nature portfolio

Peer Review File

Unbiased mapping of cereblon neosubstrate landscape by high-throughput proteomics

Corresponding Author: Zoran Rankovic

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript presents a method for identifying neosubstrate proteins of molecular glue-degraders (MGDs) using integrated proteomics and ubiquitinomics analysis of cells before and after drug treatment. Using known neo-substrates as a model, the authors performed condition studies and successfully identified nearly 50 new proteins that are thought to be neo-substrates, using two main types of cell lines and about 100 CRBN-ligands. The analysis of neo-substrates based on the differences in the chemical structures of various CRBN ligands is also largely consistent with existing data, and it can be thought that the analysis method presented in this study is versatile. Considering the huge amount of data, the analysis is carried out thoughtfully, and the structure of the manuscript is generally satisfactory. However, the analysis of ubiquitination sites in particular is relatively weak, and this should be improved. This paper would be a good fit for publication in Nature Communications with the following revisions addressed.

Reviewer suggestions:
- The current definition of neo-substrate is that it interacts directly with CRBN in an MDG-dependent manner and is degraded in the cell. In this study, the authors are attempting to prove direct interaction by biotinylation using TurboID-CRBN, but TurboID is unsuitable for proving neo-substrates because it also biotinylates indirect complex proteins. The authors need to prove direct drug-dependent interaction for the neo-substrate candidates they have discovered, especially for representative proteins such as KDM4B and VCL.

- Interestingly, in this study, proteins without G-loops were also shown as candidates for neo-substrates. The authors have shown that they may be degraded by complex formation with true neo-substrates, but the data seems to indicate that the behaviour differs depending on the cell type. As proteins without G-loops are very important when considering neo-substrates, the authors need to use a new figure to show proteins with and without G-loops.

- In this study, the authors performed ubiquitinomics analysis. Surprisingly, they found 20 sites on VCL that are ubiquitinated in an MDG-dependent manner. If there is structural information for VCL, plotting the sites of ubiquitination would be important for considering MDG-dependent ubiquitination. Furthermore, this data would be interesting for the sites of MDG-dependent ubiquitination of other neo-substrate candidates. Understanding the sites of MDG-dependent ubiquitination is equally important as understanding neo-substrate candidates. There is much to understand, such as whether these ubiquitination sites change depending on the type of MDG or the cell type. As the analysis data in this study should include these ubiquitination sites, the authors need to add new figures, results and discussions that will help us understand the differences in the types of MDG and cell types for the ubiquitination sites of known neo-substrates and new neo-substrate candidates.

- SALL4 has been reported as a neo-substrate in Huh-7 cells, but it was not detected in this study. The authors need to explain why.

- In this study, CRBN overexpression was carried out in HEK293 cells. Generally, it is not difficult to establish stable overexpression strains in Huh-7 cells using lentivirus-based gene transfer systems. Why did the authors not carry out overexpression experiments in Huh-7 cells? I think the authors need to explain why they did not do this in Huh-7 cells.

Reviewer #2

(Remarks to the Author)
Steger, Nishiguchi, Wu and Schwalb et al present a comprehensive profiling of CRBN based molecular glue degraders in proteomics studies that include total protein level characterization, ubiquitomics. Combination of these methods was able to identify novel CRBN neosubstrates as well as enabled deeper characterization of KDM4B, VCL and G3BP2.

The study is of high interest to the field of small molecule induced proximity, the ubiquitination profiling is very informative. I fully support the publication with the following questions:

How many novel imid substrates were identified in this study? Can authors perhaps compare this to published studies?

Selection of compounds - page 5 - how did the authors include statistical significance in the cutoff?

page 6 - how were the cell lines selected Huh-7 and NB-4?

page 7 - are ubiquitination sites different between distinct degraders that degrade same targets?

Page 13 CUL4A is ubiquitinated - does that suggest an auto regulatory function of the cullin ligases?

Is there a biological relevance to degrade G3BP2?

Does G3BP2 or KDM4 contain a classical G-loop degron?

How would the authors define or select direct CRBN neosubstrates as opposed to "collateral" degraded or ubiquitinated substrates?

