# Native Mass Spectrometry of Complexes Formed by Molecular Glues Revealed Stoichiometric Rearrangement of the DCAF15 E3 Ligase

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## Abstract

In this application of native mass spectrometry (nMS) to investigate complexes formed by molecular glues (MGs), we have demonstrated its efficiency in delineating stoichiometric rearrangements of E3 ligases that occur during targeted protein degradation (TPD). MGs stabilise interactions between an E3 ligase and a protein of interest (POI) targeted for degradation, and these ternary interactions are challenging to characterise. We have shown that nMS can unambiguously identify complexes formed between the CRBN:DDB1 E3 ligase and the POI GSPT1 upon the addition of lenalidomide, pomalidomide or thalidomide. Moreover, upon analysis of the DCAF15:DDA1:DDB1 E3 ligase, we uncovered that this complex self-associates into dimers and trimers when analysed alone, and dissociates into single copies of the complex upon the addition of MG (E7820 or indisulam) and POI RBM39, forming a 1:1:1 ternary complex. This work demonstrates the strength of nMS in TPD research, and reveals novel binding mechanisms of the DCAF15 E3 ligase.

## Body

Protein-protein interactions (PPIs) play pivotal roles in many cellular processes and are therefore regarded as promising targets for drug discovery.[1] The classic approach of targeting PPIs has been to inhibit their formation, often with the use of small molecules[2, 3] or engineered peptides.[4] Additionally, the use of PPI stabilisers has also garnered significant attention,[5] especially in the area of targeted protein degradation (TPD). Here, small molecules such as proteolysis-targeting chimeras (PROTACs)[6] or Molecular Glues (MGs)[7] are used to stabilise the interaction between an E3 ligase and a protein of interest (POI) that has been targeted for degradation by the cell (Figure 1). This draws the proteins into close spatial proximity, resulting in ubiquitination of the POI by the E3 ligase and subsequent proteasomal degradation of the POI. TPD is currently in an era of significant growth, and many innovative technologies are in development to address challenges in the approaches. These challenges include the availability of E3 ligases that can be hijacked in TPD,[8] achieving selectivity of a degrader to a specific protein,[9] and the availability of analytical methodologies to directly measure the formation of ternary complexes formed between the E3 ligase, the glue, and the POI.[10, 11]

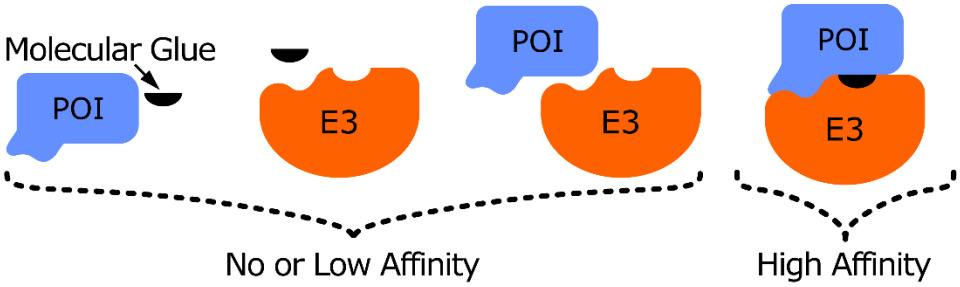


**Figure 1. Schematic depicting the binding mechanism of molecular glues (MGs). In a typical system, interactions between the POI/MG, the E3/MG or the POI/E3 have no or low affinity, whereas the combination of all three components results in a high affinity complex.**

Herein, nMS is demonstrated as a label-free, sensitive, and relatively straightforward tool for detecting the stoichiometry of intact protein complexes formed with MGs. nMS is effective in separating individual species that exist in a stoichiometric mixture, capturing transient interactions, and comparing stability of complexes.[12-14] Whilst nMS has previously been used to predict the efficacy of PROTACs[15] and to analyse complexes formed between MGs and model peptides[16], this is the first example of its application to MGs and multimeric E3 complexes. We have demonstrated its efficacy using disease relevant drugs and targets; two E3 ligase proteins (CRBN:DDB1 and DCAF15:DDA1:DDB1), five molecular glues (lenalidomide, thalidomide, pomalidomide, E7820, and indisulam), and two POIs (GSPT1 and RBM39).

