Structure of E3 ligase E6AP with a proteasome-binding site provided by substrate receptor hRpn10

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Regulated proteolysis by proteasomes involves ~800 enzymes for substrate modification with ubiquitin, including ~600 E3 ligases. We report here that E6AP/UBE3A is distinguished from other E3 ligases by having a 12 nM binding site at the proteasome contributed by substrate receptor hRpn10/PSMD4/S5a. Intrinsically disordered by itself, and previously uncharacterized, the E6AP-binding domain in hRpn10 locks into a well-defined helical structure to form an intermolecular 4-helix bundle with the E6AP AZUL, which is unique to this E3. We thus name the hRpn10 AZUL-binding domain RAZUL. We further find in human cells that loss of RAZUL by CRISPR-based gene editing leads to loss of E6AP at proteasomes. Moreover, proteasome-associated ubiquitin is reduced following E6AP knockdown or displacement from proteasomes, suggesting that E6AP ubiquitinates substrates at or for the proteasome. Altogether, our findings indicate E6AP to be a privileged E3 for the proteasome, with a dedicated, high affinity binding site contributed by hRpn10.
The 26S proteasome is a 2.5 MDa complex responsible for regulated protein degradation\(^1,2\), with substrates typically ubiquitinated by a hierarchical enzymatic cascade\(^3\). An E1 activating enzyme charges ubiquitin to become a protein modifier and transfers it to an E2 conjugating enzyme which, in concert with an E3 ligating enzyme, subsequently attaches ubiquitin to a substrate. Approximately 600 E3s exist in humans that can either accept the charged ubiquitin for direct transfer to a substrate or serve as a scaffold for ubiquitin transfer from the E2 to a substrate\(^4,5\). Following ubiquitination, receptor sites in the proteasome contributed by Rpn1, Rpn10, and Rpn13 recognize ubiquitin directly or the ubiquitin fold of shuttle factor ubiquitin-like domains\(^6,7,14\); shuttle factors bind ubiquitinated substrates by one or more ubiquitin-associated domains\(^15-17\). At the proteasome, ubiquitin chains are hydrolyzed by deubiquitinating enzymes (DUBs) Rpn11\(^18\), UCHL5/Uch37\(^19\), and Usp14\(^20-22\), as the marked substrate is translocated through an ATPase ring for entry into the hollow interior of the proteolytic core particle (CP)\(^2,23-25\). The integrity of the ubiquitin-proteasome pathway is essential for cellular homeostasis with dysfunctions linked to disease, including cancer and neurodegeneration. Inhibitors of the CP are used to treat hematologic cancers\(^26-28\) and additional proteasome subunits are being pursued as synergistic targets, including hRpn13\(^29-34\).

Hijacking of ubiquitin E3 ligase E6AP/UBE3A by high risk human papilloma virus E6 oncoprotein contributes to cervical cancer by inducing ubiquitination and in turn degradation of tumor suppressor p53\(^35\). Moreover, loss-of-function mutations in E6AP associate with Angelman syndrome\(^38-40\) and elevated gene dosage with autism spectrum disorders\(^41\). How aberrant E6AP mechanistically contributes to these neurological diseases is an active area of investigation; however, E6AP was recently found to ubiquitinate calcium- and voltage-dependent potassium channels, the dysfunction and hyperexcitability of which is associated with Angelman syndrome\(^42\). The only hit identified in parallel experiments was done with GST protein as a control.

To identify proteins that interact directly or in complex with the C-terminal hRpn10 domain, we subcloned the region spanning 305–377 in frame with GST, expressed, and purified the fusion protein from *Escherichia coli*, and bound purified GST-hRpn10\(^305-377\) to glutathione sepharose resin for incubation with lysates from 293T (human embryonic kidney epithelial) or HCT116 (colorectal carcinoma) cells. After washing, resin-bound proteins were separated by SDS-PAGE, eluted from the gel, digested with trypsin, and analyzed by mass spectrometry; parallel experiments were done with GST protein as a control. The only hit identified for either 293T or HCT116 lysate was E6AP (Supplementary Fig. 2a).

The human *UBE3A* gene encodes three isoforms generated by differential splicing\(^33\) that vary at the extreme N-terminus (Supplementary Fig. 2b). To test whether E6AP binds to the hRpn10 C-terminal region directly, we incubated full-length E6AP (Isoform II) with Ni-NTA resin pre-bound to His-hRpn10\(^305-377\), His-hRpn10\(^305-377\), or His-hRpn10\(^305-306\). Significantly shifted by hRpn10 addition to 15N-hRpn10\(^305-377\) bound E6AP, whereas hRpn10\(^305-306\) did not (Fig. 1b). We next added unlabeled AZUL, which is present in all three E6AP isoforms (Supplementary Fig. 2b), to 15N-hRpn10\(^305-377\) and monitored the effect by 2D NMR to find hRpn10 signals shifted (Fig. 1c), indicating binding. We assigned both the free and AZUL-bound state, as described in “Methods”, and quantified the changes to find D327–M356 perturbed (Supplementary Fig. 2c). Analogous experiments with unlabeled hRpn10\(^305-377\) and 15N-E6APAZUL, aided by previous assignments\(^45\), indicated residues in both AZUL helices to be significantly shifted by hRpn10 addition (Supplementary Fig. 2d, e). Altogether, these experiments indicate direct binding between E6APAZUL and hRpn10\(^305-377\), consistent with recent publications implicating AZUL and hRpn10 to E6AP interaction with the proteasome\(^44,46\).

To assess the strength of hRpn10\(^305-377\)-AZUL interaction, we used isothermal titration calorimetry (ITC) with the AZUL added incrementally to hRpn10\(^305-377\); a \(K_D\) value of 11.6 \pm 3.3 nM was measured (Fig. 1d and Supplementary Fig. 2f), indicating similar strength to hRpn13 interaction with the proteasome\(^34,35\). Surface plasmon resonance (SPR) similarly revealed a \(K_D\) value of 8.1 + 1.4 nM for GST-hRpn10\(^305-377\) binding to AZUL (Fig. 1d and Supplementary Fig. 2g).

To test whether the hRpn10 C-terminal region is required for interaction with endogenous E6AP in cells, lysates from HCT116 cells expressing either myc-hRpn10\(^305-306\) or myc-hRpn10\(^1-306\) were subjected to immunoprecipitation with anti-myc nanobody-coupled agarose. Co-immunoprecipitation of E6AP was observed with full-length but not truncated...
hRpn10 (Fig. 1e). We further co-expressed HA-E6AP with either hRpn10 construct and immunoprecipitated E6AP with anti-HA antibodies to find co-immunoprecipitation of full-length, but not truncated, hRpn10 (Fig. 1f).