Substrates with possible non G-loop degrons are exciting. There was considerable effort on KDM4B, however is VCL similarly directly degraded?

Transfection experiments with KDM4B are not described in the methods?

Reviewer #3

(Remarks to the Author)
In this manuscript "Unbiased mapping of cereblon neosubstrate landscape by high-throughput proteomics" by Steger et al., the authors highlight a medium-to-high throughput mass spectrometry-based method for profiling degrader activity across a proteome.
In this method, the authors test 100 compounds in two cell lines and identify many known as well as previously not yet identified potential neosubstrates of CRBN. Several follow-up controls are done within this larger set including an earlier time point as well as ubiquitomics to gain confidence that these are degradation targets of CRBN. The authors then choose two new neosubstrate targets, G3BP2 and KDM4B, to do more extensive testing demonstrating induced proximity to CRBN, CRBN dependency for target loss, and loss is not due to mRNA changes. This works highlights a new workflow and scale for unbiased screening of degraders and add to the growing field of identified CRBN neosubstrates. It will very valuable for the field and I have a few comments for consideration:
1. Sample prep, cost, time, resolution (number of proteins identified), and data processing are all challenges of HTS mass spectrometry and prevent profiling of large numbers of compounds. The authors state 720 LC/MS runs were performed. Could they please elaborate how they have helped solve these significant challenges with their new method? Could it be readily applied to now screen 1000s of compounds?
2. Several preprints are available looking at the degradable space by CRBN glues.
https://www.biorxiv.org/content/10.1101/2024.09.11.612438v2
https://www.biorxiv.org/content/10.1101/2024.10.07.616933v1
I could not immediately see a lot of overlap of new neosubstrates between this target list and those which have approached it through orthogonal methods, yet all of the manuscripts have found the same known neosubstrates. Given that these preprints are out and may or may not publish soon, could the authors please comment on any overlap of new targets, and if there is not any, why this might be.
3. The overexpression of CRBN to find new targets seems very artificial as there could be several biological reasons why targets are revealed only in this setting and they could easily not be direct targets of CRBN. The more meaningful control here should have profiling in cell lines that have drastically different endogenous levels of CRBN. This can even be done within a similar lineage.
4. Related to the above, authors need to show in Figure 1 a Western blot of the endogenous CRBN levels between Huh-7 and NB-4. In targets that were degraded in only one line or the other, (Page 11, ZBTB7B and CSNK1E, is this due to CRBN expression differences between the lines or any changes in mRNA?
5. The authors did TurboID to show increased proximity of target to CRBN upon presence of degrader, but it would significantly enhance the manuscript to show ternary complex formation more definitively for either G3BP2 or KDM4B using any host of biochemical or biophysical assays. Perhaps it is easiest with the domain mapped for KDM4B?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed all of my concerns.

Reviewer #2

(Remarks to the Author)
All my questions were fully addressed and I support the publication.

Reviewer #3

(Remarks to the Author)
Great work by authors to address all of the reviewers comments and address these with changes and new experiments! The revised manuscript ready well and is very thorough. No further comments for consideration.

We thank all reviewers for their careful evaluation of our manuscript and their insightful feedback. We have revised the manuscript accordingly and addressed all major points of concern. Please find our detailed responses below.

Reviewer #1 (Remarks to the Author):

This manuscript presents a method for identifying neosubstrate proteins of molecular glue-degraders (MGDs) using integrated proteomics and ubiquitinomics analysis of cells before and after drug treatment. Using known neo-substrates as a model, the authors performed condition studies and successfully identified nearly 50 new proteins that are thought to be neo-substrates, using two main types of cell lines and about 100 CRBN-ligands. The analysis of neo-substrates based on the differences in the chemical structures of various CRBN ligands is also largely consistent with existing data, and it can be thought that the analysis method presented in this study is versatile. Considering the huge amount of data, the analysis is carried out thoughtfully, and the structure of the manuscript is generally satisfactory. However, the analysis of ubiquitination sites in particular is relatively weak, and this should be improved. This paper would be a good fit for publication in Nature Communications with the following revisions addressed.

Thank you for the overall positive feedback! Please find a detailed response to the raised criticism below.

