Supporting Information

# Native Mass Spectrometry of Complexes Formed by Molecular Glues Reveals Stoichiometric Rearrangement of E3 Ligases

Cara Jackson[1], Rebecca Beveridge[1]*

[1]Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, United Kingdom

*Correspondence: Rebecca.beveridge@strath.ac.uk

## Supplementary Methods

### Expression and purification of E3 ligases and target proteins

Cereblon (amino acids 40-442) with an N-terminal 6xHis-TEV tag and DDB1 (amino acids 1-1140 with replacement of 396-705 by GNGNSG) were co-expressed in SF9 insect cell. After lysis in 50 mM HEPES/NaOH, 500 mM NaCl, 1 mM TCEP, Complete protease inhibitor 1 tab / 100mL, 10 mM imidazole, pH 8.0 and centrifugation to clarify the lysate, the cereblon/DDB1 complex (hereafter CRBN/DDB1) were captured on HisPur Ni-NTA resin and eluted with a step-wise gradient with imidazole. The eluted sample was treated with TEV protease to remove the His-tag and dialyzed overnight to remove imidazole. The dialyzed sample was then passed back on the Ni-NTA resin to re-capture uncleaved samples and separated His-tag while cleaved CRBN/DDB1 passed through the resin unbound. CRBN/DDB1 was then further purified using a HiTrap Q HP ion-exchange column with a salt-gradient and size-exclusion chromatography using HiLoad Superdex 26/600 in 10 mM Hepes, 240 mM NaCl, 1 mM TCEP, pH 7.0.

GSPT1 (amino acids 300-496) containing N-terminal 6xHis-MBP-TEV and C-terminal Avi tags was co-expressed with BirA in *E. coli* BL21-CodonPlus (DE3)-RIPL. The soluble fraction generated by cell lysis in 50 mM HEPES / NaOH, 300 mM NaCl, cOmplete protease inhibitor EDTA free 1 tab / 100 mL, 1 mM TCEP, 10% glycerol, pH 7.5 and centrifugation, was passed through HisPur Ni-NTA resin to capture the target protein. Proteins were eluted from the Ni-NTA resin using an imidazole gradient and were subjected to TEV protease cleavage while dialyzing overnight to remove imidazole. The dialyzed sample was then re-applied on the Ni-NTA resin to separate tag-cleaved GSPT1 from other species. GSPT1 was further purified by heparin chromatography, and by size-exclusion chromatography in 50 mM HEPES / NaOH,150 mM NaCl,1 mM TCEP, pH 7.5.

DCAF15 (amino acids 1-600 with an internal deletion of 276-383) with N-terminal 6xHis-TEV, DDB1 (amino acids 1-1140 with replacement of 396-705 by GNGNSG), and DDA1 (amino acids 1-102) were

co-expressed in SF9 cells. Cells were lysed in 25 mM Tris-HCl, 300 mM NaCl, 10% Glycerol, 1 mM TCEP, 20 mM imidazole, cOmplete protease inhibitor EDTA free 1 tab / 100 mL, 0.1 mg Benzonase/1L culture, pH 7.5 by a high-pressure homogenizer (500 bar, 3 passes) and the soluble fraction was separated by high-speed centrifugation. A HisTrap HP column was used as the first step to purify the DCAF15/DDB1/DDA1 complex from the soluble lysate. Samples eluted from the first column using an imidazole gradient were then further purified by ion-exchange chromatography, followed by size-exclusion chromatography in 25 mM Tris/HCl, 300 mM NaCl, 1 mM TCEP, pH 7.5.

RBM39 (amino acids 235-331) containing N-terminal 6xHis-TEV-Avi was expressed in *E. coli* BL21 (CP). Cell pellets were re-suspended in the lysis buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 1 mM TCEP) supplemented with 0.5 mM PMSF and sonicated to generate cell lysates which was then clarified by high-speed centrifugation. The supernatant was applied on a Ni-NTA Superflow resin and the bound proteins were eluted with a step-wise imidazole gradient. The eluted proteins were treated with TEV protease to release the His-Tag and was dialyzed overnight to remove imidazole. The digested and dialyzed sample treated with BirA in the presence of biotin, magnesium chloride, and ATP overnight at 4 °C to produce biotinylated RBM39. The sample was then reloaded on a Ni-NTA column to capture uncleaved proteins and liberated His tag, leaving biotinylated and tag-cleaved RBM39 in the flow-through. This fraction was collected, concentrated, and purified by size-exclusion chromatography in 25 mM HEPES pH7.5, 300 mM NaCl, 1 mM TCEP.