Altogether, these data indicate that the E6AP AZUL is a strong interaction partner of hRpn10305–377 and we henceforth refer to this domain in Rpn10 as RAZUL (Rpn10 AZUL-binding domain, Fig. 1g).
E6AP protein levels depend on hRpn10. To test the significance of the RAZUL:AZUL interaction, we considered generating a full hRpn10 knockout cell line. However, we found that hRpn10 knockdown by siRNA resulted in loss of proteasome components hRpn8 and hRpn11 associating with proteasome ATPase Rpt3 (Fig. 2a), consistent with an early report that Rpn10 is necessary for base-lid interactions in the yeast proteasome56. To avoid such proteasome defects, we utilized CRISPR/Cas9 to generate a cell line in which hRpn10 lacks RAZUL but retains intact VWA and UIMs (see “Methods”). Deletion of RAZUL was assayed by immunoblotting, as demonstrated for clones 13 and 14 (Fig. 2b); we henceforth refer to these cell lines as ∆RAZUL.

Lower levels of truncated hRpn10 were consistently observed in ∆RAZUL cells compared with the full-length protein in WT cells, with clone 14 producing less protein than clone 13 (Fig. 2b, second panel). Corresponding with the lower levels, association of truncated hRpn10 with proteasome immunoprecipitated by anti-Rpt3 antibodies was reduced in the ∆RAZUL cell lines compared with HCT116 cells (Fig. 2c, lanes 1, 3, and 7). Moreover, co-immunoprecipitation with Rpt3 of lid components hRpn8 and hRpn11, and not of base component Rpn2, was similarly reduced (Fig. 2c, lanes 1, 3, and 7). Expression of either myc-ΔRpn10 or myc-ΔRPN10 rescued the association of these lid components in both ∆RAZUL cell lines (Fig. 2c, lanes 4, 5, 8, and 9). This finding establishes that RAZUL is not necessary for lid association with the proteasome base, and that levels of hRpn10 correlate with levels of proteasome lid-base association.

In addition to hRpn10 being important for proteasome base-lid assembly, we unexpectedly found that E6AP levels correlated with hRpn10 ∆RAZUL levels (Fig. 2b, top panel). To test whether the reduced protein levels are due to protein degradation, WT and ∆RAZUL cells were treated with 10 μM MG132 for 4 h to inhibit the proteasome. As expected, MG132 treatment caused ubiquitinated proteins to accumulate in both cell lysates (Supplementary Fig. 3). We assayed E6AP levels in cells with siRNA knockdown of hRpn10 to similarly find correlation (Fig. 3a). Loss of E6AP by siRNA treatment however had no effect on hRpn10 levels (Fig. 3b). We attempted to rescue E6AP levels in ∆RAZUL cells (clone 14) by expressing myc-hRpn10 or myc-hRpn10ΔRAZUL, however, no increase in endogenous E6AP protein was observed for either condition (Fig. 3c). We further interrogated this effect in WT or ∆RAZUL cells by expressing E6AP and hRpn10 constructs
either independently or in combination. Exogenous expression of E6AP was reduced in ΔRAZUL (clone 13) compared with WT cells (Fig. 3d, lane 2 vs. lane 4). Exogenous E6AP levels were boosted however in ΔRAZUL cells (clone 13) when either truncated (lane 5) or full-length (lane 6) hRpn10 was co-transfected with E6AP (Fig. 3d); thus the effect is not dependent on the RAZUL:AZUL interaction, however full-length hRpn10 had a stronger effect on E6AP levels than truncated hRpn10. Altogether, these data link hRpn10 to E6AP production with possible spatial or temporal regulation that allows rescue of E6AP when the two proteins are co-expressed.

E6AP AZUL binds to hRpn10 RAZUL at the proteasome. We used ΔRAZUL cells to test whether the hRpn10 RAZUL recruits E6AP to the proteasome, boosting hRpn10 levels by transient transfection. Proteasomes from lysates of WT and ΔRAZUL cells (clone 13) expressing myc-tagged hRpn10 full-length or ΔRAZUL protein were immunoprecipitated with Rpt3 antibodies and immunoblotted for hRpn10, E6AP, or proteasome component hRpn2 (as a control). E6AP co-immunoprecipitated with proteasomes from WT (Fig. 4a, lane 2 and 3), but not ΔRAZUL (Fig. 4a, lane 5) cells. Expression of full-length (lane 7) but not RAZUL-truncated hRpn10 (lane 6) resulted in observable E6AP co-immunoprecipitation with proteasomes isolated from ΔRAZUL cells (Fig. 4a); we attribute the lower amounts of E6AP co-immunoprecipitated with proteasomes of hRpn10full-length_expressing ΔRAZUL cells to the reduced abundance of endogenous E6AP in this cell line, as described above (Fig. 2b). This experiment indicates that the hRpn10 RAZUL recruits E6AP to the proteasome.

We further tested whether RAZUL could compete with the proteasome for E6AP binding. Proteasomes immunoprecipitated from HCT116 cells overexpressing myc-hRpn10 RAZUL were immunoprobed for E6AP and compared with empty vector transfected cells and an IgG control. Expression of RAZUL caused E6AP to be lost from the proteasome (Fig. 4b).

To test the impact of E6AP at the proteasome, Rpt3 immunoprecipitates from lysates of cells transfected with a scrambled control or siRNA against E6AP were immunoblotted for ubiquitin. While no major change in total ubiquitin levels was observed at the level of whole cell extract (WCE), a reduction in bulk ubiquitin was apparent at the proteasome following E6AP loss, particularly for higher molecular weight species (Fig. 4c). To test whether displacing E6AP from the proteasome has a similar effect on ubiquitin levels, we expressed RAZUL to compete E6AP away from endogenous hRpn10. Ubiquitin associating with the hRpn2 E6AP Cyp B myc-tag hRpn10

| a | b | c | d |
|---|---|---|---|
| hRpn10 siRNA | E6AP siRNA | myc-hRpn10 | HA-E6AP |
| #5 | #6 | #7 | #8 |
| E6AP | hRpn10 | Cyp B | hRpn2 |
| β-actin | β-actin | β-actin | β-actin |