Reviewer suggestions:
- The current definition of neo-substrate is that it interacts directly with CRBN in an MDG-dependent manner and is degraded in the cell. In this study, the authors are attempting to prove direct interaction by biotinylation using TurboID-CRBN, but TurboID is unsuitable for proving neo-substrates because it also biotinylates indirect complex proteins. The authors need to prove direct drug-dependent interaction for the neo-substrate candidates they have discovered, especially for representative proteins such as KDM4B and VCL.

Thank you for the suggestion. To address this point, we have incorporated below text and the new figures in the revised manuscript (pages 23 and 26, respectively):

"TurboID-based proximity labeling confirmed SJ41564-induced ternary complex formation in HEK293 cells (Fig. 5e). To further validate this interaction, we developed an AlphaScreen assay using a GST-KDM4B fragment containing the PHD and tandem TUDOR domains (PHD-DTD) and recombinant His-CRBN-DDB1. SJ41564 induced dose-dependent ternary complex formation with an EC50 of 4.7 µM, confirming direct interaction between the glue, CRBN and KDM4B (Fig. 5f). No signal was observed with N-methylated negative control compound SJ48108, consistent with its inability to bind CRBN."

"To identify the specific region of VCL directly involved in the ternary complex formation, we generated Caki-1 cell lines stably overexpressing FLAG-tagged constructs of each of the five distinct VCL domains (A-E), and assessed their protein levels by immunoblotting (Supplementary Fig. 19a-c). Upon the treatment with SJ46479 we observed changes only in the level of D1, while the other four constructs remained unaffected (Supplementary Fig. 19b), suggesting that D1 domain is the one involved in the direct interaction of VCL with the neomorphic SJ46479 -CRBN surface. Interestingly, while the level of D1 was markedly low in

untreated cells, it was found significantly increased in cells treated with either SJ46479, or the proteasome inhibitor MG132 (Supplementary Fig. 19c). This suggests that, while unstable on its own due to proteasomal degradation, D1 is stabilized by the SJ46479 induced complexation with CRBN. We speculate that the three ubiquitination sites in D1 fragment (K170, K216, and K219), out of over 20 identified in the full-length protein (Fig. 5i, Supplementary Fig. 18), are not effectively ubiquitinated when in the ternary complex with SJ46479-CRBN, rendering SJ46479 incapable of inducing degradation and thereby resulting in D1 protein stabilization."

- Interestingly, in this study, proteins without G-loops were also shown as candidates for neo-substrates. The authors have shown that they may be degraded by complex formation with true neo-substrates, but the data seems to indicate that the behaviour differs depending on the cell type. As proteins without G-loops are very important when considering neo-substrates, the authors need to use a new figure to show proteins with and without G-loops.

This is a very valid point. We conducted an analysis to identify GSPT1-like G-loops and present the result in Figure 3d. We added a detailed description of the analysis to the 'Methods' section. Additionally, we have highlighted neosubstrates containing a G-loop in the heatmap shown in Figure 4b (and Supplementary Figure 13).

- In this study, the authors performed ubiquitinomics analysis. Surprisingly, they found 20 sites on VCL that are ubiquitinated in an MDG-dependent manner. If there is structural information for VCL, plotting the sites of ubiquitination would be important for considering MDG-dependent ubiquitination.

We highlighted the ubiquitinated residues and corresponding identified peptide sequences on an AlphaFold-predicted structure of VCL (Figure S18 in the revised manuscript). While a ternary complex structure would be necessary to draw definitive conclusions about the orientation of VCL within the complex and to elucidate the mechanistic details on its ubiquitination (as shown in DOI: 10.1126/sciadv.ado6492), our analysis offers preliminary insights and highlights a ubiquitination hotspot. We have made minor revisions to the relevant section of the 'Results and Discussion' accordingly.

Furthermore, this data would be interesting for the sites of MDG-dependent ubiquitination of other neo-substrate candidates. Understanding the sites of MDG-dependent ubiquitination is equally important as understanding neo-substrate candidates. There is much to understand, such as whether these ubiquitination sites change depending on the type of MDG or the cell type. As the analysis data in this study should include these ubiquitination sites, the authors need to add new figures, results and discussions that will help us understand the differences in the types of MDG and cell types for the ubiquitination sites of known neo-substrates and new neo-substrate candidates.