## Supplementary Figures

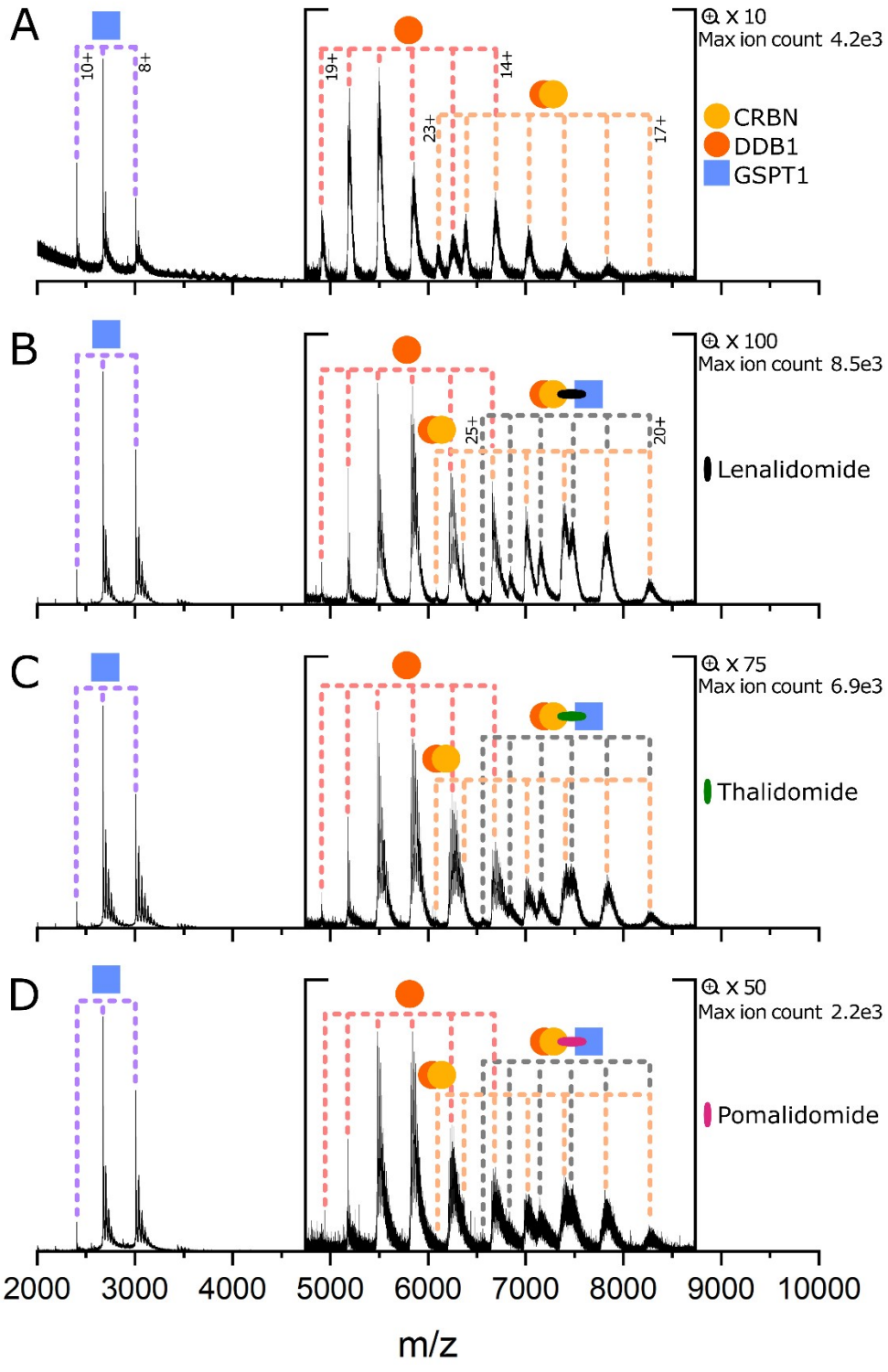



**Figure S1.** GSPT1 (5 µM) and CRBN:DDB1 (5 µM) in the absence of glue (A), presence of lenalidomide (100 µM) (B), thalidomide (100 µM) (C), and pomalidomide (100 µM) (D).