Fig. 3 E6AP levels depend on hRpn10. hRpn10 (a) or E6AP (b) was knocked down in HCT116 cells by four different siRNAs and the cell lysates immunoprocessed as indicated. Mock and scrambled control samples are included. β-actin is used as a loading control in a and d. c Lysates from HCT116 or clone 14 cells expressing myc-hRpn10 constructs were immunoprocessed as indicated. d Lysates from HCT116 or clone 13 cells expressing HA-E6AP and/or myc-hRpn10 constructs were immunoprocessed as indicated. a–d Antibodies used for immunoprocessing are indicated to the left of each panel. Source data are provided as a Source Data file.
Fig. 4 hRpn10 RAZUL contributes E6AP to the proteasome. a Immunoblots of Rpt3 immunoprecipitates or WCE from HCT116 or clone 13 lysates expressing myc-hRpn10 constructs. An asterisk *** indicates heavy chain antibody. Cyclophilin B (Cyp B) is used as a loading control for WCE samples in a-c and hRpn2 as a positive control for the immunoprecipitation. IgG controls are included. b, d Immunoblots of Rpt3 or IgG (control) immunoprecipitates or WCE of lysates from HCT116 cells transfected with empty vector (as a control) or myc-hRpn10 RAZUL. c Immunoblots of Rpt3 immunoprecipitates or WCE from lysates of HCT116 cells transfected with a scrambled control or siRNA against E6AP. a-d Antibodies used for immunoprobing are indicated to the left of each panel. e Pull-down assay for a commercially available mixture of His6-tagged, non-cleavable K48-linked Ub2/Ub4 with incubation of human 26S proteasome; however, the remaining portion of hRpn10, including the ubiquitin-binding UIM portion, is missing from reported structures51. To test directly whether the RAZUL:AZUL interaction observed for the isolated domain complex is maintained in the intact proteasome, we used NMR, which is ideal for flexible systems64,65. We next tested whether E6AP impacts the affinity of the 26S proteasome for ubiquitin chains by using an in vitro assay. 26S proteasome was added alone or with equimolar E6AP to Ni-NTA resin pre-bound to a commercially available mixture of non-hydrolyzable K48-linked Ub2/Ub4. The amount of proteasome retained on the resin following extensive washing was unaffected by the presence of E6AP (Fig. 4e, lane 7 compared with lane 6). Moreover, E6AP was retained on the ubiquitin-bound resin when proteasome was present (Fig. 4e, lane 7) and barely observable without proteasome (Fig. 4e, lane 8). Thus, the loss of ubiquitinated protein at the proteasome following E6AP knockdown (Fig. 4c) is not caused by a reduction of E6AP-bound proteasome affinity for ubiquitin chains.

hRpn10 is assembled into the proteasome RP at equimolar stoichiometry through its N-terminal VWA domain, which is well-defined in cryoelectron microscopy structures of the proteasome; however, the remaining portion of hRpn10, including the ubiquitin-binding UIM portion, is missing from reported cryoelectron microscopy densities57–59 due to flexibility51. To test directly whether the RAZUL:AZUL interaction observed for the isolated domain complex is maintained in the intact proteasome, we used NMR, which is ideal for flexible systems64,65. We added equimolar 13C-AZUL to RAZUL or 26S proteasome (Supplementary Fig. 4a) and acquired a 1D 13C-edited, 1H NMR experiment for comparison with free 13C-AZUL. Binding to RAZUL induced shifting (Supplementary Fig. 4b, middle vs.
to RAZUL and this architecture is maintained in the RAZUL-bound state, with a backbone r.m.s.d. between the free and complexed state, with a backbone r.m.s.d. of 0.3 Å (Supplementary Fig. 6c, d). Overall, our NMR data indicate that RAZUL switches from a poorly ordered state to a well-defined helical state following AZUL binding. We interrogated this finding further by circular dichroism (CD) spectroscopy, as done previously for Rap8069,70. CD measurements indicated 7% and 46% helicity, respectively, for unbound RAZUL (blue) and AZUL (green), and a theoretical spectrum (gray dashed line) for the mixture with markedly less spectral features of helicity compared with the recorded experimental spectrum (orange, Fig. 5d). Overall, 35% helicity is indicated from the experimental CD data recorded on the complex, consistent with the 36% helicity determined by NMR (Fig. 5b). The AZUL secondary structure is unaltered by binding to RAZUL (Supplementary Fig. 6a), leading us to conclude that the observed difference between the theoretical and experimental CD spectra reflects increased helicity for RAZUL, consistent with the NMR data (for example, Fig. 5c).

Structure of the RAZUL:AZUL complex. At the molecular interface, a 4-helix bundle is formed by two pairs of helices from AZUL and RAZUL stacking against each other (Fig. 5b). RAZUL α1 is centered between the two AZUL helices by hydrophobic interactions involving F334, L335, V338, and L339 as well as L342 and V345 from the RAZUL α1 α2 loop (Fig. 6a, b). These residues interact with A29, 133, and Y37 from AZUL H1 and A69 and L73 from AZUL H2 (Fig. 6a, b). From the 310-helix, V328 and M329 form hydrophobic interactions with AZUL A29, L73, and Y76 (Fig. 6c), capping the hydrophobic contact surface formed by RAZUL α1. RAZUL α2 is more peripheral compared with α1, with A351, I352, A355, M356, and L359 interacting with A67, L70 and L73 from AZUL H2 (Fig. 6d).

The RAZUL N-terminal end (E322–D327) is rich in acidic residues (Supplementary Fig. 1a) and proximal to the positively charged AZUL N-terminal end, which includes K25 and R26 (Fig. 6e). These AZUL residues contribute three hydrogen bonds with the AZUL R26 sidechain in 80% of calculated structures, as well as hydrophobic contacts with AZUL K25 and R26 (Fig. 6c). We tested whether adding a bulky phosphate group at this location could be deleterious for AZUL binding by synthesizing RAZUL peptides that span E322 and R26 (Fig. 6e). These AZUL residues contribute three hydrogen bonds with the AZUL α1 α2 loop (Fig. 6a, b). These residues interact with A29, 133, and Y37 from AZUL H1 and A69 and L73 from AZUL H2 (Fig. 6a, b). From the 310-helix, V328 and M329 form hydrophobic interactions with AZUL A29, L73, and Y76 (Fig. 6c), capping the hydrophobic contact surface formed by RAZUL α1. RAZUL α2 is more peripheral compared with α1, with A351, I352, A355, M356, and L359 interacting with A67, L70 and L73 from AZUL H2 (Fig. 6d).

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Table 1 Structural statistics for the RAZUL:AZUL complex.

| NMR distance and dihedral constraints | RAZUL | AZUL |
|-----------------------------|-------|------|
| NOE-derived distance constraints |     |      |
| Intramolecular | 705  | 1084 |
| Intra-residue | 386  | 412  |
| Inter-residue | 319  | 672  |
| Sequential (|j| – |j| = 1) | 192  | 257  |
| Nonsequential (|j| – |j| > 1) | 127  | 415  |
| Intermolecular |     | 217  |
| Hydrogen bonds | 15   | 22   |
| Total dihedral angle constraints | 66   | 110  |
| ϕ | 33   | 55   |
| ψ | 33   | 55   |
| Structure statistics |     |      |
| Violations (mean and s.d.) |     |      |
| Distance constraints (Å) | 0    | 0    |
| Dihedral angle constraints (°) | 0    | 0    |
| Max. dihedral angle violation (°) | <5   | <5   |
| Max. distance constraint violation (Å) | <0.3 | <0.3 |
| Deviations from idealized geometry |     |      |
| Bond lengths (Å) | 0.002 ± 0.000 | 0.002 ± 0.000 |
| Bond angles (°) | 0.416 ± 0.008 | 0.416 ± 0.008 |
| Improper (°) | 0.283 ± 0.014 | 0.283 ± 0.014 |
| Average pairwise r.m.s.d.* (Å) | 0.59 ± 0.11 | 0.59 ± 0.11 |
| Backbone atoms | 1.24 ± 0.17 | 1.24 ± 0.17 |
| Heavy atoms |     |      |

*Pairwise r.m.s.d. for the 15 lowest energy structures for V328-A360 from RAZUL and K25-L82 from AZUL.

bottom panel), as exemplified by methyl groups of M24 and M63 (Fig. 4f, left panel) and 133, L75, and L82 (Fig. 4f, right panel). The spectrum acquired with proteasome added closely mimicked that with only RAZUL added (Fig. 4f and Supplementary Fig. 4b), indicating that AZUL binding to RAZUL occurs identically at the proteasome. Compared with the spectrum recorded with only RAZUL added, the AZUL signals were broadened by proteasome addition, but not to the extent expected for a 2.5 MDA complex66,67. Rather, the appearance of proteasome-bound AZUL signals provides strong support for the AZUL:RAZUL subcomplex being tethered to but not docked against the rest of the 26S proteasome.