Thank you for these valuable suggestions. A more detailed analysis of glue-induced ubiquitination sites uncovered many interesting findings. We observed that the same lysine residues on known neosubstrates are ubiquitinated in response to various MGDs. Among these, we identified key lysine residues acting as "priming sites" that contribute most significantly to protein degradation Interestingly, while these sites are ubiquitinated following treatment with multiple compounds, protein degradation—or detectable downregulation—only occurs when the ubiquitination signal (measured as induced log2 fold change) exceeds a specific threshold.

We illustrated these findings in Figures 1f-g of the revised manuscript and updated the 'Results and Discussion' section accordingly.

Additionally, we have added a similar analysis to Figure 3 (new Figure 3c), but this time extending the analysis to the different cell lines tested. This analysis again demonstrated that identical lysine residues are ubiquitinated on neosubstrates across cell lines following treatment with a specific MGD compound. Furthermore, ubiquitination at defined priming sites consistently emerged as the strongest determinant of protein degradation, reinforcing the functional relevance of site-specific ubiquitination in mediating neosubstrate turnover.

- SALL4 has been reported as a neo-substrate in Huh-7 cells, but it was not detected in this study. The authors need to explain why.

Unfortunately, we did not quantify SALL4 in any of our HuH-7 cell experiments, likely due to its very low expression in this cell type. However, we agree that assessing whether SALL4 is degraded by the tested compounds is an important question, given its known role as a key liability of CRBN-targeting MGDs.

We therefore retested 10 selected compounds from each the IMiD and PG core series in the human testicular embryonal carcinoma cell line SUSA, which express high levels of SALL4. Strikingly, 9 out of 10 tested PG-based compounds failed to downregulate SALL4, while all 10 tested IMiDs induced strong SALL4 downregulation.

These results are presented in Figure 4c of the revised manuscript.

- In this study, CRBN overexpression was carried out in HEK293 cells. Generally, it is not difficult to establish stable overexpression strains in Huh-7 cells using lentivirus-based gene transfer systems. Why did the authors not carry out overexpression experiments in Huh-7 cells? I think the authors need to explain why they did not do this in Huh-7 cells.

Indeed, it is true that immortalized cell types can generally be readily transduced with lentivirus for constitutive protein expression. The use of HEK293 cells with CRBN overexpression was a practical decision, as we had previously generated this line. While we believe that the enhanced sensitivity to MGDs conferred by CRBN overexpression is applicable to other cell types (e.g. MM1S cells, doi: 10.1182/blood.2019000789), generating a CRBN-overexpressing HuH-7 line to re-screen the compounds is beyond the scope of this study.

The primary objective of this experiment was to assess whether proteins that failed in our Huh7 validation assays could be confirmed as neosubstrates in cells overexpressing CRBN. For this reason, we used HEK293 cells and primarily focussed on these neosubstrate candidates, successfully validating them in most cases. To distinguish CRBN-specific effects from general differences in protein expression between HuH-7 and HEK293 cells, we performed analyses in both parental and CRBN-overexpressing HEK293 cells.

We acknowledge the existence of cell type-specific responses to MGDs, as highlighted in our primary screen (Huh-7 vs NB-4, see supplementary Figure 8c). Some of the additional neosubstrates identified in HEK293 are indeed cell line specific. However, as noted above, the primary scope of this experiment was to validate candidate neosubstrates identified in Huh-7/NB-4 screens.

Reviewer #2 (Remarks to the Author):

Steger, Nishiguchi, Wu and Schwalb et al present a comprehensive profiling of CRBN based molecular glue degraders in proteomics studies that include total protein level characterization, ubiquitomics. Combination of these methods was able to identify novel CRBN neosubstrates as well as enabled deeper characterization of KDM4B, VCL and G3BP2.

The study is of high interest to the field of small molecule induced proximity, the ubiquitination profiling is very informative.

Thank you for your assessment and for the positive feedback!

I fully support the publication with the following questions:

How many novel imid substrates were identified in this study? Can authors perhaps compare this to published studies?