Made infamous as the teratogenic morning sickness medication, the immunomodulatory drug (IMiD) thalidomide has gained newfound therapeutic use in the treatment of multiple myeloma.[17] Thalidomide and its derivatives lenalidomide and pomalidomide act as molecular glues between the E3 ubiquitin ligase consisting of cereblon and damaged DNA binding protein 1 (CRBN:DDB1), and various target POIs. In addition to these IMiD MGs, an additional group named the splicing inhibitor sulfonamides (SPLAMs) are becoming widely used in clinical trials, either as a single agent or in combination with other treatments.[18] E7820 and indisulam are the most documented SPLAMs, used to recruit RNA binding protein 39 (RBM39) for degradation by the E3 ubiquitin ligase consisting of DDB1 and CUL4 associated factor 15, DET1 and DDB1 associated 1, and damaged DNA binding protein 1 (DCAF15:DDA1:DDB1). RBM39 and DCAF15 are known to have a low affinity for each other, but SPLAM molecular glues can greatly enhance the strength of this interaction.[19]

We first sought to investigate the complexes formed by the IMiD MGs lenalidomide, thalidomide and pomalidomide with the CRBN:DDB1 E3 ligase and the GSPT1 POI. We sprayed CRBN:DDB1 and GSPT1 (5µM each) in a mixture from 100 mM ammonium acetate (AmAc) in the absence and presence of the MG lenalidomide (Figure 2A and B, respectively). AmAc is the most popular solvent in nMS as it is volatile and provides the required pH (6.8) for native protein analysis.[20] In the absence of MGs, no interactions are observed between GSPT1 and CRBN:DDB1 (Figure 2A). Here, CRBN:DDB1 presents in charge states 17+ to 23+, monomeric DDB1 presents in charge states 14+ to 19+ and the POI GSPT1 presents in three charge states, from 8+ to 10+. Upon the addition of lenalidomide (Figure 2B), new peaks corresponding to the E3:MG:POI complex can be observed in six charge states from 20+ to 25+. The same is observed upon the addition of additional MGs thalidomide and pomalidomide (Figure S1). No peaks corresponding to binary species (E3:MG or POI:MG) were observed in the mixture of all three components (Figure 2B, S1), nor when CRBN:DDB1 + MG was sprayed in the absence of GSPT1 (Figure S2). IMiDs have previously been shown to bind CRBN with weak $K_D$s of 10-65 µM, which we would not expect to observe with nMS.[21] The measured mass of all proteins used in this study is given in Table S1.