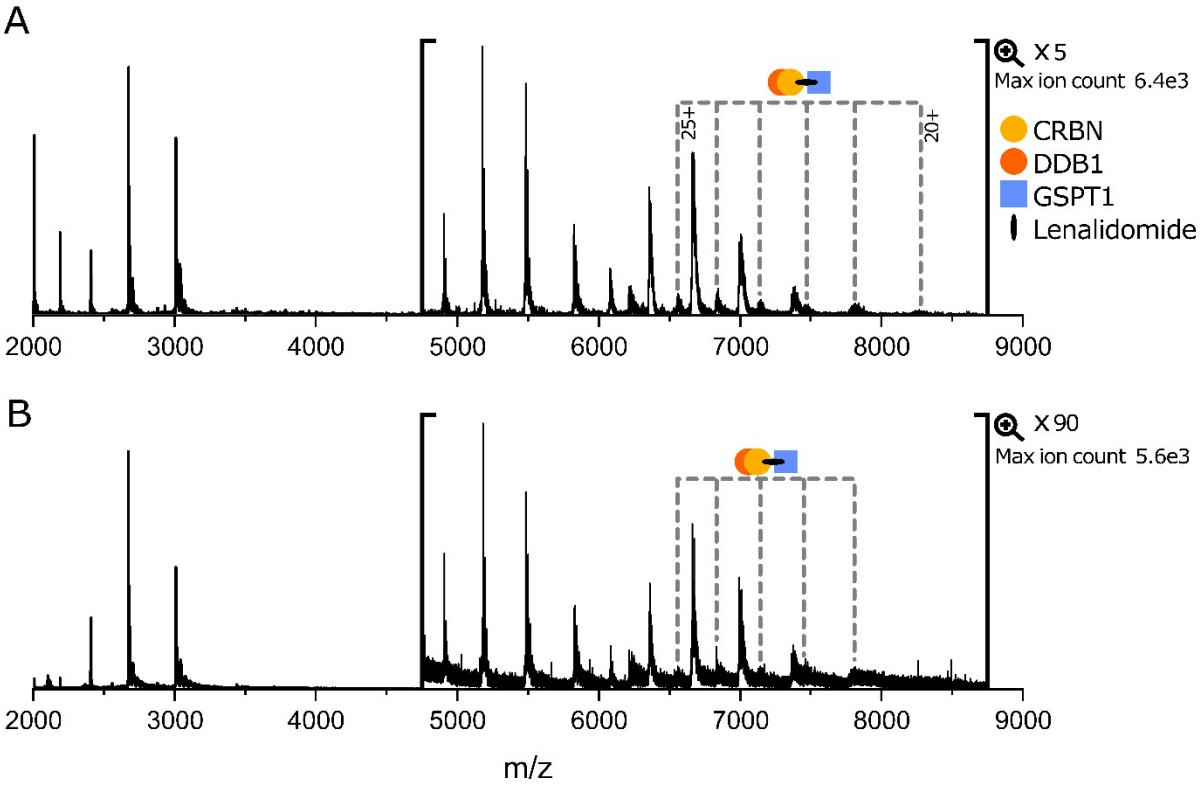



**Figure S2.** (A) GSPT1 + CRBN:DDB1 + lenalidomide (50 µM), (B) GSPT1 + CRBN:DDB1 + lenalidomide (5 µM). Protein concentrations are 5 µM and samples are in 100 mM AmAc containing 0.5% DMSO.

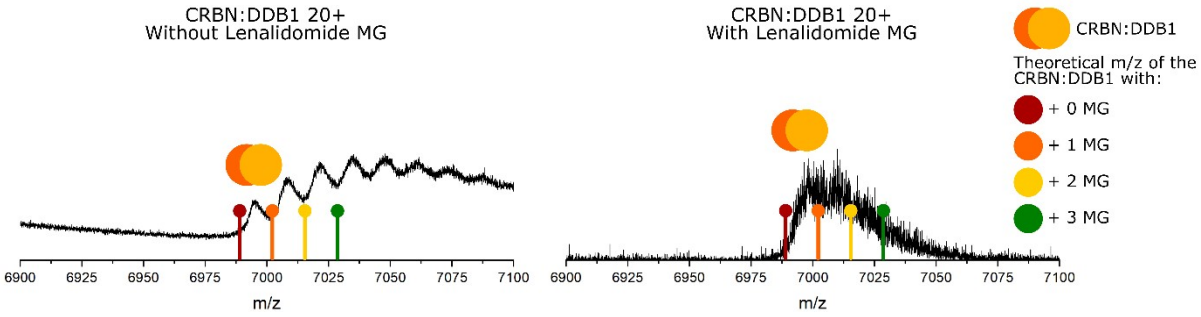



**Figure S3.** CRBN:DDB1 (5µM) in the absence and presence of lenalidomide (100 µM). The 20+ charge state peak has been focused on. The m/z at the leftmost of each peak is used to compare to the theoretical mass, as m/z values over the rest of the peak correspond to the species plus various adducts gained during desolvation. The m/z of both peaks aligns with the theoretical m/z of CRBN:DDB1 with no MG bound (6990 m/z). The width of the peak does not allow to definitively rule out lenalidomide binding, but at least some portion of CRBN:DDB1 exists in the unbound form. The adducts on the peak without Lenalidomide correspond to a molecular mass of 276 Da (accurate mass measured by collision induced dissociation).

