

A dihydrouracil CRBN ligand mitigates IMiD associated safety liabilities in heterobifunctional targeted protein degrader

Corresponding Author: Dr Kevin Moreau

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)

Overall the paper is well written with a lot of cell based and proteomic studies. However, I got a feeling that paper was written in a hurry with little consideration to overall presentation of figures which could have been much better. Also, my main concern is that out of 7 main figures, 5 have been devoted to show that conventional IMiDs (lenalidomide, pomalidomide, thalidomide, etc) or PROTACs based on these can degrade Ikaros and Aiolos, which is a well known fact. There are a lot of publications to show this: <https://pubmed.ncbi.nlm.nih.gov/24328678/>, <https://www.nature.com/articles/bcj201566>, etc. I could be very naive if I am missing something novel here and would really appreciate if the authors can enlighten me more about this point and bring this out in the intro- or discussion section. Figure 6 and 7 shows some novelty where they identified the dihydrouracil CRBN binder that spares Ikaros and Aiolos whether as molecular glues or as PROTACs.

1. For Fig. 1.B and similar figures subsequently, the authors should use a consistent color code for lenalidomide, Pomalidomide, etc. If iberdomide is green in one, then please do not use the same color for other IMiDs. It will be easy for the readers while reading.
2. Please use contrasting colors to make out the differences between the compounds for all HiBiT degradation assays. Also, please clearly state what does the Y-axis for all the HiBiT degradation assays refer to. Although normalized to DMSO control but putting a label "% of DMSO" in Y-axis with scale ranging from 0 to negative 100 does not make sense. It should be the protein levels or related readings on the y-axis. Please use appropriate label.
3. For fig.2, please make the scale showing logFC bigger as it's very small compared to the actual figure.
4. Please make better use of the real estate in Fig.5. The whole figure panel has been devoted to two chemical structures. It's a suggestion and not a critic.
5. For Fig.6, the authors try to show that compound 2 does not cause any degradation of Ikaros or Aiolos and no off-targets were detected in deep global proteomics experiment. Although, compound 2 and thalidomide are not much different physiochemically (table 2), have the authors done cell permeability assay to assess the compound 2 concentration in the cell, just to be sure that the lack of degradation is not because of compound 2 being less permeable as compared to thalidomide.
6. For fig.6F, please make the LogFC scale bigger for visibility.
7. For fig.7A, more labeling is needed. Please label the residues that are shown as sticks. Label the PDB code and which structure is what, etc. Although some of the info is given in the legends, it would be better for the readers if the figure is labeled as much as possible. Also the corresponding text (line 314-318) explaining about the structure should be well written. For e.g. "with the DHU mimicking thalidomide's glutarimide ring, forming the same hydrogen bonds to the backbone

carbonyl and NH groups as seen in the equivalent thalidomide crystal structure", it is not clear what residues/chemical group we are talking about as nothing is mentioned/labeled in the Fig.7A. since the authors have taken so much effort to obtain the crystal for the complex, they should take time to explain some of the structural observations in the text.

8. For Fig. 7C, clearly the protac 6 does not degrade Aiolos and SALL4 as compared to ZXH-3-26. However, the authors should mention around line 352-359, that protac 6 is less efficient as compared to ZXH-3-26 by a factor of atleast more than 10 in degrading BRD4 if we compare Fig.7C (i) and (ii).

9. For supplementary fig.5, more labeling is needed. Please mention the ITC parameters obtained here, rather than putting it in the methods section. Could the authors comment on the nature of the graph in Supp Fig.A, where its positive at the beginning and becomes negative later on. maybe change in enthalpy? Also, how to infer $n=0.6$? are there more than 1 binding sites?

10. In relation to point 9 above, could the authors provide the gel of a final purified product of DDB1/CRBN? Could the authors mention whether S75 or S200 was used? Also, I am curious to know whether authors got a stoichiometric DDB1/CRBN after doing only gel filtration because there is always an IEX step associated to obtain a pure stoichiometric DDB1/CRBN after gel filtration step.