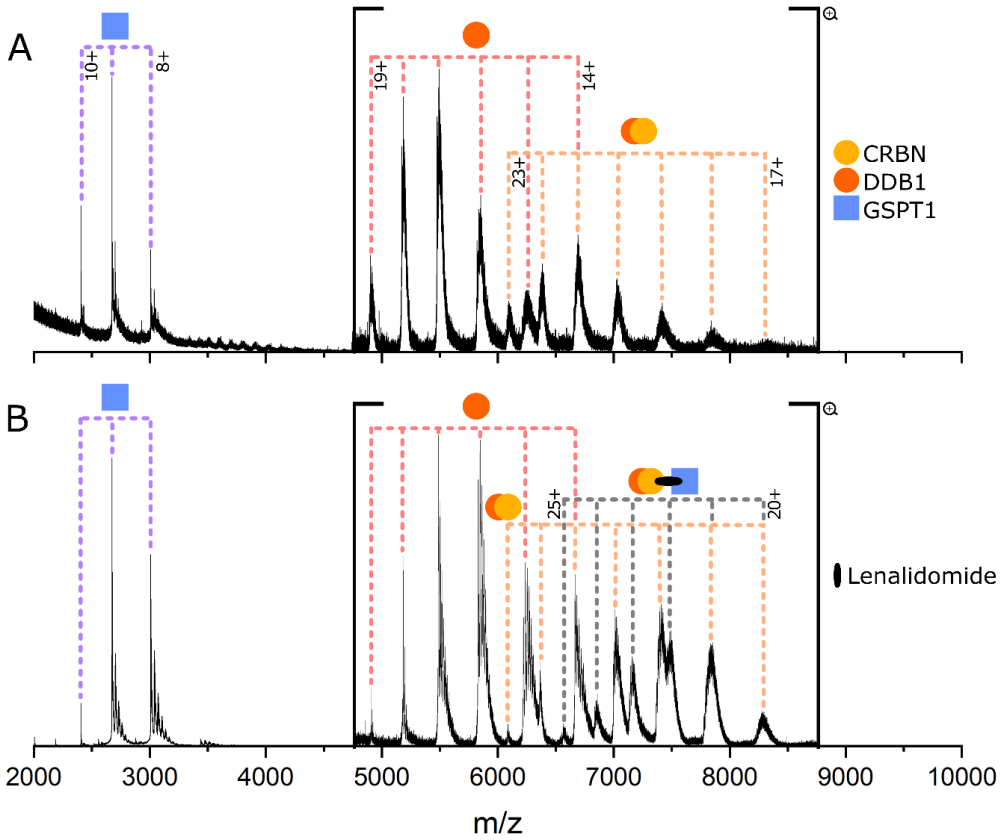


**Figure 2. (A) GSPT1 + CRBN:DDB1 (B) GSPT1 + CRBN:DDB1 + lenalidomide. Protein concentrations are 5 µM and lenalidomide is 100 µM when present.**

We next turned our attention to the DCAF15-targeting SPLAM MG E7820 (Figure 3). We used the DCAF15:DDA1:DDB1 complex lacking the proline-rich, atrophin-homology domain of DCAF15 (amino acids 276–380) which has been used in previous studies, and we refer to here as the DCAF15 complex.[19] The POI used is the RRM2 domain of RBM39, which we refer to as RBM39 hereafter. In this case, the control experiment containing RBM39 (4+ to 6+) and the DCAF15 complex in the absence of MG yielded extremely surprising results. Monomeric DCAF15 complex (charge states 19+ to 24+) is only observed to a very low extent, and most of the signal intensity of this species corresponds to dimers and trimers of the DCAF15 complex (Figure 3A). The dimer of the DCAF15 complex presents in nine charge states from 29+ to 37+ and the trimer also presents in nine charge states, 36+ to 44+. Unbound DDB1 is also present in charge states 14+ to 18+, and the DDA1:DDB1 dimer is present in charge states 15+ to 17+.

