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A RING E3–substrate complex poised for ubiquitin-like protein transfer: structural insights into cullin-RING ligases

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Abstract

How RING E3 ligases mediate E2-to-substrate ubiquitin-like protein (UBL) transfer remains unknown. Here we address how the RING E3 RBX1 positions NEDD8's E2 (UBC12) and substrate (CUL1). We find that existing structures are incompatible with CUL1 NEDD8ylation and report a new conformation of RBX1 that places UBC12 adjacent to CUL1. We propose RING domain rotation as a general mechanism for UBL transfer for the largest family of E3s.

E2 and E3 enzymes covalently link ubiquitin-like proteins (UBLs) to their protein targets. The largest family of E3s comprises the RING cohort, which bind both a UBL-loaded E2 and a protein substrate targeted for UBL transfer[1]. Although structures have indicated how RING E3s bind E2s and recruit substrates, to date no structure has revealed a RING domain and substrate orientation that explains how UBL transfer can occur[1–4].

Much knowledge of RING E3s derives from studies of the modular multiprotein cullin-RING ligases (CRLs)[1]. Prototypic CRLs, "SCFs", comprise the scaffold protein CUL1, the RING protein RBX1, the adaptor SKP1, and an F-box protein that both binds SKP1 and recruits substrates for RBX1-mediated ubiquitination[1,5,6]. In all three structures containing CUL1–RBX1, RBX1's N-terminal strand binds CUL1 through participation in an

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Author Contributions

MFC designed, performed, and analyzed experiments and wrote the manuscript. DCS designed, performed and analyzed experiments. DMD designed and analyzed experiments. CRRG designed and performed experiments. RWK designed and analyzed experiments. IK designed and performed experiments. BAS advised and assisted on all aspects of the project and wrote the manuscript.

Competing financial interest statement

The authors declare no competing financial interests.

intermolecular β-sheet, and its C-terminal RING packs against the WHB portion of the CUL1 C-terminal domain (CTD)[7,8]. The RBX1 RING has been implicated in three distinct E3 activities. First, RBX1 is an E3 for ligation of the UBL NEDD8 to CUL1 (ref. 9). Here, RBX1's RING binds the NEDD8 E2 UBC12 (also known as UBE2M), and the RBX1-associated CUL1 is the substrate. RBX1 is sufficient as an E3 for NEDD8ylation, although a second E3, DCN1, potentiates this reaction[10–12]. Second, NEDD8 ligation favors a conformational change that switches on SCF ubiquitin E3 activities[13–15]. The NEDD8-modified CUL1 assembles with SKP1–F-box protein–substrate, RBX1 binds a ubiquitin-charged E2 such as UBCH5 or CDC34, and ubiquitin is ligated to a substrate[15,16]. Finally, RBX1 binds another ubiquitin-charged CDC34, and ubiquitin is transferred to Lys48 on a substrate-linked ubiquitin. This final step is repeated in a processive manner to build a Lys48-linked polyubiquitin chain targeting the substrate for 26S proteasomal degradation[17]. Indeed, ~20% of all ubiquitin-proteasomal degradation is estimated to result from E3 activities of NEDD8-activated CRLs[18]. Nonetheless, to date none of the 7 RBX1 structures is in a conformation demonstrating any of its UBL ligase functions[7,8,13,19].

We set out to understand an active CUL1–RBX1 conformation for the first E3 function of NEDD8ylation. Prior studies indicated that the canonical E2-binding surface of RBX1's RING recruits UBC12 (ref. 20). Furthermore, the conventional RING-binding surface of yeast Ubc12 has been implicated in activation by yeast Rbx1 (ref. 12). Therefore, we made a model of a CUL1–RBX1–UBC12 complex based on structures of UBC12, cullin–RBX1 complexes, and other E2s bound to other RING domain proteins (Supplementary Fig. 1)[2–4,7]. We validated the model using mutagenesis and NMR. Mutations designed to disrupt the predicted RBX1–UBC12 interface impaired NEDD8 transfer to CUL1 (Fig. 1a). Furthermore, titration of RBX1's RING domain into a ^{15}N-labeled form of UBC12's catalytic domain resulted in chemical shift differences localized primarily to the canonical RING-binding surface (Fig. 1b, Supplementary Fig. 1, Supplementary Table 1)