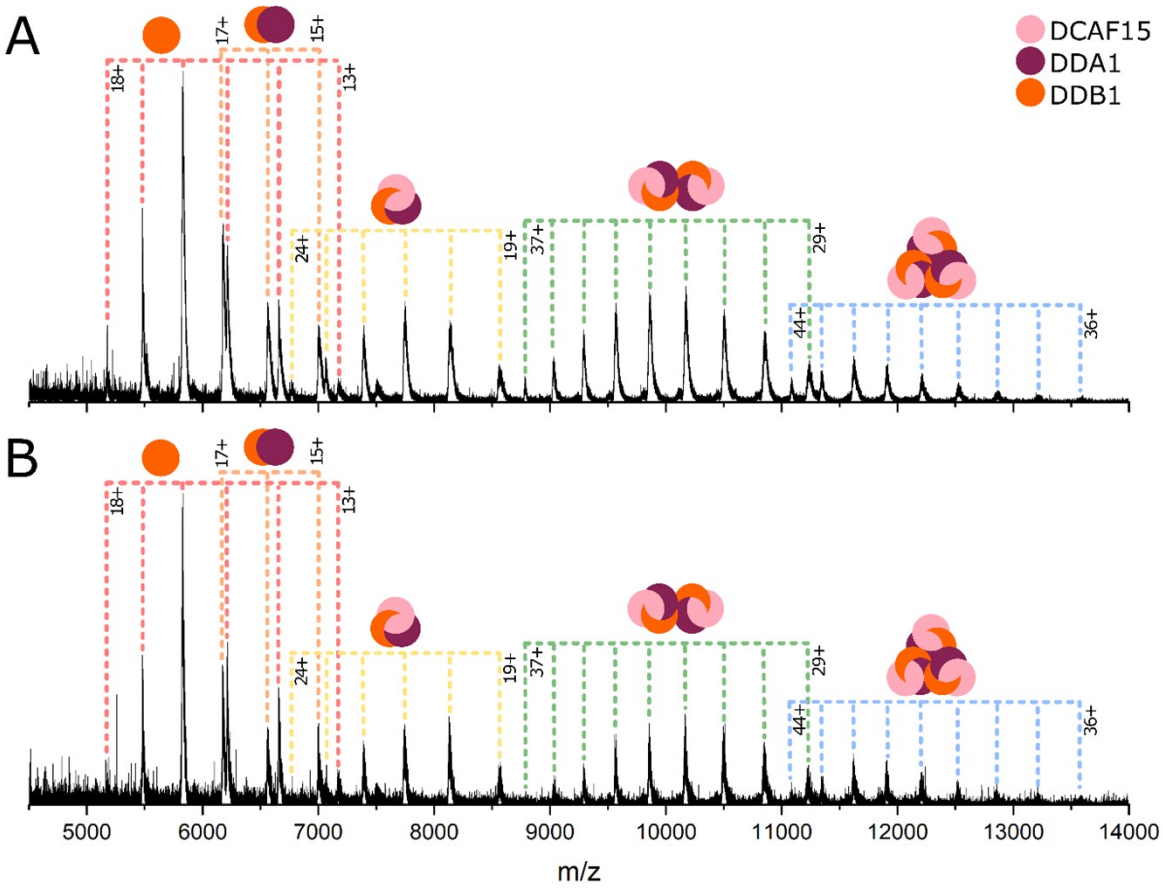



**Figure S4.** DCAF15 complex at 5 µM (A) and 2.5 µM (B). All species are present at the same relative intensity at both concentrations, with an overall reduction in intensity for the lower concentration, as expected.