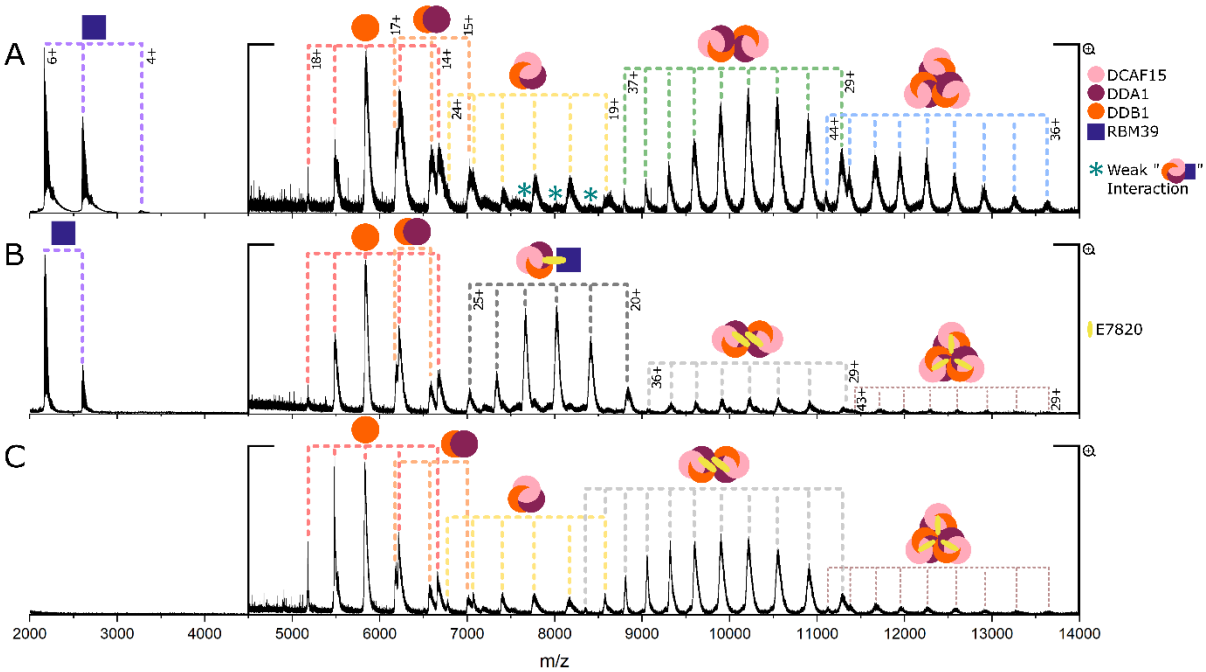


**Figure 3. (A) RBM39 + DCAF15 complex (DCF15:DDA1:DDB1). (B) RBM39 + DCAF15 complex + E7820. (C) DCAF15 complex + E7820. Protein concentrations are 5 µM and E7820 is 100 µM when present.**

To identify whether this multimerization is concentration dependent, we sprayed the DCAF15 complex alone at concentrations of 5 µM and 2.5 µM (Figure S3) which yielded very similar complex distributions. This indicated that formation of the dimers and trimers is not concentration dependent. Upon close observation, peaks can also be identified corresponding to the 1:1 complex between the DCAF15 complex and RBM39, as indicated by asterisks in Figures 3, S4 and S5. This is in agreement with the literature, as RBM39 is known to be a native substrate of the DCAF15 complex, and a weak interaction of 4-6 µM has previously been measured between the two species.[19]

Upon addition of the MG E7820 (Figure 3B) to the DCAF15 complex and the RBM39, a stoichiometric rearrangement of the DCAF15 complex occurs and the main signal intensity now corresponds to a single copy of the complex bound to E7820 and RBM39 in a 1:1:1 stoichiometry (20+ to 25+). Low amounts of dimeric DCAF15 complex remain present bound to two molecules of E7820 (Figure 3B), and the trimeric complex is almost completely eradicated. To investigate whether this stoichiometric rearrangement of the DCAF15 complex is due to the MG or the POI, the DCAF15 complex and E7820 were sprayed together in the absence of RBM39. In this case, the DCAF15 complex is mainly observed as dimers bound to E7820 in a 2:2 stoichiometry (Figure 3C and S6). Very low signal for the trimer of the DCAF15 complex is observed bound to three E7820 molecules (3:3 complex), but the intensity of the trimer is much lower than for the DCAF15 complex in the absence of MG (Figures 3A and S4). We therefore hypothesise that the MG destabilises the DCAF15 complex trimer and causes preference for

the dimer. No interaction was seen between the monomeric DCAF15 complex and E7820, which has been previously measured to have a $K_D > 50$ µM.[22] This is a weak interaction that we wouldn't expect to observe with nMS, but the fact that we see the 2:2 complex suggests that the MGs bind at the interface of the dimer and have a stronger interaction than with the monomer (Figure S6).

As a complementary approach to nMS and to consolidate our findings, size exclusion chromatography (SEC) was employed to compare the interactions of the DCAF15 complex, RBM39 and E7820 (Figure 4). We separately analysed the DCAF15 complex and RBM39 (Figure 4 A and B respectively), and the DCAF15 complex mixed with RBM39 in the absence (C) and presence (D) of E7820.