Although valid for RBX1–UBC12 interactions, the CUL1–RBX1–UBC12 model presents a topological problem: a large ~30 Å gap separates the catalytic Cys111 of UBC12 and the NEDD8 acceptor Lys720 on CUL1 (Fig. 2a). To address whether this CUL1–RBX1 conformation is competent for NEDD8 transfer, we generated conformationally-restrained disulfide-linked versions of CUL1–RBX1. Because of minor differences among the three published CUL1–RBX1 crystal structures[7,8], three closely-related sets of cysteine pairs were designed to capitalize on proximity of CUL1's Cterminus with helix-3 of RBX1 (Supplementary Fig. 2). The cysteine-pair locations varied by one residue in either CUL1 or RBX1, and all became at least partially disulfide-linked upon exposure of solutions to air overnight. We assayed NEDD8 transfer to CUL1, either in the presence or absence of the disulfide links between CUL1 and RBX1. CUL1 that is disulfide-linked to RBX1 was not NEDD8ylated. However, under the same conditions, CUL1 is readily modified by NEDD8 in non-disulfide-linked CUL1–RBX1 complexes either remaining after air exposure (eg pairs B and C), or in reduced samples (Fig. 2b). Similar results were also obtained for a complex between RBX1 and CUL1's isolated C-terminal domain (CUL1^{CTD}) (Supplementary Fig. 2). Lack of CUL1 NEDD8ylation upon disulfide-linking CUL1 and RBX1 is not due to a defect in RBX1 catalysis of UBC12-mediated NEDD8ylation per se, because under the same conditions the disulfide-constrained CUL1~RBX1 (where '~'

indicates covalent bonding) complex stimulates NEDD8 transfer from UBC12 to the nonspecific nucleophile hydroxylamine (Supplementary Fig. 3). Thus, restraining proximity of CUL1's C-terminal region and RBX1's RING domain impairs NEDD8 transfer specifically to the substrate CUL1. We conclude that (1) the CUL1–RBX1 conformation in existing crystal structures is incompatible with NEDD8ylation, and (2) it is likely that CUL1, RBX1, or both undergo substantial conformational changes during NEDD8 ligation to CUL1.

We reasoned that cullin–RBX1 complexes likely exist in conformations other than those seen previously, and in efforts to observe an alternative form we obtained a crystal structure of $CUL1^{CTD}$ in complex with RBX1. The structure reveals a new conformation, apparently favored by this construct under our crystallization conditions, which explains how an RBX1-associated UBC12 can become juxtaposed with CUL1's NEDD8 acceptor Lys720 (Fig. 3, Supplementary Fig. 4, 5, Supplementary Table 2). Comparison to previous CUL–RBX1 structures[7,8,19] reveals a striking repositioning of the RBX1 RING away from CUL1. The new conformation is achieved by a dramatic extension of the RBX1 linker between the N-terminal CUL1-binding strand and C-terminal RING involving ~60° rigid-body rotation about a hinge centered at residues Val38/Val39 (Fig. 3d, Supplementary Fig. 6), a region we previously speculated, based on much data, would adopt multiple conformations[13]. Superposition of the new $CUL1^{CTD}$ structure with the corresponding regions of prior CUL1–RBX1 structures also reveals slight relative displacement of the WHB subdomain. This is due to a ~10° tilting at the base of helix-29. Notably, comparison to prior structures of $CUL5^{CTD}$–RBX1 and NEDD8~$CUL5^{CTD}$–RBX1 revealed that the corresponding hinges undergo dramatic reorientation upon NEDD8 ligation (Supplementary Fig. 6)[13]. Thus, although not previously observed, it seems likely that our structure exists within the normal range of CUL1–RBX1 conformations.

Docking E2–RING structures[2–4] onto the new $CUL1^{CTD}$–RBX1 structure entirely closes the gap between an E2 active site and the CUL1 acceptor Lys720. In fact, if a model is made with UBC12, the gap is slightly over-closed. However, a model using a prior CUL1 structure complexed with the new RBX1 structure places the UBC12 catalytic Cys111 ~3 Å from the ε-amino group of CUL1's NEDD8 acceptor Lys720 (Fig. 3e).