11. Fig.3D legend, the legends for 3D (i) and (ii) have been swapped.

Reviewer #2

(Remarks to the Author)

The authors of this manuscript address a critical concern regarding the safety of IMiD-based PROTACs, which have increasing numbers entering clinical trials. IMiDs target Cereblon and lead to degradation of IMiD neosubstrates, such as Ikaros, Aiolos, and SALL4, which causes adverse effects; heterobifunctional IMiD-based PROTACs may, therefore, also be expected to have potential safety issues given their ability to engage CRBN through the same IMiD substructure as well as the intended protein of interest. This manuscript describes the author's work to:

1-Develop new in vitro assays in hematopoietic stem and progenitor cells and a CRBN knock-in transgenic mouse model which could form the basis of a screening cascade to profile potential safety concerns with emerging IMiD-based PROTACs during pre-clinical development

2-Identify an alternative CRBN ligand which can avoid the safety liabilities associated with the IMiD substructures.

It is not in my area of expertise to address any technical aspects of the in vitro assays or the in vivo model. However, I appreciate the authors' methodical approach to compare a panel of reported IMiD-based PROTACs with known IMiDs (e.g. thalidomide, lenalinomide, and pomalidomide) and their ability to degrade neosubstrates – first via a nanoluciferase/HiBIT system and then via a high-throughput (mass spec) Proteomics approach. They move on to a proliferation assay in primary HSPCs (controlled by a cell line where CRBN is genetically deleted by CRISPR) and then a I391V transgenic mouse in which IMiDs are pharmacologically active, so they can observe neosubstrate degradation and lymphopenia. Throughout these studies, the authors note various selectivities in behaviors of the reported PROTACs, underlining the value of the proposed screening cascade to identify where off-target degradation of neosubstrates would be of concern, which cannot be easily predicted.

I was particularly interested in the authors' identification of new CRBN ligands. They first conducted a virtual screen based on reported uracil-containing CRBN ligands, shown to bind CRBN at the same site as thalidomide, and then an HTRF-based screen of a focused DHU motif library, upon which they identified Compound 2. Compound 2 has an IC50 of 155 nM by HTRF (or is it 183 nM? Please see requested changes below) and Kd of 216 nM by ITC. It also has reduced neosubstrate degradation and reduced changes in hematopoietic stem and progenitor cell differentiation. The authors then used Compound 2 as a new CRBN-handle to replace the glutarimide IMiD-based PROTACs to compare its behavior. They also solved a high-resolution X-ray structure showing that it binds similarly to CRBN compared to thalidomide. They then advise that the indole C3 position is favorable for growth when designing new PROTACs and test this theory by modifying a known BRD4 PROTAC to use the DHU motif instead of an IMiD handle. This new compound (6) demonstrated a better safety profile than the original PROTAC with the IMiD.

The manuscript and experiments are well done and I recommend it for publication, if the following questions and/or small edit requests can be addressed:

1. Please correct the discrepancy of IC50 = 155nM for compound 2 as reported in text line 256 vs Table 2 and Figure 5, with 183 nM.

2. Add color notations to the caption for Figure 7A (X-ray structure) to clearly indicate compound 2 vs. thalidomide (blue/gray) in the figure.

3. Although the CRBN protein from the published thalidomide co-ligand structure has been removed from the figure, can the authors please comment in the main text as to whether there were any rearrangements of protein backbone with the two ligands (thalidomide vs. Cmpd 2)? i.e. Does the binding pocket change to accommodate the new ligand or are they

identical? This is perhaps relevant to rationalize why the molecular glue MoA of thalidomide leading to neosubstrate degradation is not applicable for Compound 2. Do the authors have a biophysical rationale as to why compound 2 would not induce the same effects, leading to a safer compound? They even remark (lines 393-96) that the DHU motif alone is not responsible for the selectivity. Is the selectivity just due to the N-1 motif?

4. The ITC data in supplementary Figure 5 demonstrates that the interaction of compound 2 with CRBN is endothermic, which is unusual. Do the authors have a reasoning for this? Is it disrupting the DDB1 complex? Did they try using a truncated CRBN-only construct similar to the one used for X-ray for comparison? Or, perhaps, this relates to my comment #3—a change in the CRBN backbone structure compared to apo-CRBN that is less favorable thermodynamically?

5. If #4 cannot be rationalized, could the authors conduct the ITC experiment with thalidomide for comparison to see if it is also endothermic?

6. Please include the HTRF curves for IC50 determination of Compound 2 in the supplemental data.

7. For the ITC data (supplementary Figure 5), can the authors please adjust the text in the title to indicate that CRBN/DDB1 is used?