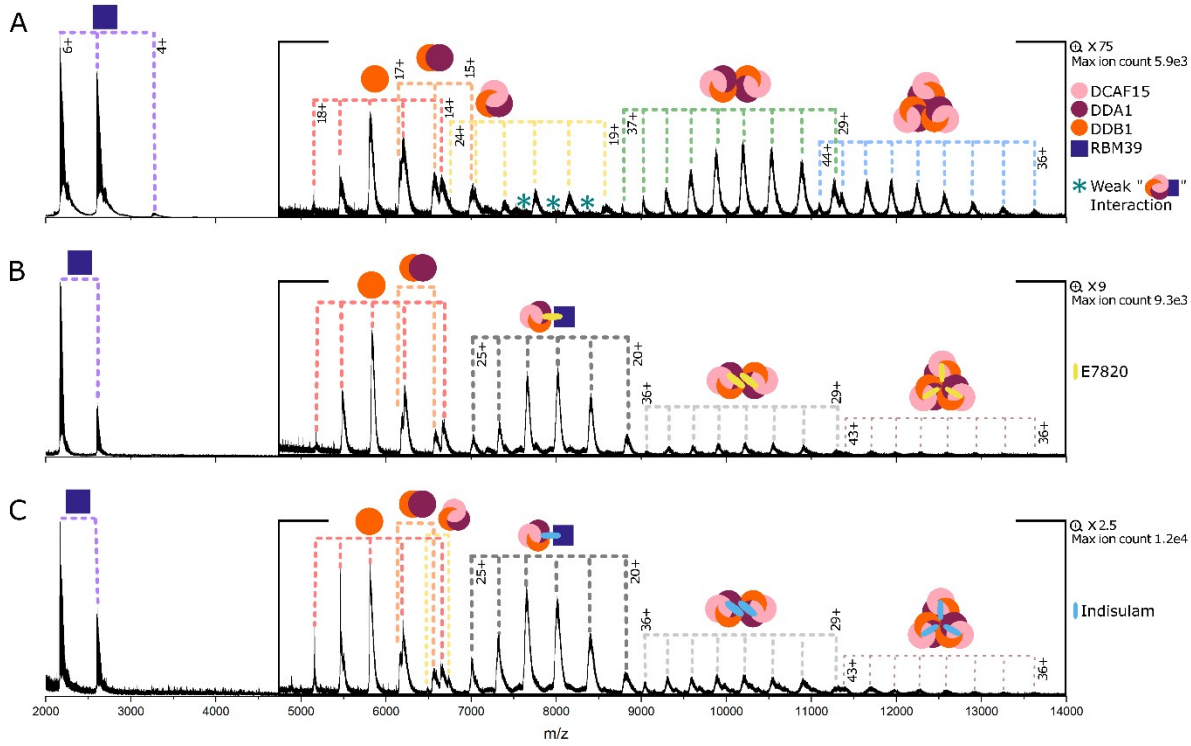



**Figure S5.** Mixture of RBM39 (5µM) and DCAF15 complex (5 µM) in the absence (A) and presence of E7820 (100 µM) (B) and indisulam (100 µM) (C).

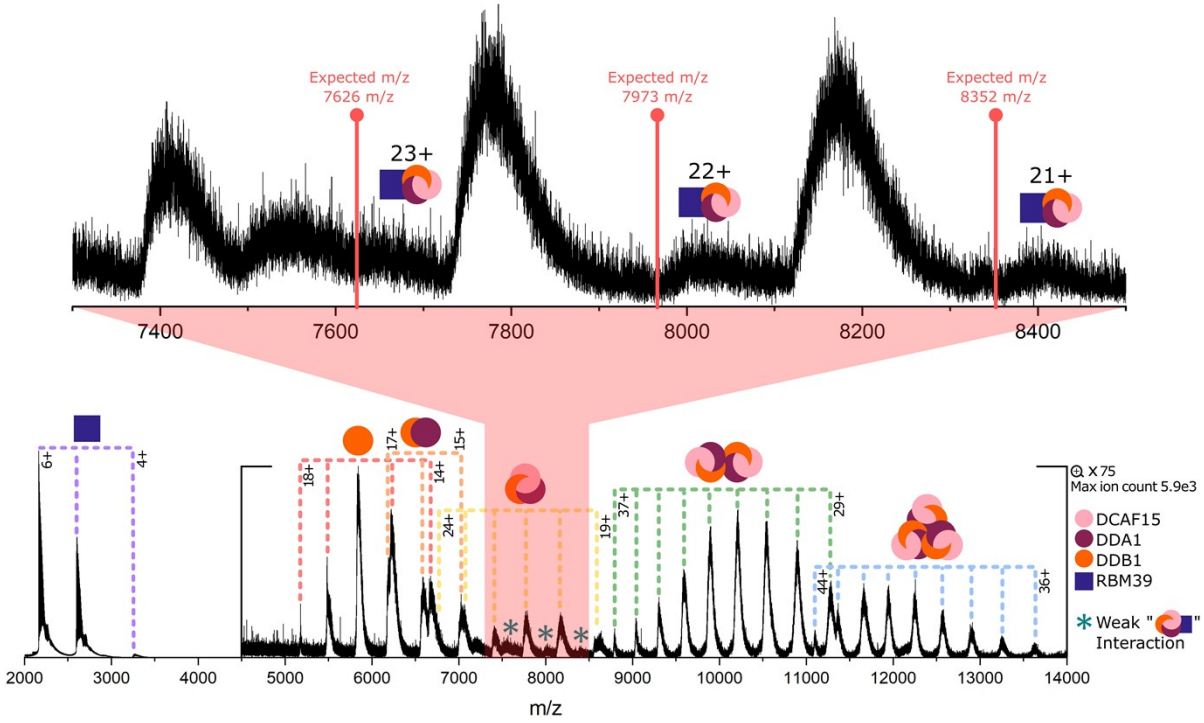



**Figure S6.** Mixture of RBM39 (5µM) and DCAF15 complex (5µM) in the absence of E7820 showing low intensity peaks corresponding to the weak DCAF15 complex RBM39 interaction.