E6AP induces helicity in RAZUL. We used NMR to solve the structure of the RAZUL:AZUL complex, as described previously64,67 and in “Methods”, with the data summarized in Table 1. We recorded 13C-half-filtered NOESY experiments on samples of the complex with one protein 13C labeled and the other unlabeled to measure unambiguous intermolecular interactions (Supplementary Fig. 5). Altogether, 217 interactions between AZUL and RAZUL were identified (Table 1). The 15 lowest energy structures converged with a root-mean-square deviation (r.m.s.d.) of 0.59 Å (Fig. 5a).

Unbound AZUL comprises two helices (H1 and H2) and a Zn finger45 and this architecture is maintained in the RAZUL-bound state, with a backbone r.m.s.d. between the free and complexed structures of 1.3 Å (Supplementary Fig. 6a). RAZUL binds the AZUL helices from the opposite direction compared with the Zn finger (Fig. 5b). In this complex, two α-helices are formed in RAZUL that span P332–N341 (α1) and E350–S361 (α2), with an angle of 158.5° between the two helical axes. Directly N-terminal to RAZUL α1 is a single turn of a 310-helix that spans V328–Q330 (Fig. 5b). We submitted the atomic coordinates of the RAZUL:AZUL complex to the Dali server68 to find no similarities with either AZUL or RAZUL, indicating that RAZUL is unique and not previously described.

Comparisons of our NMR data acquired on free and AZUL-bound RAZUL indicate that RAZUL acquires helicity upon binding to AZUL. Carbonyl and Cα values when compared with random coil taking into account amino acid type yields a chemical shift index (CSI) that informs on secondary structure; these values shift to reflect greater helicity for RAZUL when bound to AZUL (Fig. 5c and Supplementary Fig. 6b). Moreover, intramolecular interactions characteristic of helicity were observed following AZUL addition, but not for free RAZUL (Supplementary Fig. 6c, d). Overall, our NMR data indicate that RAZUL switches from a poorly ordered state to a well-defined helical state following AZUL binding. We interrogated this finding further by circular dichroism (CD) spectroscopy, as done previously for Rap8069,70. CD measurements indicated 7% and 46% helicity, respectively, for unbound RAZUL (blue) and AZUL (green), and a theoretical spectrum (gray dashed line) for the mixture with markedly less spectral features of helicity compared with the recorded experimental spectrum (orange, Fig. 5d). Overall, 35% helicity is indicated from the experimental CD data recorded on the complex, consistent with the 36% helicity determined by NMR (Fig. 5b). The AZUL secondary structure is unaltered by binding to RAZUL (Supplementary Fig. 6a), leading us to conclude that the observed difference between the theoretical and experimental CD spectra reflects increased helicity for RAZUL, consistent with the NMR data (for example, Fig. 5c).
hRpn10 (1–364) in ΔRAZUL cells (Fig. 6g, lane 9). This finding is consistent with the ITC data indicating equivalent AZUL affinity for hRpn10322–366 (Fig. 6f).

Altogether, these data indicate that the hRpn10 RAZUL forms an independent structural domain that contributes E6AP to the proteasome.

Discussion

Approximately 600 ubiquitin E3 ligases exist in humans. We report here that proteasome substrate receptor hRpn10 evolved a 12 nM affinity binding domain for recruiting E6AP to the proteasome through the N-terminal AZUL domain, which is a unique feature of E6AP (Fig. 7). This result establishes E6AP as a privileged E3 ligase for the proteasome with the possibility of direct coupling of its ligase activity to proteasome activity. This finding provides new foundational knowledge that impacts future studies aimed at addressing the role of E6AP in cervical cancer, Angelman syndrome, and autism. Our results also redefine the current models of the 26S proteasome to include a dedicated binding domain in hRpn10 that has until now been characterized despite the discovery of Rpn10 as a proteasome substrate receptor over 2.5 decades ago72. This domain may have remained elusive in earlier studies in part due to its disordered state when unbound and its absence in fungi.

The induced folding of RAZUL upon binding to E6AP is similar to the coupled folding reported in previous studies73,74, such as the N-terminal transactivation (TAD) domain of p53, which exchanges between a disordered and partially helical conformation when unbound75–77 and forms a stable amphipathic α-helix when complexed with the E3 ligase Mdm2 78. Increasing intrinsic helicity of the p53 TAD domain yields stronger binding to Mdm2 in vitro and in cells79, suggesting that RAZUL could potentially be engineered to increase E6AP occupancy at the proteasome in future work or alternatively for E6AP targeting.

In contrast to hRpn10, the other two proteasome receptors, Rpn1 and Rpn13, contribute binding sites for DUBs11,22,80–82. Proximity between substrate receptors and DUBs is no doubt
**Fig. 6 RAZUL:AZUL forms an intermolecular 4-helix bundle.** Regions displaying AZUL contacts with RAZUL α1 (a, b), the 3₁₀-helix (c), and α2 (d) showing selected interacting sidechain atoms with oxygen, nitrogen, and sulfur colored red, blue, and yellow, respectively. The structure in e is displayed as a ribbon diagram to better view the 3₁₀-helix with hydrogen bonds included as red dashed lines. The structures in a, b, and d depict helices as cylinders for simplicity. e Electrostatic surface diagram for AZUL to highlight acidic (red) and basic (blue) regions. The bound RAZUL is displayed with cylindrical helices and sticks for N-terminal acidic residues with oxygen colored red. f ITC analyses for AZUL-binding hRpn10322–366 without or with Y326 phosphorylated. g Immunoprecipitation of Rpt3 immunoprecipitates or WCE from HCT116 or clone 13 lysates expressing indicated myc-hRpn10 constructs, including with the AZUL domain of E3 ligase E6AP (green), thus recruiting E6AP to show selected interacting sidechain atoms with oxygen, nitrogen, and sulfur colored red, blue, and yellow, respectively. The structure in e is displayed as a ribbon diagram to better view the 3₁₀-helix with hydrogen bonds included as red dashed lines. The structures in a, b, and d depict helices as cylinders for simplicity. e Electrostatic surface diagram for AZUL to highlight acidic (red) and basic (blue) regions. The bound RAZUL is displayed with cylindrical helices and sticks for N-terminal acidic residues with oxygen colored red. f ITC analyses for AZUL-binding hRpn10322–366 without or with Y326 phosphorylated. g Immunoprecipitation of Rpt3 immunoprecipitates or WCE from HCT116 or clone 13 lysates expressing indicated myc-hRpn10 constructs, including with the AZUL (ΔRAZUL) or the C-terminal T3 residues deleted (1–364) or with hRpn10 Y326F substituted with phenylalanine (Y326F). Cyclophilin B (Cyp B) is used as a loading control for WCE samples. Antibodies used for immunoprecipitation are indicated to the left of each panel. An asterisk ** indicates heavy chain antibody. Source data are provided as a Source Data file.