We tested the model in two ways. First, we introduced individual cysteines into Ubc12 and CUL1 and tested their crosslinking by the homobifunctional sulfhydryl crosslinker Bis-Maleimidoethane (**Methods**, Fig. 3f). It was necessary to incorporate individual cysteines into a cysteine-free version of the structurally-superimposable Ubc12 from budding yeast[12] because we were unable to express a cysteine-less version of human UBC12. The Ubc12 and CUL1 mutants harboring cysteines predicted by the model to be in close proximity were crosslinked preferentially (Fig. 3f). Second, we tested *in vitro* NEDD8ylation by UBC12 mutants with Ala substitutions for Arg116 and Leu145, which are at the predicted interface with CUL1 but have not previously been implicated in UBL transfer (Fig. 3g). Indeed, the mutants are defective at NEDD8 transfer to CUL1. Furthermore, the relevance of the new RBX1 conformation for CUL1 NEDD8ylation is supported by much prior data. First, in the new structure, the 5-residue linker between the N-terminal CUL1-binding strand and the C-terminal RING domain extends 15.6 Å (Supplementary Fig. 6). If only a single residue were

to be deleted, the remaining 4 residues could only span a maximum Cα-to-Cα distance of 14.4 Å, which would be insufficient for the RING domain to acquire the structurally-observed position. Indeed, deletion of one RBX1 residue at the Val38/Val39 hinge almost completely abrogates CUL1 NEDD8ylation[13]. Second, assaying CUL1 NEDD8ylation upon proline-scanning mutagenesis of the RBX1 hinge revealed the most substantial defect for a V39P mutation, which would restrict rotation about the structurally-observed hinge[13]. Notably, the V39P-induced NEDD8ylation defect was rescued by upstream insertion of a single glycine, which would restore the ability of the RBX1 RING domain to be positioned as in our new structure. Finally, the CAND1 NEDD8ylation inhibitor binds the previously-observed CUL1–RBX1 interface[8]. Thus, CAND1 may prevent rotation of the RBX1 RING domain to the new, catalytically-relevant conformation to inhibit NEDD8ylation.