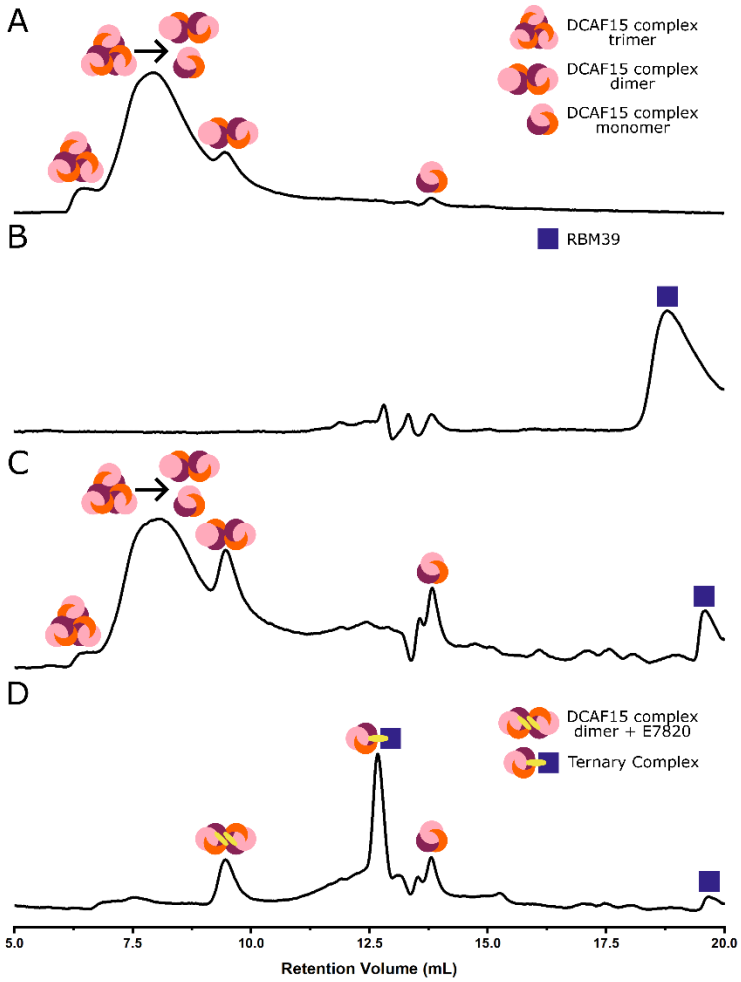


**Figure 4. Size exclusion chromatography of (A) DCAF15 complex, (B) RBM39, (C) DCAF15 complex + RBM39, (D) DCAF15 complex + RBM39 + E7820.**

For the DCAF15 complex alone, we attribute the peaks at retention volumes of 6.5 and 9 mL 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 monomer of the DCAF15 complex arrives at 13.5 mL. The peak eluting at 18-19 mL is assigned as unbound RBM39 (Figure 4B). When the proteins are mixed in the absence of E7820 (Figure 4C), there is a similar distribution of the DCAF15 complexes. However, upon addition of E7820 (Figure 4D), 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 we assign as being the 1:1:1 DCAF15:E7820:RBM39 complex. There is also a large reduction in the intensity of the trimer peak, as well as the peak corresponding to the dissociation products of the trimer. Overall, this set of experiments demonstrates that the DCAF15 complex exists mainly as dimer and trimers in the absence of E7820 and RBM39. This is not altered by the presence of RBM39, and only slightly by the binding of E7820 which reduces the amount of trimeric DCAF15 complex. However, upon mixing all three components, the multimers of the DCAF15 complex dissociate into monomers, and the DCAF15:E7820:RBM39 becomes the most prominent species. Equivalent data for the indisulam MG is shown in Figure S4.

In summary, nMS has been demonstrated as an asset for the determination of MG ternary complex formation, successfully and clearly showing the presence of two ternary complexes between disease relevant proteins. It is a sensitive, fast, label-free technique requiring low sample consumption. This work has shown that nMS can show these complexes with intact proteins, in addition to the model peptides which have previously been used.[16] nMS was able to directly show the existence of the IMiD ternary complexes, which will be beneficial in the screening of small molecule libraries for further glues that modulate this interaction.

An unexpected and important outcome of this study was that the DCAF15 complex self-associates into dimers and trimers, which are disrupted only in the presence of E7820/indisulam and RBM39. Despite this E3 complex being extensively studied in structural biology due to its potential role in TPD, oligomerisation has not previously been reported, to our knowledge. Regulation of alternative E3 ligases, including UBR5 [23] has been suggested to occur via self-association, and this is a potential explanation for the oligomerisation of the DCAF15 complex. Only upon addition of E7820/indisulam and RBM39 does the DCAF15 complex dissociate into single copies of the complex, instead binding to MG and RBM39 in a 1:1:1 stoichiometry. Such findings are paramount in understanding E3 ligases for their manipulation in TPD, and we expect that analysis of complexes via nMS will eventually be routine in guiding drug design.