**Fig. 7 Model of hRpn10 RAZUL recruitment of E6AP to the 26S proteasome.** Proteasome substrate receptors Rpn1, Rpn10, and Rpn13 recognize ubiquitin chains (yellow) through their T1 site (blue), UIM region (blue), and Pru domain (blue), respectively. The Rpn10 C-terminal RAZUL is intrinsically disordered (left panel), but folds to form a 4-helix bundle with the AZUL domain of E3 ligase E6AP (green), thus recruiting E6AP to the proteasome (right panel). Zn, light blue.

Conducive to ubiquitin recycling and the chain removal needed for substrate translocation into the proteolytic CP. Although it does not contribute a DUB-binding site, Rpn10 is proximal to the proteasomal DUB Rpn1157,58. Why would the proteasome be benefitted by physically linking a substrate receptor to a ubiquitin E3 ligase? One possibility is to spatially and temporally link its activity and its substrates with the proteasome. One model based on previous studies is that E6AP directly acts on proteasome subunits, considering that E6AP is reported to ubiquitinate proteasomal DUB Rpn1157,58 and affinity for ubiquitinated proteins88; however, yeast lack both RAZUL and E6AP (Supplementary Fig. 1a) suggesting Rpn10 ubiquitination has redundant mechanisms or is independent of E6AP. In agreement with this, we found no evidence of altered levels or a molecular weight increase indicative of ubiquitination for hRpn10 following E6AP knockdown in HCT116 cells (Fig. 3b), although it remains possible that such activity requires induction by a specific cellular event or is cell type specific.
Another possibility is that E6AP localization to the proteasome via hRpn10 RAZUL serves in the broader context to allow additional ubiquitin chains to be added to protein substrates, providing higher affinity and in turn, more efficient degradation. Various models have been proposed to explain how different chain lengths/linkages affect the degradation rate of proteins, with multiple short ubiquitin chains shown to have higher efficiency of degradation than a single long chain. Multiple ubiquitin chains on a substrate may more readily enable multiplexed points of contact with receptor sites and associated/nearby DUBs around the degradation channel and thereby enable more efficient substrate translocation. As proteolysis by the proteasome requires a flexible initiation sequence to engage the ATPase ring of the RP98-99, premature deubiquitination without substrate engagement with the ATPase ring can lead to release of a substrate prior to degradation. Substrates with multiple ubiquitin chains have higher affinity for the proteasome and are deubiquitinated more slowly, favoring them for proteolysis by the proteasome, as observed by single molecule experiments.60

E6AP interacting with the proteasome through the Rpn10 RAZUL would be ideally situated to modify substrates in this context, with proximity to substrates recruited by the Rpn10 UIMs or associated shuttle factors. This model is supported by our finding that E6AP knockdown or displacement from the proteasome reduces ubiquitin co-immunoprecipitating with the proteasome, despite largely unchanged ubiquitin levels in the WCE. It has also been demonstrated that ubiquitin chains consisting of K11/K48 branched chains are more efficiently degraded by the proteasome than K11-linked chains.62 E6AP is known to catalyze K48 linkages98-101, however its specificity has not been studied extensively. It is possible that E6AP could add K48-linked ubiquitin (or other linkages, as yet to be determined) to existing K11-linked chains, in order to enhance degradation of substrates. A role for ubiquitin chain remodeling has been proposed for UBE3C102, which is the first ubiquitin ligase reported to physically interact with the proteasome. The UBE3C binding site is likely somewhere in the proteasome base subcomplex102,103, however, its location has yet to be elucidated, and its recruitment to the proteasome appears to be assisted by structurally impaired substrate.83

As human E6AP isoform 3 localizes to the nucleus in an hRpn10- and AZUL-dependent manner44, it may be that the E6AP’s function in the nucleus is primarily related to its association with hRpn10. By contrast, the decreased affinity for E6AP when RAZUL is phosphorylated at Y326 may imply that certain cellular contexts require less E6AP associated with the proteasome (Fig. 7). For example, the identification of Y326 phosphorylation in Jukat cells (immortalized human T lymphocyte) may be significant given that immune cell activation is tightly regulated by ubiquitination104. E6AP has been reported to interact with Lck and Blk105, which are immune cell-specific tyrosine kinases at the plasma membrane involved in T cell receptor (TCR) or B cell antigen receptor (BCR) mediated activation, respectively. It is conceivable that upon TCR or BCR activation, an immune cell-specific kinase is recruited to hRpn10 via E6AP, and that subsequent phosphorylation of hRpn10 leads to the reduction of E6AP at the proteasome, and in turn, delayed degradation of receptor components, allowing an elongated timeframe of activation. This mode of regulation could allow a fine-tuning of receptor activation by regulating E6AP activity at the proteasome in a very specific cellular location and context. While future studies will elucidate the intricacies of E6AP function at the proteasome, our findings provide insight into the uniqueness of E6AP as an E3 ligase with a dedicated binding site at the proteasome and protein abundance that correlates with proteasome substrate receptor hRpn10. Our finding that E6AP binds a domain of hRpn10 not present in yeast in combination with our discovery that E6AP levels depend on hRpn10 suggests that these two proteins evolved together to have linked functions, underscoring the importance of E6AP at the proteasome. These results provide a new foundation towards understanding the role of E6AP in its associated disease states.

