens DDB1 binding (lanes 4, 5 and 8, 9), the double mutations completely ablate complex formation (lanes 6 and 10). Third, the double DxR box in two consecutive WD40 blades are also found in two fission yeast WD40 proteins, Cdt2 and Dos1 (also known as Clr8) (Fig. 3c), which are known to function with yeast DDB1–Cul4, and its close relative Rik1–Cul4, E3 complexes[19,24,25]. The double DxR motif, therefore, probably underlies an evolutionarily conserved mechanism for docking the substrate-recruiting module to the DDB1–CUL4A ubiquitin ligase machinery.

The presence of a conserved motif shared among the DCAF proteins predicts a common docking site on DDB1. The overall

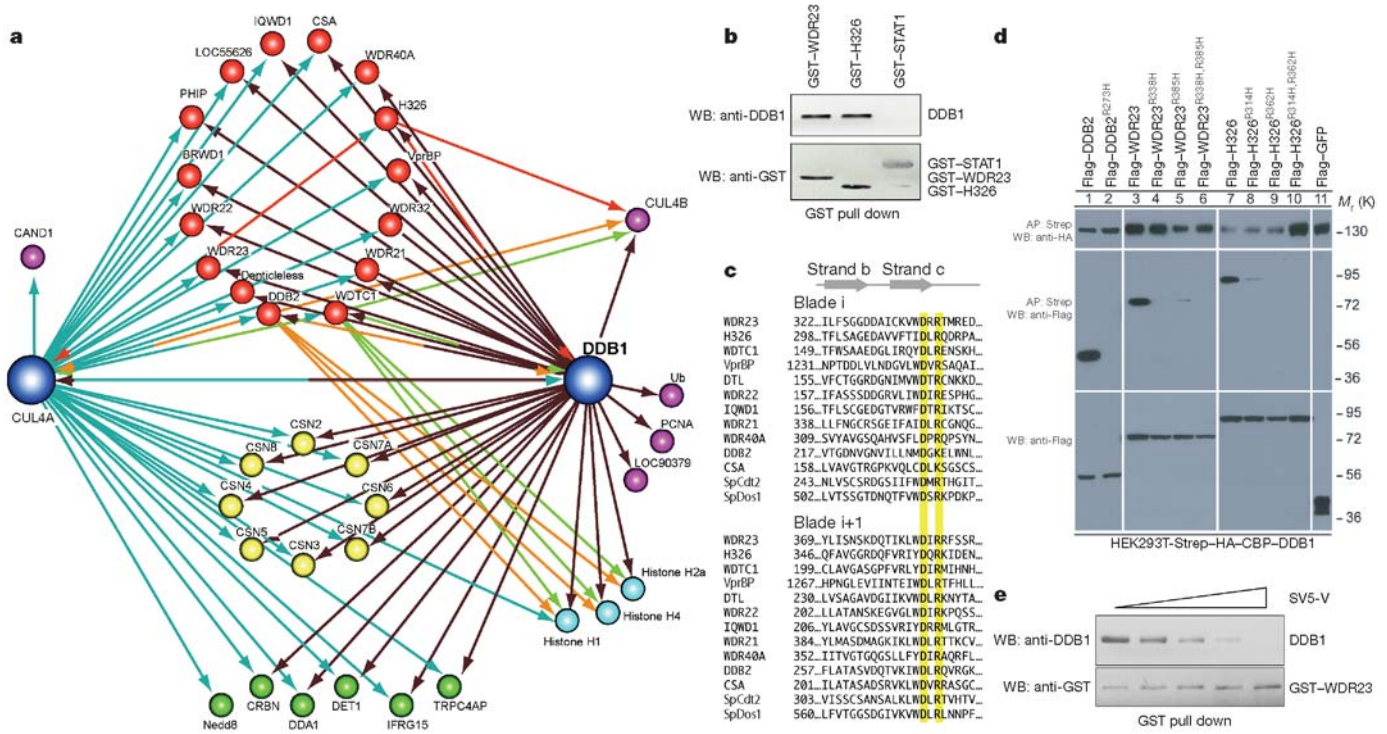

Figure 3 | Identification of a novel family of WD40 proteins as the potential substrate receptors for the DDB1–CUL4A E3 machinery. a, Mammalian protein interaction network of the DDB1–CUL4A ubiquitin ligases established by tandem-affinity analyses of CUL4A, DDB1, DDB2, WDTC1 and H326. The novel family of DDB1–CUL4A-associated WD40 domain proteins (DCAF proteins) is shown in red. Arrows represent unidirectional (single arrowhead) and bidirectional (double arrowhead) interactions. **b**, *In vitro* GST pull-down assay followed by western blot (WB) analysis showing the direct interactions between DDB1 and two DCAF proteins, WDR23 (DCAF11) and H326 (DCAF8), with STAT1 as a negative control. **c**, Sequence alignment showing the DxR motifs (highlighted in yellow) conserved in two consecutive β-propeller blades of the DCAF proteins identified in our studies and two yeast WD40 proteins known to bind the DDB1(Rik1)–Cul4A complex. The two β-strands preceding the DxR motifs are indicated. **d**, Mutagenesis of the arginine residues in the double DxR box of two representative DCAF proteins impairs DDB1 binding. Interactions between DDB1 and the wild-type and mutant DCAF proteins were assessed by co-affinity purification (AP) using streptavidin (DDB1 pull down) and western blot (WB) analysis using anti-Flag antibodies (DCAF proteins) (middle panel, lanes 1–10). DDB1 purification efficiency and the expression of the DCAF proteins were monitored as shown in the top and bottom panels, respectively. Flag–GFP was used as a negative control. **e**, Competition between SV5-V and WDR23 (DCAF11) for DDB1 binding. The experiments shown in panel **b** were repeated with increasing amounts of purified SV5-V protein. A molar ratio of 1:2 (DDB1:SV5-V) was reached in the final lane.