Taken together with previous results, our new data provide the first structural view of a RING E3–substrate complex in a conformation compatible with UBL transfer. Our disulfide-trapping experiments now show that previously observed closed conformations cannot account for RBX1-mediated NEDD8ylation. Instead, we propose that an active conformation is achieved largely by striking rotation of the RBX1 RING domain even in the absence of any other factors, reflecting inherent flexibility of RBX1. Although there may be cases where other mechanisms facilitate UBL-transfer, it seems likely that many other RING E3s will in the future be found to work similarly, with rotation of RING domains playing major roles in bridging gaps to substrates and mediating UBL ligation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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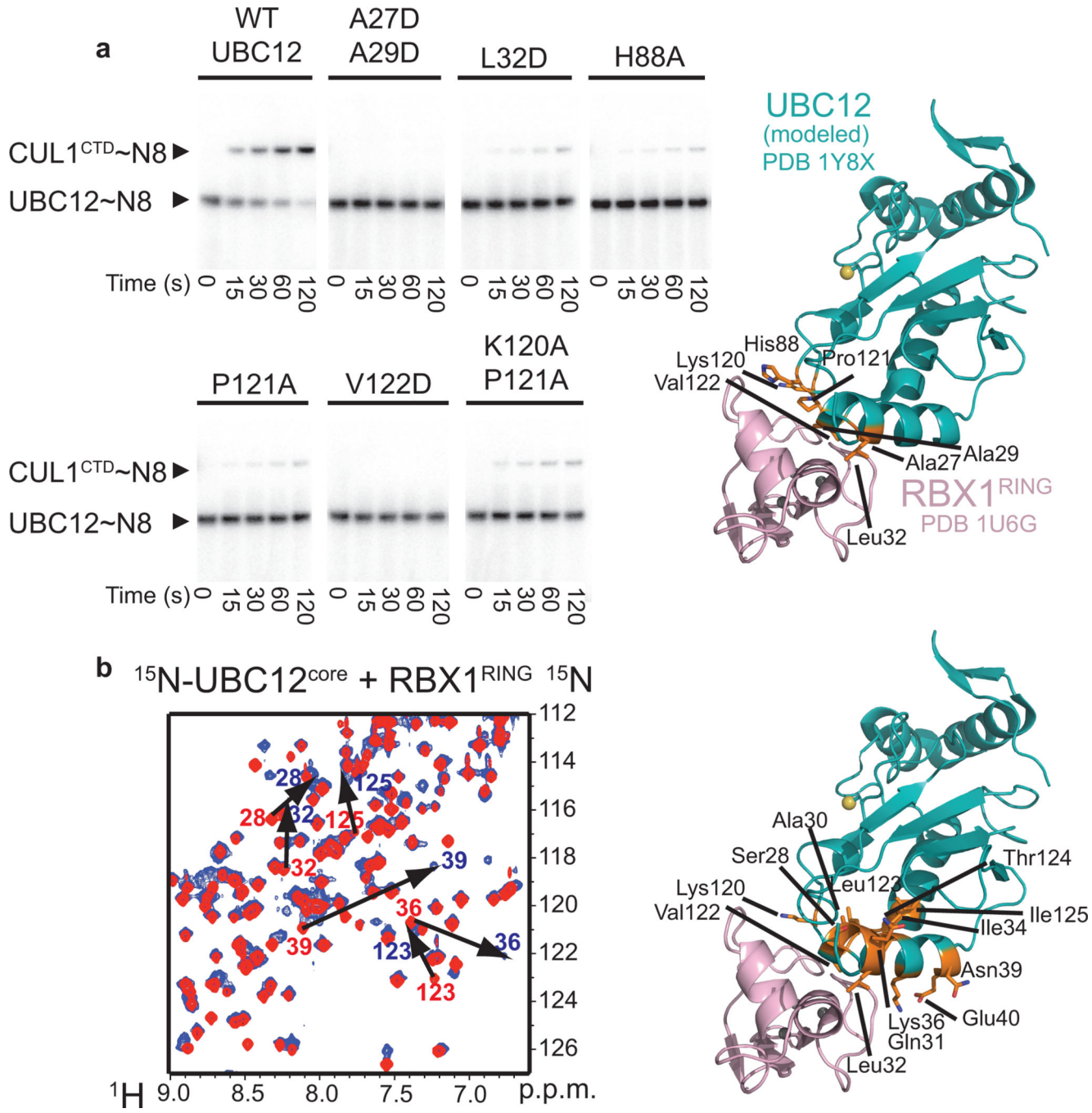

Figure 1.

UBC12–RBX1 interactions. (**a**) WT and mutant UBC12-mediated CUL1 NEDD8ylation *in vitro*. Mutated residues are orange on UBC12 (otherwise cyan)–RBX1 (pink) model. (**b**) 2D HSQC [^{15}N-^{1}H] spectra of ^{15}N-UBC12^{core} in the absence (red) and presence (blue) of equimolar RBX1^{RING}. A subset of shifted resonances are labeled in the spectra. Residues with chemical shift differences greater than one standard deviation above the mean are labeled and colored orange on UBC12 (otherwise cyan)–RBX1 (pink) model.