**Methods**

**Protein sample preparation for biophysics experiments.** Human Rpn1093–377 was subcloned between the EcoRI and Xhol restriction sites of the pjGEX-6P3 vector (GE Healthcare 28954651) in frame with an N-terminal glutathione-S-transferase (GST) and a Prescision protease cleavage site, by using pET11d vector containing His6-hRpn10full-length (a gift from Dr Funio Hanaoka) and the appropriate primer pairs (Supplementary Table 1). The plasmid was transformed into E. coli strain Rosetta 2 (DE3) (MilliporeSigma 71400) with selection by ampicillin and chloramphenicol. The transformed cells were grown at 37 °C to OD600 of 0.5–0.6 and protein expression induced overnight at 17 °C by addition of 0.4 mM IPTG. The cells were harvested, frozen in liquid nitrogen and stored at −80 °C for 4–h, followed by resuspension in buffer 1 (50 mM Tris at pH 7.2, 300 mM NaCl, 5 mM DTT) supplemented with protease inhibitor cocktail tablets (Roche Diagnostics 11836153001). The cells were lysed by sonication and spun down at 27,000 × g for 30 min, after which the supernatant was incubated with pre-washed glutathione sepharose protein (GE Healthcare 17075605) for 3 h. After extensive washing in buffer 1, hRpn1093–377 was either separated from GST and the resin by cleaving with Prescision protease (GE Healthcare 27083401) in buffer 2 (10 mM MOPS at pH 6.5, 50 mM NaCl, 5 mM DTT, and 10 μM zinc sulfate), or eluted from the resin with the GST-tag intact by using buffer 3 (50 mM Tris at pH 7.2, 300 mM NaCl, 5 mM DTT, 20 mM glutathione). Further purification was achieved by size exclusion chromatography on a FPLC system AKTA pure (GE Healthcare) using a HiLoad 16/60 Superdex 75 (for samples with no GST-tag) or Superdex 200 (for GST-tagged protein) prep grade column in buffer 2 or 3. E6AP AZUL, spanning amino acids 24 to 87, was subcloned between the Ndel and SacI restriction sites of the pET28a (+) vector (MilliporeSigma 69984) in frame with an N-terminal poly histidine tag and a thrombin cleavage site, by using p4054 HA-E6AP isoform I (Addgene plasmid #8658 given by Dr Peter Howley)106 and the appropriate primer pairs (Supplementary Table 1). The plasmid was transformed into E. coli strain BL21 (DE3) (Thermo Fisher Scientific C600003) with kanamycin selection. The transformed cells were grown at 37 °C to OD600 of 0.5–0.6 and protein expression induced at 17 °C overnight by 0.4 mM IPTG. At the time of induction, zinc sulfate was added to a final concentration of 20 μM. The cells were harvested, frozen in liquid nitrogen and stored at −80 °C for 4–h, followed by resuspension in buffer 4 (10 mM MOPS at pH 7.2, 300 mM NaCl, 5 mM 2-mercaptoethanol, 10 μM zinc sulfate) supplemented with EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics 11836170001). The cells were lysed by sonication and spun down at 27,000 × g for 30 min. The supernatant was incubated with pre-washed Ni-NTA agarose resin (Quagen 302563) for 1 h at 4 °C. After extensive washing in buffer 4, E6AP AZUL was separated from the His-tag and the resin by cleaving with thrombin in buffer 5 (10 mM MOPS at pH 6.5, 50 mM NaCl, 5 mM 2-mercaptoethanol, and 10 μM zinc sulfate). Further purification was achieved by size exclusion chromatography on an FPLC system AKTA pure (GE Healthcare) using a HiLoad 16/60 Superdex 75 prep grade column in buffer 5. E6AP AZUL was subcloned between the Ndel and EcoRI restriction sites of the pET11d vector (MilliporeSigma 69960) in frame with an N-terminal poly histidine tag and a thrombin cleavage site, by using pET11d vector containing His6-hRpn10full-length (a gift from Dr Funio Hanaoka) and the appropriate primer pairs (Supplementary Table 1). The plasmid was transformed into E. coli strain BL21 (DE3) (Thermo Fisher Scientific C600003) and purified in an identical manner as N-terminal His-tagged E6AP AZUL, but eluted from the resin with the His-tag intact using elution buffer 6 (10 mM MOPS at pH 7.2, 50 mM NaCl, 5 mM 2-mercaptoethanol, and 250 mM imidazole), and further purified by size exclusion chromatography on a FPLC system AKTA pure (GE Healthcare) with a HiLoad 16/60 Superdex 75 prep grade column in buffer 5. 15N ammonium chloride, 31P glucose, and 1H2O were used for isotopic labeling. All NMR samples were validated by mass spectrometry. 26S proteasome (human) was purchased (Envio Life Sciences, Inc. BML-PW9310).

**Peptide synthesis.** hRpn10132–366 peptide without or with Y326 phosphorylated was synthesized on a Liberty Blue Microwave peptide synthesizer (CEM Corporation) using Fmoc chemistry. To avoid oxidation, Met residues in the sequence of HVR were substituted by isotopic norleucine. The following modifications were introduced to the published protocol for high efficiency peptide synthesis117. The coupling with N,N′-disopropylcarbodiimide (DIC)/ethyl 2-cyano-2-(hydroxymethyl) acetate (OXYMA) was performed for 4 min at 90 °C for all residues except Cys and His, for which the reaction was carried out for 10 min at 50 °C. Removal of the Fmoc group was conducted at 90 °C for 2 min for sequences containing no Cys.
GSTM-pulldown experiment and sample preparation for mass spectrometry. HCT116 and 293T cells were purchased from American Type Culture Collection (ATCC, CRL-3216). The GST-hRpn10 constructs were expressed in the insect cell line Sf9 (Invitrogen, Carlsbad, CA). Cells were grown in a 37 °C humidified atmosphere of 5% CO2. Cells were collected, washed twice with PBS, and lysed in 1% Triton-X buffer (50 mM Tris at pH 7.5, 150 mM NaCl, and 1 mM PMSF), supplemented with protease inhibitor cocktail (Roche Diagnostics 11836153001). Total protein concentration in the lysate was determined by the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific 23225). Overall, 2 nmoi of purified GST-tagged hRpn10 was added to 200 mL of pre-washed glutathione sepharose resin for 3 h, washed once with buffer 7 (50 mM Tris at pH 7.5, 150 mM NaCl, 2 mM DTT, and 1.0% v/v Triton-X-100), and the resin next incubated with 1.2 mL of cell lysate for 3 h. Unbound protein was removed by washing three times in buffer buffer 7, after which resin-bound proteins were eluted, subjected to electrophoresis on a 4–20% NaDodSO4–PAGE Bis-Tris gel (Thermo Fisher Scientific NP0322), and visualized by Coomassie staining.

For each of the six lanes (GST-hRpn10–377; GST-hRpn10–537; GST-EDAP AZUL; GST-hRpn10–322 peptide; GST-hRpn10–232; GST-hRpn10–142), the region above 51 kDa was cut into 12 bands that were placed individually into 1.5 mL eppendorf tubes. Each gel band was then further cut into small pieces and destained using 50% acetic acid/25 mM NH4HCO3 at pH 8. After removal of the organic solvent, gel pieces were dried by vacuum centrifugation for 1 h. Trypsin (20 ng mL−1) in 25 mM NH4HCO3 at pH 8, after addition of 3% formic acid, was added to each sample (50 mL) and incubated on ice for 1 h. A total of 25 mM ammonium bicarbonate were added to completely saturate the bands for overnight incubation at 37°C. Peptides were extracted in 70% acetonitrile and 5% formic acid using bath sonication and supernatant solutions were dried in a speed vacuum. Samples were desalted utilizing Pierce C18 spin columns (Thermo Fisher Scientific), dried, and resuspended in 0.1% TFA prior to mass spectrometry analysis.