architecture of the virally hijacked DDB1–CUL4A–ROC1 complex strongly suggests that such a site might be located on the BPA–BPC double-propeller fold of DDB1, probably occupied by SV5-V, even though the viral protein does not have a WD40 domain. In fact, in an *in vitro* binding assay, association of DDB1 and two representative DCAF proteins, WDR23 (DCAF11) and WDTC1 (DCAF9) (data not shown), can be stoichiometrically competed off by increasing amounts of purified SV5-V protein (Fig. 3e). This *in vitro* result is consistent with a previous report showing that the same viral protein can compete with DDB2 for binding with DDB1[26]. It is interesting to note that, in order to anchor itself to DDB1, the viral protein not only binds to the surface of the BPA–BPC double-propeller fold of DDB1, but also inserts an entire helix into the large pocket of the DDB1 double-propeller fold. Although the size and secondary structure elements of a regular WD40 domain do not allow it to do so, it remains possible that some DCAF proteins might use sequence regions outside their WD40 domains to bind to the pocket of the DDB1 double-propeller fold, thereby further securing their associations with the CUL4A adaptor. Such an assembly mode could be used by the few DCAF proteins that lack a perfect double DxR box motif. Exactly how the DCAF proteins are docked to DDB1 awaits future structural studies.

As gene expression varies in different cell types and under different cellular states, the DCAF proteins identified in our studies are not expected to represent the complete family of substrate receptors of the DDB1–CUL4A E3 in human cells. Having established the structural and molecular basis of the assembly of the E3 machinery, our studies pave the road for a future understanding of the functions of the DDB1–CUL4A complex in eukaryotic biology and in human diseases.

METHODS

Protein preparation. Human CUL4A and ROC1 were co-expressed and purified from *Escherichia coli* and mixed with previously purified DDB1 and SV5-V[17]. WDR23 (DCAF11) (amino-acid residues 39–546) and H326 (DCAF8) (amino-acid residues 177–555) were overexpressed and purified from *E. coli* as GST-fusion proteins.

Crystallography. The VDC-complex crystals were grown at 4 °C by the hanging-drop diffusion method. The crystals grew from 100 mM NaHEPES, 7–9% (w/v) PEG 4000, 10% (v/v) isopropanol, 5 mM dithiothreitol, pH 8.0. A native data set was collected at the 8.2.2 beamline at the Advanced Light Source in Berkeley, California, using a crystal flash-frozen in the crystallization buffer supplemented with 20% (v/v) 2-methyl-2,4-pentanediol (MPD) at −170 °C. The crystal forms in space group $C222_1$, with $a = 83.46$ Å, $b = 203.19$ Å, $c = 424.87$ Å, and one complex in the asymmetric unit. For further details, see Supplementary Methods and Supplementary Fig. 5.

Tandem-affinity purification. Tandem-affinity purification (TAP) and mass spectrometry analyses of the DDB1 and CUL4A protein complexes were performed using HEK293T cells stably expressing the TAP-tagged constructs. The detailed protocol has been described previously[20].

Co-affinity purification and GST pull-down assay. Co-affinity purifications were performed using HEK293T cells stably expressing pGLUE-DDB1. Cells were transfected with different Flag-tagged DCAF proteins using $CaPO_4$. Forty-eight hours post-transfection, cells were lysed using stringent radio-immunoprecipitation buffer (RIPA; 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Igepal CA-630, 0.25% (w/v) deoxycholic acid, 2 mM EDTA, 1 mM NaF, 50 mM β-glycerophosphate). Lysates were cleared by centrifugation and affinity purification was performed using sepharose–streptavidin resin (Amersham). Anti-Flag M2 antibody (F1804; Sigma) and anti-haemagglutinin (HA) antibody (3F10; Roche) were used to detect the Flag-tagged DCAF proteins and DDB1, respectively. Previously reported procedures[18] were followed for the *in vitro* GST pull-down experiments except that the final samples were analysed by western blot analysis using an anti-DDB1 antibody (ab13562; abcam).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.A. and T.L. contributed equally to this work.

Author Information Structure coordinates for the DDB1–CUL4A–ROC1–SV5-V complex have been deposited in the Protein Data Bank under accession number 2HYE. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to N.Z. (nzheng@u.washington.edu) or R.T.M. (rtmoon@u.washington.edu).