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## References

1. Scott, D.E., A.R. Bayly, C. Abell, and J. Skidmore, *Small molecules, big targets: drug discovery faces the protein–protein interaction challenge.* Nature Reviews Drug Discovery, 2016. **15**(8): p. 533-550.
2. Gorczynski, M.J., J. Grembecka, Y. Zhou, Y. Kong, L. Roudaia, M.G. Douvas, M. Newman, I. Bielnicka, G. Baber, T. Corpora, J. Shi, M. Sridharan, R. Lilien, B.R. Donald, N.A. Speck, M.L. Brown, and J.H. Bushweller, *Allosteric Inhibition of the Protein-Protein Interaction between the Leukemia-Associated Proteins Runx1 and CBFβ.* Chemistry & Biology, 2007. **14**(10): p. 1186-1197.
3. Yin, X., C. Giap, J.S. Lazo, and E.V. Prochownik, *Low molecular weight inhibitors of Myc–Max interaction and function.* Oncogene, 2003. **22**(40): p. 6151-6159.
4. Dietrich, L., B. Rathmer, K. Ewan, T. Bange, S. Heinrichs, T.C. Dale, D. Schade, and T.N. Grossmann, *Cell Permeable Stapled Peptide Inhibitor of Wnt Signaling that Targets β-Catenin Protein-Protein Interactions.* Cell Chemical Biology, 2017. **24**(8): p. 958-968.e5.
5. Thiel, P., M. Kaiser, and C. Ottmann, *Small-Molecule Stabilization of Protein–Protein Interactions: An Underestimated Concept in Drug Discovery?* Angewandte Chemie International Edition, 2012. **51**(9): p. 2012-2018.
6. Sakamoto, K.M., K.B. Kim, A. Kumagai, F. Mercurio, C.M. Crews, and R.J. Deshaies, *Protacs: Chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation.* Proceedings of the National Academy of Sciences, 2001. **98**(15): p. 8554-8559.
7. Słabicki, M., Z. Kozicka, G. Petzold, Y.-D. Li, M. Manojkumar, R.D. Bunker, K.A. Donovan, Q.L. Sievers, J. Koeppel, D. Suchyta, A.S. Sperling, E.C. Fink, J.A. Gasser, L.R. Wang, S.M. Corsello, R.S. Sellar, M. Jan, D. Gillingham, C. Scholl, S. Fröhling, T.R. Golub, E.S. Fischer, N.H. Thomä, and B.L. Ebert, *The CDK inhibitor CR8 acts as a molecular glue degrader that depletes cyclin K.* Nature, 2020. **585**(7824): p. 293-297.
8. Kramer, L.T. and X. Zhang, *Expanding the landscape of E3 ligases for targeted protein degradation.* Current Research in Chemical Biology, 2022. **2**: p. 100020.
9. Smith, B.E., S.L. Wang, S. Jaime-Figueroa, A. Harbin, J. Wang, B.D. Hamman, and C.M. Crews, *Differential PROTAC substrate specificity dictated by orientation of recruited E3 ligase.* Nature Communications, 2019. **10**(1): p. 131.
10. Gadd, M.S., A. Testa, X. Lucas, K.-H. Chan, W. Chen, D.J. Lamont, M. Zengerle, and A. Ciulli, *Structural basis of PROTAC cooperative recognition for selective protein degradation.* Nature Chemical Biology, 2017. **13**(5): p. 514-521.
11. Roy, M.J., S. Winkler, S.J. Hughes, C. Whitworth, M. Galant, W. Farnaby, K. Rumpel, and A. Ciulli, *SPR-Measured Dissociation Kinetics of PROTAC Ternary Complexes Influence Target Degradation Rate.* ACS Chemical Biology, 2019. **14**(3): p. 361-368.