Mass spectrometry. Peptides were analyzed on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The desalted tryptic peptide was loaded onto an Acclaim PepMap 100 C18 LC column (Thermo Fisher Scientific) utilizing a Thermofisher Easy nLC 1000 system (Thermo Fisher Scientific) connected to the Q Exactive mass spectrometer. The peptides were eluted with a 5–48% gradient of acetonitrile with 0.1% formic acid over 55 min with a flow rate of 300 nL min−1. The raw MS data were collected and analyzed in Proteome Discoverer 2.2 (Thermo Fisher Scientific) with Sequest HT software and was searched against the Human Proteome database.

His pull-down assays. His pull-down assays were performed as described previously21,22,23. Briefly, 2 nmoi of purified His-tagged hRpn10(full-length, hRpn10Δ377, or hRpn10Δ537) was added to 10 mL of pre-washed Ni-NTA agarose resin (Qiagen, Valencia, CA) equilibrated in buffer 5. The resin was then incubated with 200 pmol of E6AP (UBPBio K1411) for 1 h and unbound protein removed by extensive washing with buffer 5. Resin-bound proteins were eluted and subjected to SDS-PAGE followed by visualization with Coomassie staining.

For pull-down assays testing whether E6AP impacts the affinity of the 26S proteasome for ubiquitin chains, 70 pmol of a commercially available mixture provided by His-tagged, non-cleavable K48-linked Ub2/Ub4 (UBPBio D1701) was added to 200 μg of His-hRpn10Δ377, His-hRpn10Δ537, and His-hRpn10Δ232 agarose resin each with 100 μL of Ni-NTA agarose resin (Qiagen, Valencia, CA) equilibrated in buffer 5. The mixtures were then incubated with 10 μg of human erythrocyte 26S proteasome (Enzo Life Sciences, Inc., BML-PW9310) alone, 10 μg of 26S proteasome with equimolar of E6AP (UBPBio K1411), or 10 μg of E6AP alone for 1 h and unbound protein removed by extensive washing with buffer 8. A total of 10 nmoi of human 26S proteasome or E6AP was added to 200 μL of Ni-NTA agarose resin (Qiagen, Valencia, CA) equilibrated in buffer 5 and incubated extensively with buffer 8, as negative controls. Resin-bound proteins were eluted and subjected to SDS-PAGE followed by visualization with Coomassie staining.

I TC and SPR binding affinity experiments. ITC was performed at 25 °C on a MicroCal ITC200 system. hRpn10–377, E6AP AZUL, and hRpn10–537–322–366 peptide were synthesized and purified as described previously24. Eighteen 2 μL aliquots of 0.462 mM E6AP AZUL were injected at 1000 rpm into a calorimeter cell (volume 200.7 μL) that contained 0.0405 mM hRpn10–377–377. For measuring interaction between hRpn10–377–377 and peptide E6AP AZUL, eighteen 2 μL aliquots of 0.110 mM E6AP AZUL were injected at 1000 rpm into a calorimeter cell (volume 200.7 μL) that contained 0.011 mM hRpn10–377–377 without or with Y326 phosphorylated. Blank experiments were performed by replacing protein samples with buffer and this blank data was subtracted from the experimental data during analysis. The integrated interaction heat values were normalized to the function of the molar ratio of E6AP AZUL to hRpn10–377–377.

SPR experiments were recorded for GST-tagged hRpn10–377 and E6AP AZUL with a Biacore T200 system (GE Healthcare). Utilizing the GST capture kit, GST-hRpn10 constructs were immobilized on a CM5 sensor chip (GE Healthcare) with 10 nM zinc sulfate. Single cycle kinetic experiments were performed using five injections (30 μL min−1) of increasing concentration of protein (5–50 nM) passed over the sensor chip for 150 s association, followed by a 420 s dissociation. The experiments were repeated in triplicate. Buffer and reference subtracted kinetic constants (k on and k off) and binding affinities (K d) were determined utilizing the Biacore T200 evaluation software (GE Healthcare).

CD experiments. Far-UV range CD spectra (240–190 nm) of 10 μM hRpn10–377, 10 μM E6AP AZUL, the mixture of 10 μM hRpn10–377 and 10 μM E6AP AZUL, and buffer 9 (10 mM MOPS, 10 mM NaCl, 10 mM DTT, 10 μM zinc sulfate at pH 6.5, as a control) were recorded on a Jasco J-1500 CD spectrometer (Tokio, Japan) using a quartz cuvette with 1.0 mm path length and temperature controlled at 25 ± 0.1°C. All spectra were collected continuously at a scan speed of 200 nm/min and averaged over accumulation of three spectra. The buffer spectrum was subtracted from the protein spectra during data analyses. The molar ellipticity θ (in deg cm2 dmol−1) was calculated from the measured machine units m° in millidegrees at wavelength λ using the Eq. 1.

\[
\theta = \frac{m°}{10^3 \text{C} \lambda L}
\]
assembly of the left and right homology arms with the P2A-puromycin cassette. The left and right homology arms were amplified using PCR from 293T genomic DNA and mRNA, as described in the previous study (Table 1). PCR fragments of the expected size were generated from clones 13 and 14 using primers homologous to the homology arms (Supplementary Table 1). Fragment PCR products were purified using a PCR purification kit (Qiagen) and cloned into the vector (Life Technologies K457501) and sequenced verified.

Cell lysis and immunoprecipitations. All cells were washed twice in cold PBS (Thermo Fisher Scientific) prior to harvesting. Cells used for Rpt3 IP were harvested on ice in 1% Triton X-100 buffer (1% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF, 0.5 μg ml−1 Pepstatin A, and Roche EDTA-free protease inhibitor cocktail). Cell extracts were centrifuged for 15 min at 4 °C and 20,000 g, and the supernatant was isolated. IPs were performed overnight at 4 °C with 1–1.5 mg total protein lysate, using 4 μl Rpn3 antibody (abcam ab140515) per conditions and 0.5 μl mlgG beads (GE healthcare) bound to 1–1.5 mg for 3 h, and precipitates were washed 5–7 times with 1% Triton X-100 buffer. Immunocomplexes were heated to 95 °C for 10 min in denaturing sample buffer prior to subjection to SDS-PAGE.

Cys transferases were harvested on ice in 0.5% NP-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF, 5 μg ml−1 Pepstatin A, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM Na3VO4, and Roche EDTA-free protease inhibitor cocktail). Cell extracts were placed on ice for 30 min with extensive pipetting every 10 min. Extracts were centrifuged at 20,000 × g for 30 min at 4 °C. Supernatants were diluted 1:1 with m-lyc-trap dilution/wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF, 5 μg ml−1 Pepstatin A, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM Na3VO4, and Roche EDTA-free protease inhibitor cocktail), and 1–1.5 mg total protein lysate was incubated with 25 μL m-lyc-trap agarose (nanobody coupled) beads (chromotek) overnight at 4 °C. Nanobody-myc complexes were washed five times with m-lyc-trap dilution/wash buffer at 4 °C for 10 min in denaturing sample buffer prior to subjection to SDS-PAGE.