We compared the neosubstrates quantified in our screens with those previously reported. Of the 49 neosubstrates reported by Oleinikovas et al (https://doi.org/10.1146/annurev-pharmtox-022123-104147), 35 were detected in our primary screen (NB-4 and HuH-7 cells). After data filtering, 33 were retained, and 25 showed significant downregulation in response to at least one compound (24-hour treatment data). The results are summarized in Figure 1b and a full list of the neosubstrates along with references is provided in Table S2.
Additionally, previously reported neosubstrates identified across our entire dataset (including results from Huh-7, NB-4, and HEK293 cells, using both proteomics and ubiquitinomics) are labelled in Table S1 ('known substrates'). We made minor revisions to the 'Results and Discussion' section to improve clarity.

Selection of compounds - page 5 - how did the authors include statistical significance in the cutoff?

Thank you for this question - it made us realise that our statement related to compound selection was unclear. Our molecular glue library consists of small sub-libraries built around individual core scaffolds. Hence, to ensure that all library cores are represented in the test set we selected a centroid structure, based on physchem properties such as molecular weight, from each core scaffold. Only scaffolds associated with the GSPT1 and CK1a were excluded.

To clarify the selection process, we replaced the original statement with the following:

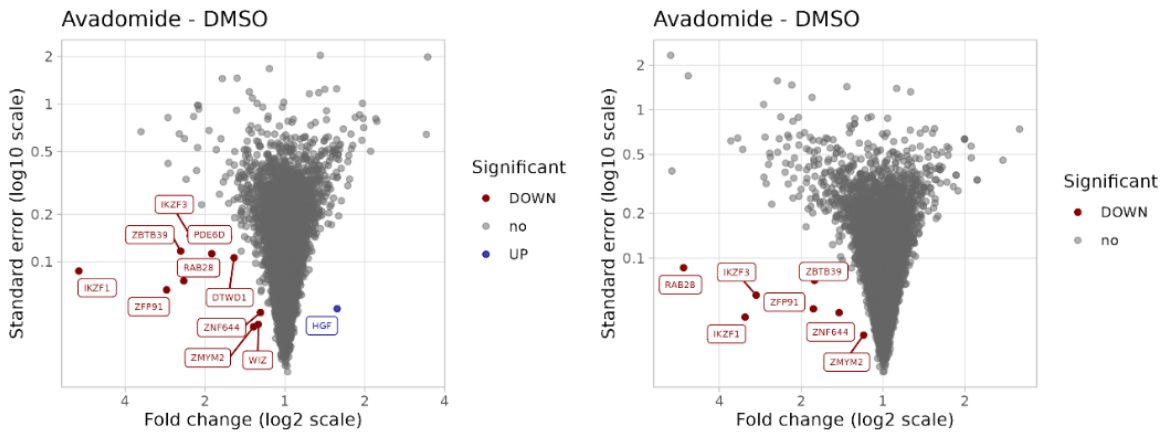
"As an initial proof of concept to our screening workflow, we selected centroids from each core scaffold, which produced a set of 100 compounds representing the molecular structure coverage of the library (Supplementary Fig. S1)."

page 6 - how were the cell lines selected Huh-7 and NB-4?

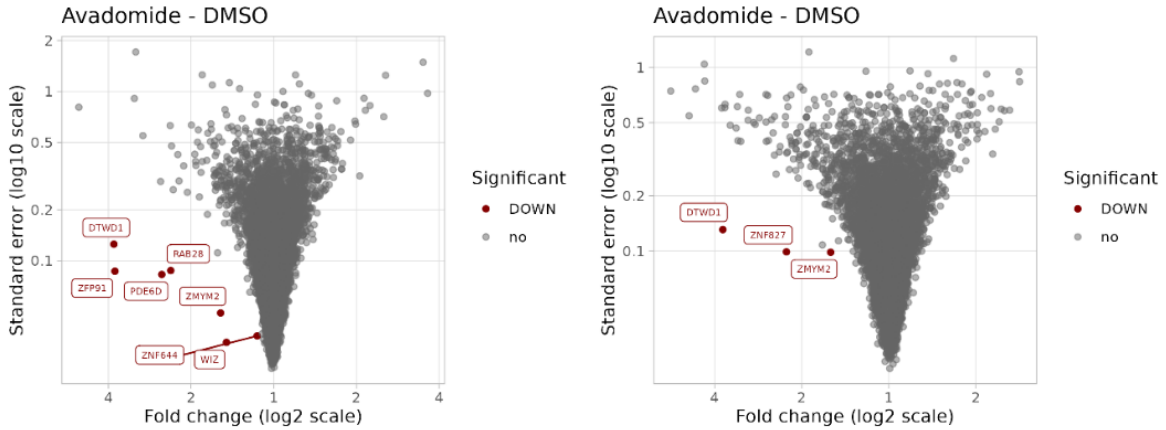
We selected the cell lines based on complementary protein expression profiles (see Supplementary Figure 1b) and their responsiveness to CRBN-based molecular glue degraders. Specifically, we evaluated two suspension cell lines (Jurkat and NB-4) and two adherent lines