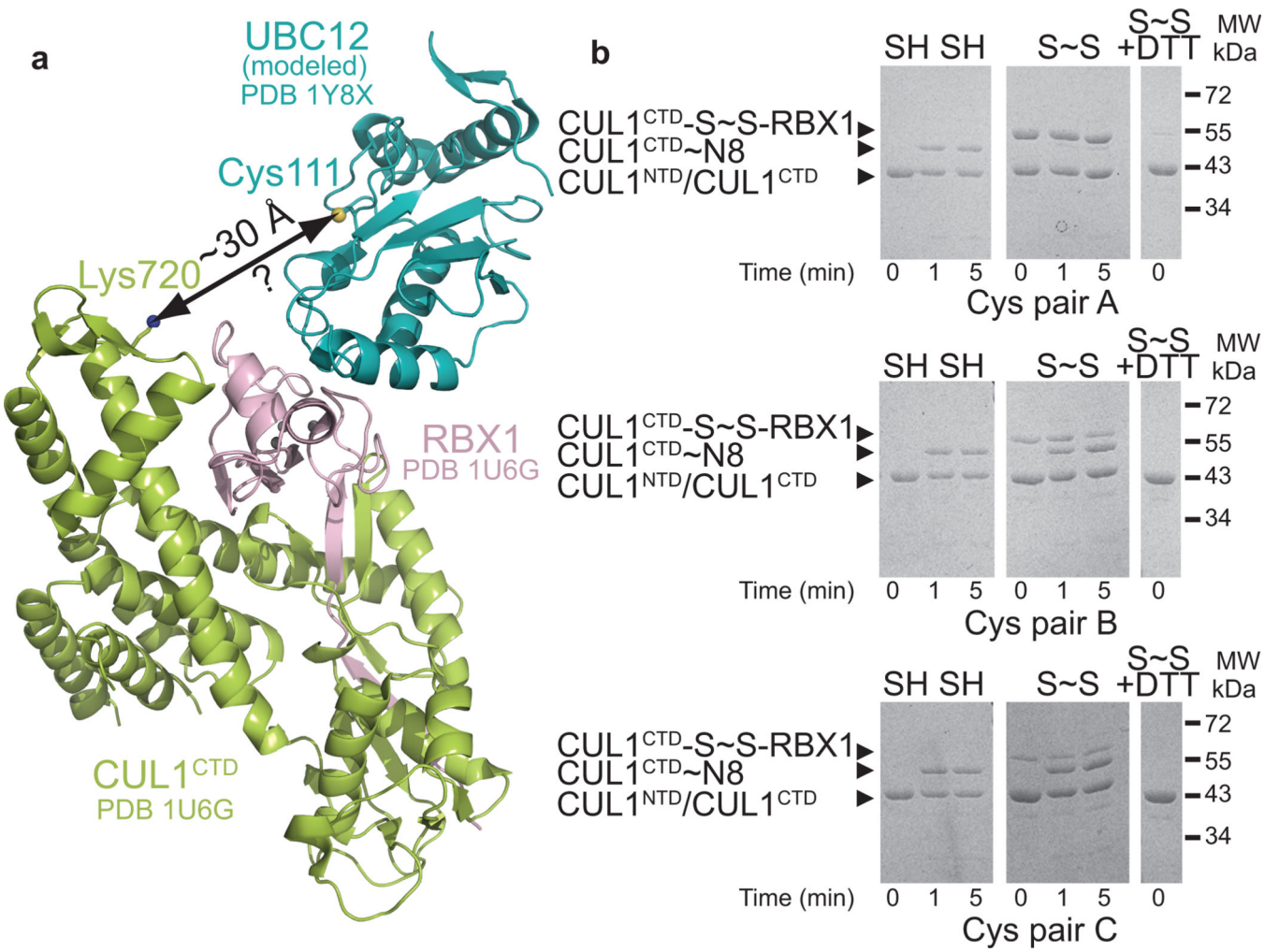

Figure 2.

Models from prior structures reveal an E2-to-substrate gap. (**a**) Structural model of $CUL1^{CTD}$–RBX1–$UBC12^{core}$ (colored light green, pink, cyan respectively) complex based on previous CUL1–RBX1 and RING–E2 structures[2–4,7,8] (Supplementary Fig. 1). CUL1 NEDD8 modification site and UBC12 catalytic cysteine are indicated by blue and yellow spheres respectively. (**b**) *In vitro* CUL1 NEDD8ylation assays using disulfide-linked (S~S) and unlinked (SH SH) split'n'coexpress CUL1–RBX1 (ref. 7). Pairs A–C refer to three distinct combinations of engineered cysteines (Supplementary Fig. 2).