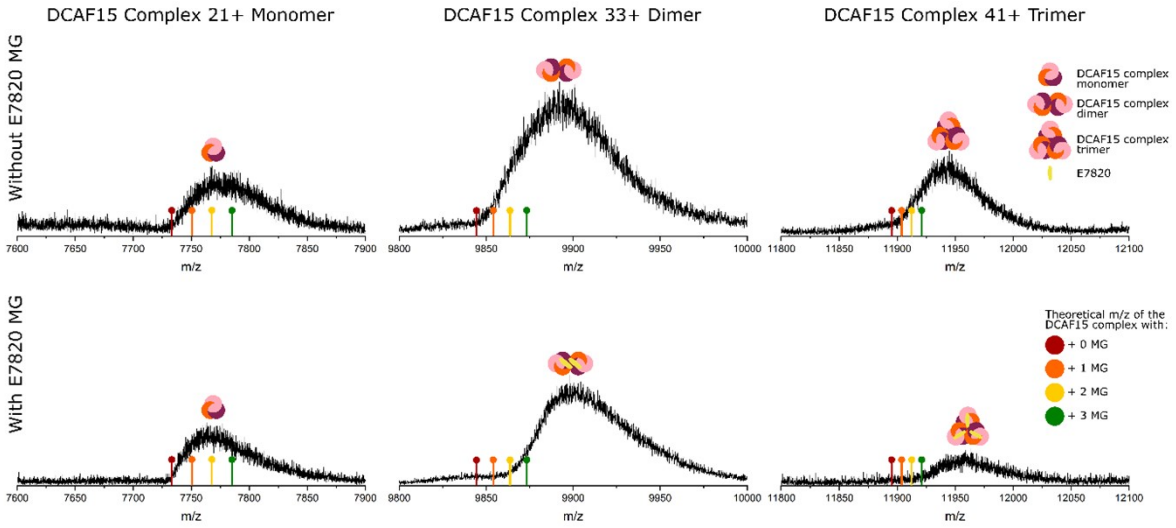



**Figure S7.** Selected peaks of RBM39 (5µM) and DCAF15:DDA1:DDB1 (5µM) in the absence (top) and presence (bottom) of E7820 (100 µM). Full spectra can be viewed in Figures 3A and C respectively in the main text. A mass shift can be observed for the dimer and trimer following E7820 addition, showing dimers and trimers of the DCAF15 complex do bind to the MG. The shift for the dimer is 20 m/z, which in charge state 33+ corresponds to a mass difference of 660 Da, approximately the molecular weight of two E7820 molecules (672 Da theoretical mass increase). The shift for the trimer is 23 m/z, which in charge state 44+ corresponds to a mass difference of 1012 Da, approximately the molecular weight of three E7820 molecules (theoretical mass shift 1008 Da).

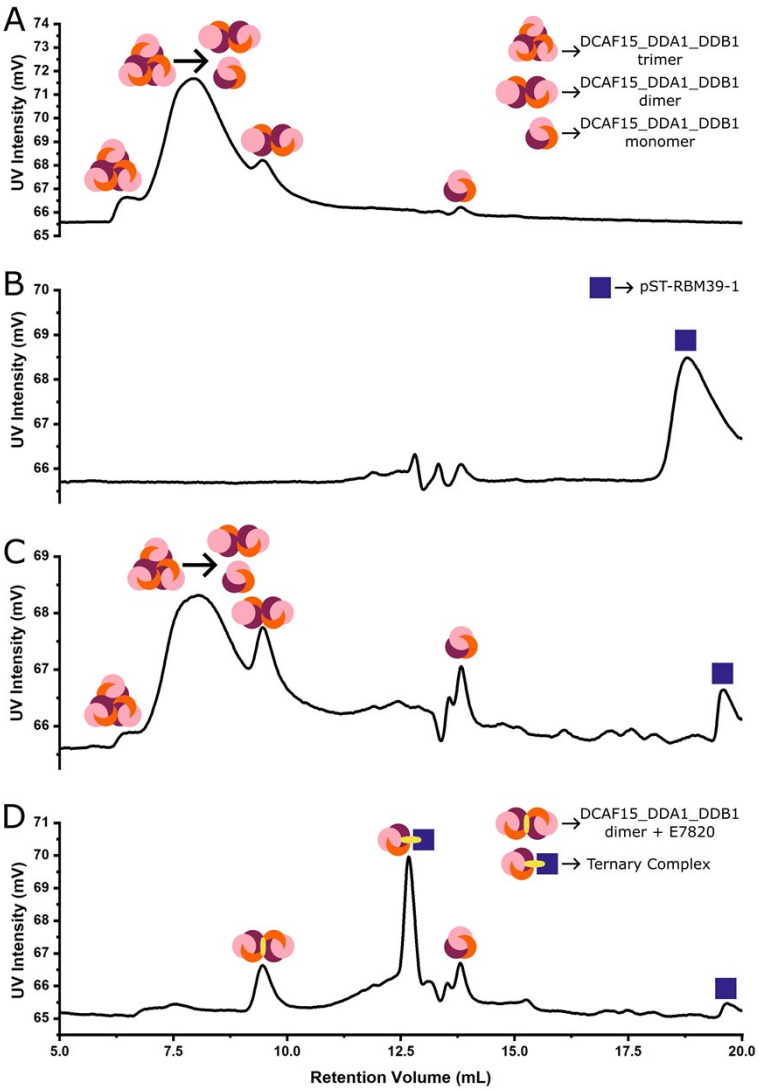



**Figure S8.** Size exclusion chromatography of (A) DCAF15 complex, (B) RBM39, (C) DCAF15 complex + RBM39, (D) DCAF15 complex + RBM39 + E7820.