In experiments not involving immunoprecipitation, cells were either harvested in 1% Triton X-100 buffer or 1% NP-40 buffer (1% Nonidet P-40, 25 mM Tris pH 7.2, 137 mM NaCl, 10% glycerol, 1 mM DTT, 5 mg ml−1 Pepstatin A, 1 mM PMSF, and Roche protease inhibitor cocktail). Lysates were centrifuged at 20,000 × g and 4 °C for 15 min and supernatants were isolated for immunoblotting.

SDS-PAGE, immunoblots, and antibodies. Protein lysates were subjected to SDS-PAGE on 4–12% NuPAGE Bis-Tris gels (Thermo Fisher Scientific) using MOPS SDS running buffer (Thermo Fisher Scientific NP0322) with MOPS SDS buffer running buffer (Thermo Fisher Scientific NP0001), except in the case of Fig. 4b, d, where MES SDS running buffer (Thermo Fisher Scientific NP0002) was used to achieve better resolution of myc-RAZUL. Proteins were transferred to 0.45 μm Amersham nitrocellulose membrane (Thermo Fisher Scientific LC2005) using a NayPge transfer buffer (Thermo Fisher Scientific NP0066) supplemented with 10% methanol. Following transfer, membranes were blocked with 5% milk in tris-buffered saline with 1% tween 20 (TBS-T). Blocked membranes were incubated with primary antibodies (diluted in 5% milk in TBS-T overnight). Membranes were washed five times in TBS-T and incubated with HRP-conjugated secondary antibodies (diluted in 5% milk in TBS-T) for 2 h. Following another set of five washes, blots were developed using HyGlo quicksparks chluminisence HRP detection reagent (Denville Scientific Inc. E2400) and HyBlot CL Autoradiography Film (Denville Scientific Inc. E3018). Primary antibodies used were: β-actin (Cell Signaling Technology 4970, 1:30,000), Cyclophilin B (Abcam ab178397, 1:10,000), Δ2H-labeled Rpn10305 (PDB) with accession number 6U19. Chemical shift assignments have been deposited in the Protein Data Bank from a previous study45.

All NMR experiments were conducted at 25 °C in buffer 10 (10 mM MOPS at pH 6.5, 30 mM NaCl, 5 mM DTT, 10 μM zinc sulfate, 1 mM pefabloc, 0.1% NaN3, and 5% H2O/95% D2O), except for 2D 1H–15N HSQC, 3D HCCH-TOSY, CCH-TOSY, CCH-TOSY-15N, 15N- and 1H–15N-half-filtered NOESY experiments, which were acquired on samples dissolved in 2H2O. Spectra were recorded on Bruker AvanceIII 600, 700, 800, or 850 MHz spectrometers equipped with cryogenically cooled probes.

All NMR data processing was performed with NMRpipe115 and spectra were visualized and analyzed with XEASY116. Secondary structures were assessed by comparing chemical shift values of Cα and C′ atoms to random coil positions to generate a CSI117 and also by the TALOS+ program118.

NMR titration experiments. In 2H, 15N HSQC experiments were recorded on samples 1 and 2. These experiments were also recorded on sample 3 to obtain free state assignments for Rpn1159–377. Unambiguous intermolecular distance constraints were obtained by using 3D 1H–15N-half-filtered NOESY experiments (100 ms mixing time) recorded on samples 1 and 2. Chemical shift assignment of hRpn11 AZUL in the free state was available from a previous study45.

1D 13C-edited, 1H NMR experiments. Three NMR samples were prepared in buffer 11 (50 mM d4-Tris pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1.5 mM ATP–YS, 10 μM zinc sulfate, 2 mM DTT, 0.5 mM pefabloc and 5% 2H2O/95% 1H2O), including 0.3 μM of free 13C labeled AZUL domain, 0.3 μM of labeled AZUL domain mixed with equimolar unlabeled AZUL, and 0.3 μM of 13C labeled AZUL domain mixed with equimolar human 26 S proteasome (Enzo Life Sciences, Inc., BML-PW9310). 200,000 1D traces of a 1H, 13C HSQC experiment119,120 were averaged for each sample at 25 °C and 850 MHz with a cryogenically cooled probe.

Structure calculation of the hRpn11 RAZUL: E6AP AZUL complex. XPLOR-NIH 2.1c121 was used on a Linux operating system to solve the complexed structure by using NOE and hydrogen bond constraints as well as backbone φ and ψ torsion angle constraints derived from TALOS+118 (Table 1). Hydrogen bonds were generated by using secondary structure assignments and NOE connectivities with defined distances from the acceptor oxygen to the donor hydrogen and nitrogen of 1.8–2.1 and 2.5–2.9 Å, respectively. Hydrogen bonds restraints were not included in the initial calculation but were in the final round of structure calculations. When calculating the structures of hRpn11 RAZUL:E6AP AZUL, intermolecular distance constraints determined from the 3D 1H–15N-half-filtered NOESY experiments were used, in addition to intramolecular constraints for hRpn11 RAZUL and E6AP AZUL that were generated from 15N or 13C NOESY spectra acquired on the complexes (Table 1). The complexed structures were calculated from 50 linear starting structures of hRpn11 RAZUL and E6AP AZUL molecules, which were subjected to 2000 steps of initial energy minimization to ensure full spatial sampling and appropriate coordinate geometry. The structures were next confined according to the input data by subjecting them to 55,000 simulated annealing steps of 0.05 ps at 3000 K, followed by 5000 cooling steps of 0.005 ps, 5000 steps of energy minimization were applied to produce the final structures, which were recorded as coordinate files. The resulting structures had no distance or dihedral angle violation greater than 0.3 Å or 5°, respectively. The 15 lowest energy structures were chosen for visualization and statistical analyses. Structure evaluation was performed with the program PROCHECK-NMR, the percentage of residues in the most favored, additionally allowed and disallowed regions is 95.4, 4.5, 0.1, and 0.0, respectively. Visualization was performed with MOLMOL123 and PyMOL (PyMOL Molecular Graphics System, http://www.pymol.org). The electrostatic surface of E6AP AZUL was generated by the Poisson–Boltzmann (APBS) method124,125.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Atomic coordinates for RAZUL:AZUL have been deposited in the Protein Data Bank (PDB) with accession number 6U19. Chemical shift assignments have been deposited in the Biological Magnetic Resonance Data Bank (BMRB) with accession number 27875. The source data underlying Figs. 1b, e, f, 2a–c, 3a–d, 4a–c, 5c, d, and 6g and
are available from the corresponding authors upon reasonable request.

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Author contributions
G.R.B. performed molecular biology and cell biology studies. X.C. performed CD, NMR, and structure calculations. R.C. designed and generated plasmids for making ΔRAZUL cells. M.O. and T.A. performed SPR. C.J. and T.A. performed mass spectrometry analysis. D.L.E. prepared cell lysate for GST pull-down. V.S. made and isolated clones of ΔRAZUL cells. S.G.T. performed ITC. N.I.T. synthesized peptides. K.J.W. designed experiments. G.R.B., X.C., and K.J.W. wrote/edited the manuscript.

Competing interests
The authors declare no competing interests.

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