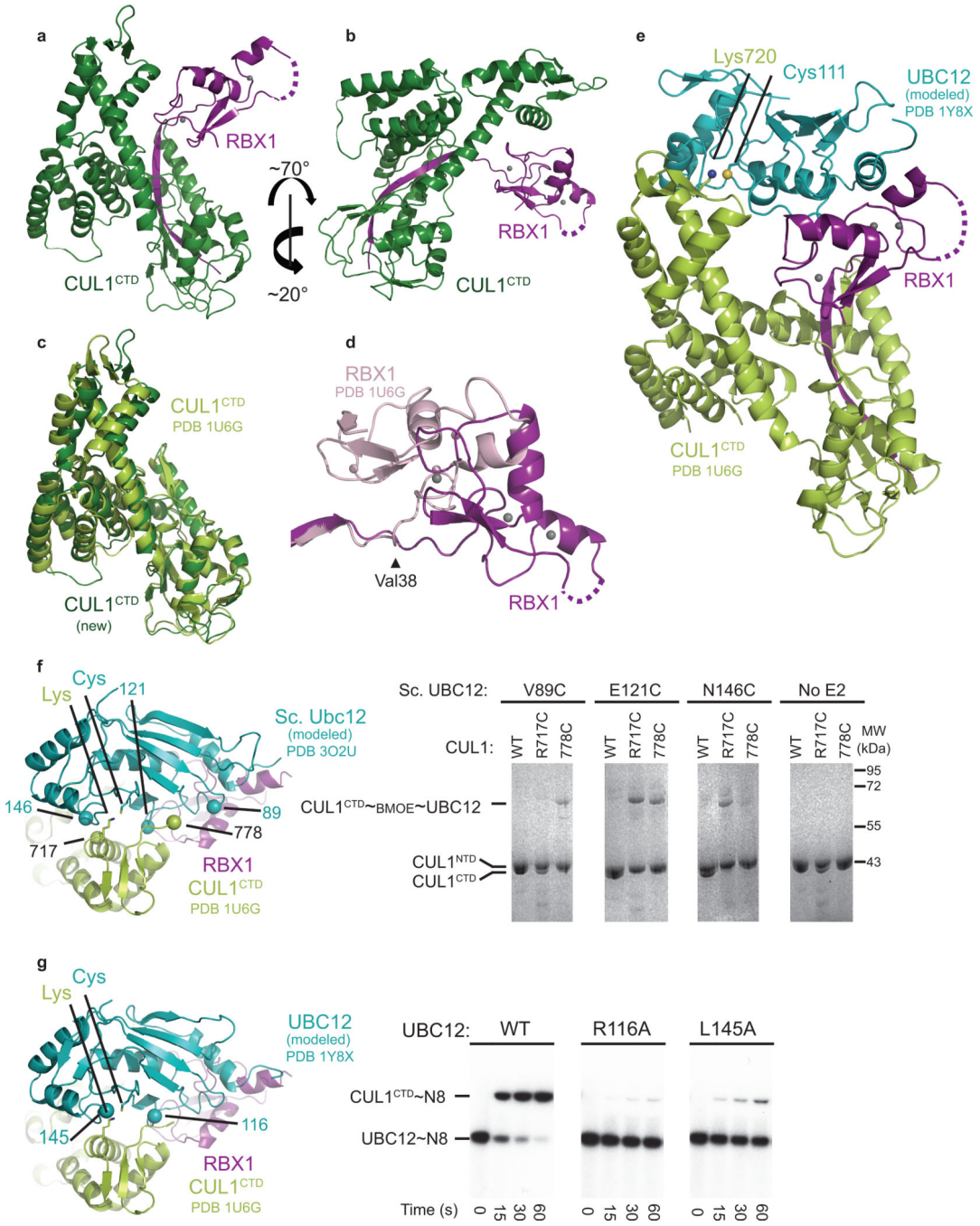

Figure 3.

Structure of CUL1^{CTD}–RBX1 in new conformation. (**a**) One copy of CUL1^{CTD} (dark green) –RBX1 (purple) from the asymmetric unit. (**b**) Same as (**a**), but rotated ~70° in y and ~20° in x. (**c**) Structural overlay with previous CUL1^{CTD} (light green) oriented similarly to (**a**)[7,8]. (**d**) Structural overlay with previous RBX1 (pink) in same orientation as (**b**). (**e**) Structural model of CUL1^{CTD}–RBX1–UBC12^{core} (light green, purple, cyan respectively) complex based on the new RBX1 conformation. (**f**) Bis-Maleimidoethane (BMOE)-crosslinking between engineered cysteine mutants of Ubc12 and CUL1^{CTD}. Residues mutated to cysteine

are numbered on the model (left, view rotated by ~70° and ~40° about the×and z-axes relative to panel **e**) and products of crosslinking reactions are shown for all possible Ubc12+CUL1–RBX1 combinations (right). Note residue numbering for yeast Ubc12. For reference, Ubc12 catalytic cysteine and CUL1 NEDD8ylation site are shown as sticks. (**g**) *In vitro* CUL1 NEDD8ylation for UBC12 mutants at the predicted interface. Mutated residues are shown on the model (left).