(A)- DCAF15 complex alone. Peaks at retention volumes of 6.5 and 9 mL are attributed as trimeric and dimeric DCAF15 complex, respectively. The large, broad peak between 7 and 9 mL is attributed as being products of trimer dissociation (dimer and monomers) that has occurred during the time course of the experiment. This would also account for the raised baseline after the dimer (i.e., after 10 mL), as this would be the dissociated monomer. The peak arriving at 13.5 mL is assigned as the monomeric DCAF15 complex. These assignments are based on the abundance of monomers, dimers and trimers in the nMS in these conditions (Figures 3A and 4A).

(B)- The peak eluting at 18-19 mL is assigned as unbound RBM39.

(C)- DCAF15 complex + RBM39 in the absence of E7820. There is a similar distribution of the DCAF15 complexes existing as dimers and trimer.

(D)- DCAF15 complex + RBM39 + E7820. There are changes to the peak distribution that is in agreement with the findings from nMS. Most importantly, a new peak is observed at 12.5 mL which is assigned as the 1:1:1 DCAF15:E7820:RBM39 complex. There is also a large reduction in the intensity of the DCAF15 trimer peak, as well as the peak corresponding to the dissociation products of the trimer.

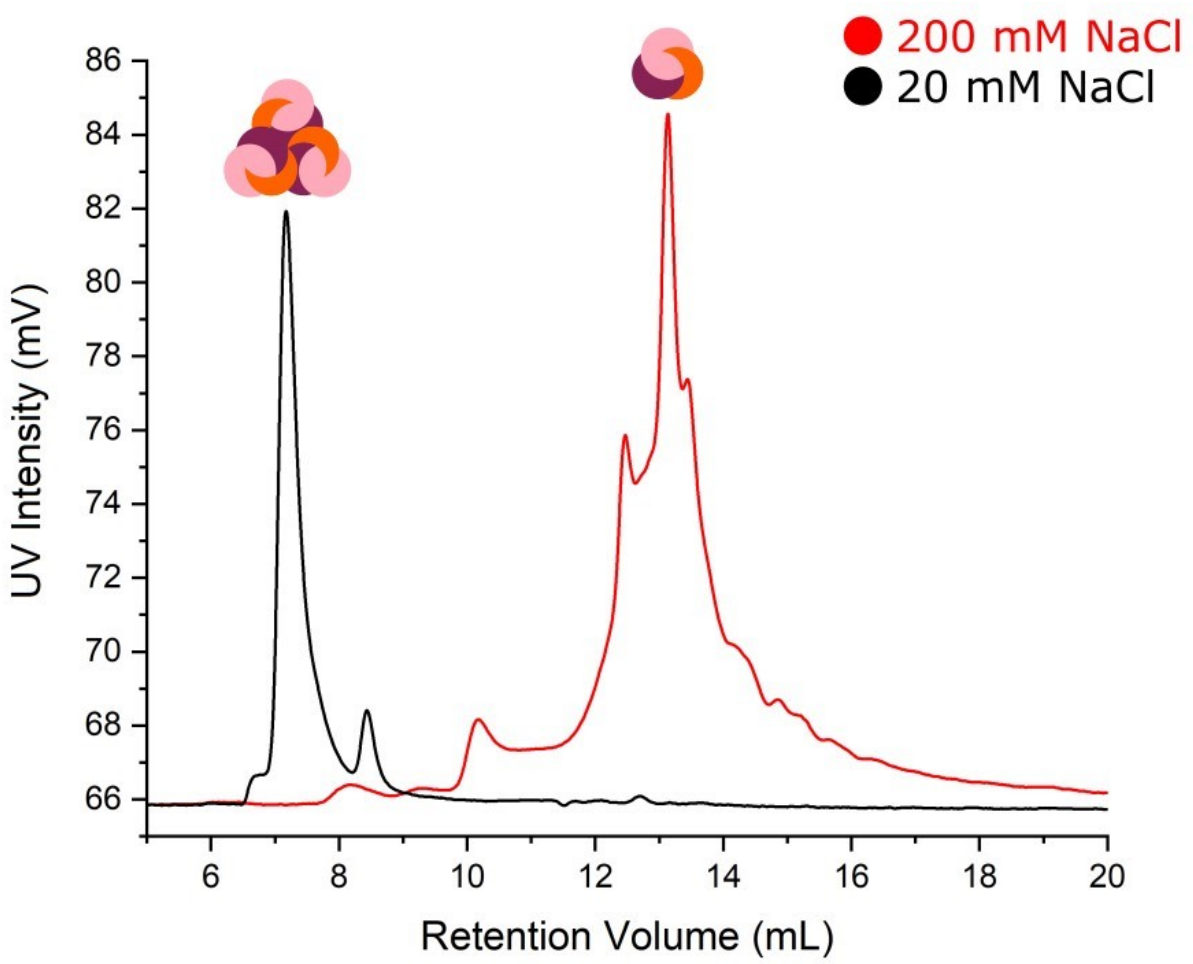



**Figure S9.** Size exclusion chromatography of the DCAF15 complex in HEPES buffer at low (black) and high (red) concentrations of NaCl. At high salt the protein elutes mainly as a monomer, and there are some shoulders on the peak that likely correspond to dissociated DDB1 or DDB1/DDA1 dimer, as observed in the nMS. At low salt concentrations there is no peak corresponding to monomeric protein, and all the protein elutes at early retention times corresponding to multimers.

## Supplementary Tables

**Table S1.** Structures and molecular weights of all glues used in the study.

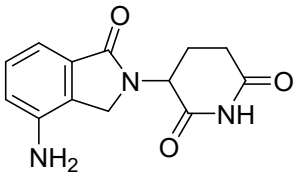
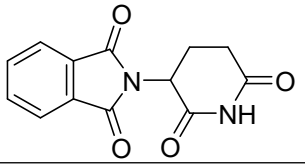
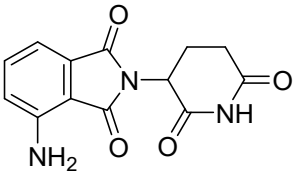