(HuH7 and A498) for their response to Avadomide using MS-based proteomics following a 5-hour treatment (Response Figure 1). Based on the number of significantly downregulated neosubstrates, we selected HuH7 and NB-4 for the primary screen. We chose not to include this dataset in the manuscript, as it is already data-rich, and the additional data would divert from the main focus of the study.

NB4 (left) vs Jurkat (right) (5 hrs avadomide)



HuH7 (left) vs A498 (right) (5 hrs avadomide)



Response Figure 1: Global proteomics of four cell lines treated with avadomide for 5 hours (top left = NB-4), top right = Jurkat, bottom left = HuH7, bottom right = A498). Significantly downregulated proteins are coloured in red.

page 7 - are ubiquitination sites different between distinct degraders that degrade same targets?

As the same point was raised be Reviewer#1, we investigated this in more detail. The short answer is yes. We included some figures in the revised manuscript to address this point (please see details above, in the 'Reviewer#1' section).

Page 13 CUL4A is ubiquitinated - does that suggest an auto regulatory function of the cullin ligases?

Since our cell-based ubiquitinomics assay does not distinguish between autoregulation and in-trans ubiquitination events, we cannot answer this question definitively. However, the fact that

CUL4A is ubiquitinated (and degraded) only in response to a subset of screening compounds suggests that the effect is unlikely to be due to autoregulation.

Is there a biological relevance to degrade G3BP2?

G3BP2 is implicated in RNA processing and stress granule formation. Its expression is altered in multiple human malignancies (see references below) and G3BP2 stress granule formation is linked to cardiovascular and neurodegenerative disorders (https://doi.org:10.1038/s41583-019-0222-5). However, we did not investigate to what extend G3BP2 degradation contributes to disease biology.

Gupta, N. et al. Stress granule-associated protein G3BP2 regulates breast tumor initiation. Proc Natl Acad Sci U S A 114, 1033-1038 (2017). https://doi.org:10.1073/pnas.1525387114 14 Zhang, H. et al. Downregulation of G3BPs inhibits the growth, migration and invasion of human lung carcinoma H1299 cells by suppressing the Src/FAKassociated signaling pathway. Cancer Gene Ther 20, 622-629 (2013). https://doi.org:10.1038/cgt.2013.62 15 Li, H. et al. MG53 suppresses tumor progression and stress granule formation by modulating G3BP2 activity in non-small cell lung cancer. Mol Cancer 20, 118 (2021). https://doi.org:10.1186/s12943-021-01418-3

Does G3BP2 or KDM4 contain a classical G-loop degron?

According to our analysis, neither G3BP2 nor KDM4B contain a classical GSPT1-like G-loop degron. Figures 3d an 4f in the revised manuscript show which of the newly identified neosubstrates contain such a motif.

We noted in the manuscript that a very recent preprint from Monte Rosa Therapeutics (Ref 56: https://doi.org/10.1101/2025.04.30.651496) elegantly demonstrated that G3BP2 interacts with CRBN via an unconventional binding site on the LON domain, bypassing the G-loop requirement.

How would the authors define or select direct CRBN neosubstrates as opposed to "collateral" degraded or ubiquitinated substrates?