12. Beveridge, R., L.G. Migas, K.A.P. Payne, N.S. Scrutton, D. Leys, and P.E. Barran, *Mass spectrometry locates local and allosteric conformational changes that occur on cofactor binding.* Nature Communications, 2016. **7**(1): p. 12163.
13. Sugiyama, M., H. Yagi, K. Ishii, L. Porcar, A. Martel, K. Oyama, M. Noda, Y. Yunoki, R. Murakami, R. Inoue, N. Sato, Y. Oba, K. Terauchi, S. Uchiyama, and K. Kato, *Structural characterization of the circadian clock protein complex composed of KaiB and KaiC by inverse contrast-matching small-angle neutron scattering.* Scientific Reports, 2016. **6**(1): p. 35567.
14. Cveticanin, J., T. Mondal, E.M. Meiering, M. Sharon, and A. Horovitz, *Insight into the Autosomal-Dominant Inheritance Pattern of SOD1-Associated ALS from Native Mass Spectrometry.* Journal of Molecular Biology, 2020. **432**(23): p. 5995-6002.
15. Beveridge, R., D. Kessler, K. Rumpel, P. Ettmayer, A. Meinhart, and T. Clausen, *Native Mass Spectrometry Can Effectively Predict PROTAC Efficacy.* ACS Central Science, 2020. **6**(7): p. 1223-1230.
16. Bellamy-Carter, J., M. Mohata, M. Falcicchio, J. Basran, Y. Higuchi, R.G. Doveston, and A.C. Leney, *Discovering protein–protein interaction stabilisers by native mass spectrometry.* Chemical Science, 2021. **12**(32): p. 10724-10731.
17. Yamamoto, J., T. Ito, Y. Yamaguchi, and H. Handa, *Discovery of CRBN as a target of thalidomide: a breakthrough for progress in the development of protein degraders.* Chemical Society Reviews, 2022. **51**(15): p. 6234-6250.
18. Milojkovic Kerklaan, B., S. Slater, M. Flynn, A. Greystoke, P.O. Witteveen, M. Megui-Roelvink, F. de Vos, E. Dean, L. Reyderman, L. Ottesen, M. Ranson, M.P.J. Lolkema, R. Plummer, R. Kristeleit, T.R.J. Evans, and J.H.M. Schellens, *A phase I, dose escalation, pharmacodynamic, pharmacokinetic, and food-effect study of α2 integrin inhibitor E7820 in patients with advanced solid tumors.* Investigational New Drugs, 2016. **34**(3): p. 329-337.
19. Du, X., O.A. Volkov, R.M. Czerwinski, H. Tan, C. Huerta, E.R. Morton, J.P. Rizzi, P.M. Wehn, R. Xu, D. Nijhawan, and E.M. Wallace, *Structural Basis and Kinetic Pathway of RBM39 Recruitment to DCAF15 by a Sulfonamide Molecular Glue E7820.* Structure, 2019. **27**(11): p. 1625-1633.e3.
20. Konermann, L., *Addressing a Common Misconception: Ammonium Acetate as Neutral pH "Buffer" for Native Electrospray Mass Spectrometry.* Journal of the American Society for Mass Spectrometry, 2017. **28**(9): p. 1827-1835.
21. Akuffo, A.A., A.Y. Alontaga, R. Metcalf, M.S. Beatty, A. Becker, J.M. McDaniel, R.S. Hesterberg, W.E. Goodheart, S. Gunawan, M. Ayaz, Y. Yang, M.R. Karim, M.E. Orobello, K. Daniel, W. Guida, J.A. Yoder, A.M. Rajadhyaksha, E. Schönbrunn, H.R. Lawrence, N.J. Lawrence, and P.K. Epling-Burnette, *Ligand-mediated protein degradation reveals functional conservation among sequence variants of the CUL4-type E3 ligase substrate receptor cereblon.* J Biol Chem, 2018. **293**(16): p. 6187-6200.
22. Bussiere, D.E., L. Xie, H. Srinivas, W. Shu, A. Burke, C. Be, J. Zhao, A. Godbole, D. King, R.G. Karki, V. Hornak, F. Xu, J. Cobb, N. Carte, A.O. Frank, A. Frommlet, P. Graff, M. Knapp, A. Fazal, B. Okram, S. Jiang, P.-Y. Michellys, R. Beckwith, H. Voshol, C. Wiesmann, J.M. Solomon, and J. Paulk, *Structural basis of indisulam-mediated RBM39 recruitment to DCAF15 E3 ligase complex.* Nature Chemical Biology, 2020. **16**(1): p. 15-23.
23. Hodáková, Z., I. Grishkovskaya, H.L. Brunner, D.L. Bolhuis, K. Belačić, A. Schleiffer, H. Kotisch, N.G. Brown, and D. Haselbach, *Cryo-EM structure of the chain-elongating E3 ligase UBR5.* bioRxiv, 2022: p. 2022.11.03.515015.