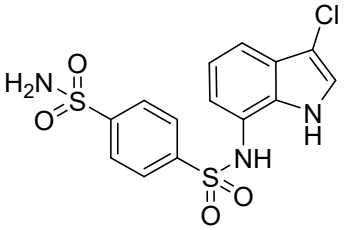
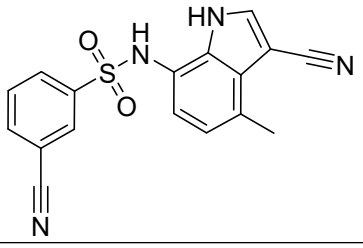
| Name | Structure | Molecular Weight (Da) |
|---|---|---|
| Lenalidomide |  | 259.2 |
| Thalidomide |  | 258.2 |
| Pomalidomide |  | 273.2 |
| Indisulam |  | 385.8 |
| E7820 |  | 336.4 |

**Table S2.** Theoretical and measured molecular weights of proteins used for IMiD experiments.

| Protein | Theoretical MW from sequence (Da) | Measured MW (Da)* |
|---|---|---|
| CRBN_DDB1 | 139 772.0 | 139 775.5 ± 5.09 |
| GSPT1 | 23 832.8 + 226 (biotin) = 24 058.8 | 24 057.7 ± 0.20 |
| DDB1 | 93 247.2 | 93 187.0 ± 7.84 |
| DDA1_DDB1 | 105 082.3 | 104 948.3 ± 4.50 |
| CRBN_DDB1: GSPT1: Lenalidomide Ternary Complex | 164 090.0 | 164 085.9 ± 12.11 |
| CRBN_DDB1: GSPT1: Thalidomide Ternary Complex | 164 089.0 | 164 089.8 ± 7.48 |
| CRBN_DDB1: GSPT1: Pomalidomide Ternary Complex | 164 104.0 | 164 096.4 ± 8.50 |

*Measured mass is the average from all labelled charge states, and the error is the standard deviation.

**Table S3.** Theoretical and measured molecular weights of proteins used for SPLAM experiments.

| Protein | Theoretical MW from sequence (Da) | Measured MW (Da)* |
|---|---|---|
| DCAF15 complex | 162 404.7 | 162 392.3 ± 18.00 |
| RBM39 | 12 758.4 + 226 (biotin) = 12 984.4 | 12 978.0 ± 0.01 |
| DDB1 | 93 247.2 | 93 187.0 ± 7.84 |
| DDA1:DDB1 | 105 082.3 | 104 948.3 ± 4.50 |
| DCAF15 complex: RBM39 : E7820 Ternary Complex | 175 725.5 | 175 748.5 ± 14.64 |
| DCAF15 complex: RBM39 : Indisulam Ternary Complex | 175 774.9 | 175 790.1 ± 8.18 |
| DCAF15 complex dimer | 324 809.4 | 324 791.7 ± 23.44 |
| DCAF15 complex trimer | 487 214.1 | 487 208.0 ± 19.35 |
| DCAF15 complex dimer + 2 E7820 | 325 482.2 | 325 463.0 ± 32.56 |
| DCAF15 complex dimer + 2 Indisulam | 325 581.0 | 325 563.7 ± 34.29 |
| DCAF15 complex trimer + 3 E7820 | 488 223.3 | 488219.8 ± 28.47 |
| DCAF15 complex trimer + 3 Indisulam | 488 371.5 | 488 380.3 ± 29.43 |

*Measured mass is the average from all labelled charge states, and the error is the standard deviation.