Our screening and validation assays do not allow to distinguish between direct degradation and indirect (or ‘collateral/bystander’) degradation events. Definitive differentiation between primary and secondary degradation would require dedicated in vitro experiments using recombinant proteins. Alternatively, individually overexpressing the proteins of interest and assessing their degradation or interaction using HiBiT or NanoBRET assays could provide clarity. Such targeted studies were recently conducted for G3BP2 and USP10 (https://doi.org/10.1101/2025.04.30.651496), identifying G3BP2 as the direct target. Reassuringly, these findings are consistent with our own conclusions.

Besides G3BP2, we highlighted several other examples of degraded protein complexes in our dataset, including the MNAT1/CDK7/CCNH complex. Based on the stronger downregulation of MNAT1 in the proteomics data and its more pronounced ubiquitination, we hypothesize that MNAT1 is the direct neosubstrate. Supporting this, only MNAT1 contains a GSPT1-like G-loop, and a recent study demonstrated recruitment of MNAT1 to CRBN using proximity labelling MS (TurboID) and NanoBRET, identifying G50 as the key residue mediating the interaction

(https://doi.org/10.1101/2024.10.07.616933). While our data cannot the unambiguously differentiate direct from indirect degradation events, the available evidence supports the validity of our data interpretation.

Substrates with possible non G-loop degrons are exciting. There was considerable effort on KDM4B, however is VCL similarly directly degraded?

While we do not currently have direct in vitro evidence demonstrating compound-dependent interaction between the recombinantly expressed proteins, we hypothesize that the interaction is direct. VCL was selectively downregulated by a subset of our degraders in a strictly CRBN-dependent manner and was identified as a specific interactor in TurboID-CRBN LC-MS experiments. Moreover, VCL exhibited strong ubiquitination at multiple lysine residues upon degrader treatment, further supporting its classification as a bona fide neosubstrate.

Transfection experiments with KDM4B are not described in the methods?

Thank you for spotting this. We added a detailed description of the methods.

Reviewer #3 (Remarks to the Author):

In this manuscript "Unbiased mapping of cereblon neosubstrate landscape by high-throughput proteomics" by Steger et al., the authors highlight a medium-to-high throughput mass spectrometry-based method for profiling degrader activity across a proteome.
In this method, the authors test 100 compounds in two cell lines and identify many known as well as previously not yet identified potential neosubstrates of CRBN. Several follow-up controls are done within this larger set including an earlier time point as well as ubiquitomics to gain confidence that these are degradation targets of CRBN. The authors then choose two new neosubstrate targets, G3BP2 and KDM4B, to do more extensive testing demonstrating induced proximity to CRBN, CRBN dependency for target loss, and loss is not due to mRNA changes. This works highlights a new workflow and scale for unbiased screening of degraders and add to the growing field of identified CRBN neosubstrates.

Thank you!

It will very valuable for the field and I have a few comments for consideration:

1. Sample prep, cost, time, resolution (number of proteins identified), and data processing are all challenges of HTS mass spectrometry and prevent profiling of large numbers of compounds. The authors state 720 LC/MS runs were performed. Could they please elaborate how they have helped solve these significant challenges with their new method? Could it be readily applied to now screen 1000s of compounds?

These are indeed important considerations.

The main limiting factor for high-throughput MS-based screening is the acquisition time, which is largely determined by the performance of the MS hardware. It is outside our reach to directly influence the development of better performing high-throughput compatible MS hardware. At

the time of our work, we employed one of the most advanced MS platforms available, achieving a throughput of approximately 25 samples per day. Based on our evaluation, this setup represented the best compromise between proteome depth and throughput.

A key strength of our approach lies in the streamlined workflow. We employed 96-well plate-based cell treatments, direct in-well protein digestion (eliminating transfer steps of the lysate), and label-free DIA in 96-well plates. The setup is highly scalable and, in principle, can be applied to screen 1,000s of compounds. The primary bottleneck remains instrument time. For example, screening 1,000 compounds under our current conditions would require approximately 5 months of instrument time.

Notably, recent advances in MS hardware are increasing sample throughput considerably (https://doi.org/10.1038/s41587-023-02099-7). This, along with DIA-compatible multiplexing technologies (e.g., https://doi.org/10.1038/s41587-022-01389-w) will enable large-scale MS-based drug screenings in the near future.

2. Several preprints are available looking at the degradable space by CRBN glues.
https://www.biorxiv.org/content/10.1101/2024.09.11.612438v2
https://www.biorxiv.org/content/10.1101/2024.10.07.616933v1
I could not immediately see a lot of overlap of new neosubstrates between this target list and those which have approached it through orthogonal methods, yet all of the manuscripts have found the same known neosubstrates. Given that these preprints are out and may or may not publish soon, could the authors please comment on any overlap of new targets, and if there is not any, why this might be.

This is certainly an important point.

We are aware of the two complementary preprints and agree that it is reassuring to see an overlap across all three studies in the identification of known neosubstrates (many of which are frequently hit targets, according to our results).

Since both preprints were posted prior to ours, we cited them in our original manuscript. For example, PPIL4 that was identified and characterized in depth in https://www.biorxiv.org/content/10.1101/2024.09.11.612438v2, was classified as 'known neosubstrate' in our study (see Figure 1 and Tables S1 and S2).

Unfortunately, neither preprint included tables of identified targets (or predicted β-hairpin G-loop proteins), which limits a systematic comparison. Nevertheless, we have quoted both manuscripts when describing specific findings, for example for the MNAT1/CDK7/CCNH complex.

To clarify which of our newly identified targets were also reported in the preprints, we closely examined their figures for potential overlap. CRBN binding partners identified in either preprint are now annotated in the revised version of Figure 3d (Sankey diagram).

3. The overexpression of CRBN to find new targets seems very artificial as there could be several biological reasons why targets are revealed only in this setting and they could easily not be direct targets of CRBN. The more meaningful control here should have profiling in cell lines that have drastically different endogenous levels of CRBN. This can even be done within a similar lineage.

We agree that CRBN overexpression is somewhat artificial. However, the primary purpose of using CRBN-overexpressing cells was to enhance sensitivity for validating candidate neosubstrates identified in our primary screens (HuH7 and NB-4). The marked increase in both protein downregulation and ubiquitination upon CRBN overexpression supports the validity of our screening strategy.

While using cell lines with varying endogenous CRBN levels could, in theory, serve for compound screening, our analysis of approximately 20 cell lines treated with CRBN-targeting MGDs showed only a partial correlation between CRBN expression and cellular response (based on the number of downregulated proteins; data not shown).

We believe that screening across multiple cell lineages is valuable in terms of substrate Id due to complementary expression profiles. However, neosubstrate ID can be significantly boosted by exogenous CRBN expression. We used HEK293 cells for this purpose, but similar effects were observed in MM1S cells in a previous study (doi: 10.1182/blood.2019000789). We therefore think that this strategy is likely transferable to other cell types when the goal is to screen for new molecular glue candidates. Of course, translating these findings into systems with native CRBN expression may require follow-up medicinal chemistry optimization.

4. Related to the above, authors need to show in Figure 1 a Western blot of the endogenous CRBN levels between Huh-7 and NB-4. In targets that were degraded in only one line or the other, (Page 11, ZBTB7B and CSNK1E, is this due to CRBN expression differences between the lines or any changes in mRNA?

Thank you for pointing this out. We realized that one of our supplementary figures (Supplementary Figure 8c) was not referenced correctly in the manuscript. As shown in that figure and described in the 'Results' section, we observed clear cell-type specific responses, even for proteins expressed at comparable levels in both cell lines, for example ZBTB7B and CSNK1E. Notably, these differences in protein degradation did not correlate with CRBN expression levels. We believe that factors such as PTMs, subcellular localization, or the expression of other components of the ubiquitin-proteasome system may contribute for these effects. We discussed these points in the 'Conclusions' section.

5. The authors did TurboID to show increased proximity of target to CRBN upon presence of degrader, but it would significantly enhance the manuscript to show ternary complex formation more definitively for either G3BP2 or KDM4B using any host of biochemical or biophysical assays. Perhaps it is easiest with the domain mapped for KDM4B?

Thank you for the suggestion. We generated a GST-KDM4B (PHD-DTD) protein construct and developed and AlphaScreen to validate the direct interaction between KDM4B, CRBN and the Glue. The results are presented in Figure 5f of the revised manuscript.