8. Can they also add language in the Supplementary methods (Page 18, Expression and purification for ITC, lines 12-13) to clarify whether or not the protein is a co-expression of two proteins to obtain the CRBN/DDB1 complex or whether it is a fusion construct? The :: notation would suggest a fusion construct, but there are two different purification tags and cleavages suggesting the former.

Reviewer #3

(Remarks to the Author)

Rodrigo-Brenni et al report the investigation of adverse effects from canonical IMiDs (lenalidomide, pomalidomide) in vitro and in vivo due to degradation of neosubstrates IKZF1/3 that represent safety liabilities, demonstrate that these same neosubstrates are degraded by certain PROTACs, and use that motivate studies to substitute thalidomide derivatives with an uracil derivative in a representative PROTAC. The most novel component of this work is the identification of a dihydrouracil derivative 2 that binds to CRBN at now submicromolar dissociation constants unlike previous dihydrouracil ligands, which opens the opportunity for this scaffold to be a more effective replacement of traditional glutarimide ligands. Although unfortunately, derivatization of the dihydrouracil derivatives elevates the dissociation constant, the ligand is demonstrated to be robust when directed against BRD4 and avoid degradation of canonical neosubstrates of thalidomide derivatives. These efforts echo prior art in the field directed at developing safer alternatives to traditional thalidomide derivatives, although does not acknowledge these efforts and their solutions and carefully selects PROTACs as examples that do have off-targets from molecular glues. There is still a place for the dihydrouracil derivative reported here due to metabolic liabilities that other glutarimide ligands possess, although its generalizability would need to be demonstrated beyond BRD4. Otherwise, the experimental data in this manuscript appear robust and confirm many of other efforts in the field that have demonstrated that degradation of IKZF1/3 have adverse effects on white blood cells and that certain PROTACs also degrade these same neosubstrates, which supports the important need for designing safer versions of thalidomide ligands for use in the clinic.

Major comments to support the authors as they revise this work:

1. The DHU moiety would need to be demonstrated for utility beyond BRD4 with similar safety profiles for it to be explored further as equivalent to other ligands used in the field.
2. A rationale for why the dihydrouracil indoline scaffold 2 has tighter binding capacity for cereblon by an order of magnitude, as compared to prior dihydrouracil scaffold 1 is not apparent from the text or structure. Description of the structure–activity relationship studies or additional insight here would be interesting.
3. Alternate solutions in other reports need to be incorporated to the introduction and this work placed in their context. These include examination of alternate linkers, benzylamide glutarimide derivatives, bump hole derivatives of thalidomide for reducing molecular glue targets, and peptidic cyclimid ligands for cereblon.
4. The IC50 values of thalidomide and compound 2 represented in Table 2 do not match those reported in the text.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

In this manuscript, Rodrigo-Brenni et al. developed a novel proteolysis-targeting chimera (PROTAC) utilizing

immunomodulatory drug (IMiD) scaffolds. While several PROTACs have been designed using IMiD-based structures to recruit Cereblon (CRBN) for targeted protein degradation, a major limitation of these PROTACs is the bystander degradation of CRBN neosubstrates, such as Ikaros and Aiolos. To mitigate these safety liabilities, this study identifies a novel dihydrouracil (DHU)-based CRBN ligand, which binds to CRBN effectively while sparing the degradation of off-target neosubstrates. When incorporated into PROTACs, exemplified by compounds targeting BRD4, the DHU scaffold enabled selective BRD4 degradation without affecting Ikaros and Aiolos. This work presents a promising strategy for developing safer and more selective CRBN-recruiting PROTACs. However, a major limitation of this study is insufficient characterization of the newly designed BRD4-targeting PROTACs in in vitro HSC models and in vivo mouse models.

The in vivo experiments using Crbn-I391V mice primarily reproduced findings from a previous report, with the exception of validating that compound "2" does not degrade Ikaros and Aiolos. As noted in Major Comment #3, the authors have not ruled out the possibility that compound "2" failed to bind to Crbn-I391V or that it exhibited different pharmacokinetics compared to existing IMiDs. Also, as detailed in Major Comment #4, it is essential to validate the efficacy of target protein degradation and the substrate specificity of compound "6" (the PROTAC developed in this study) in the in vivo model. Thus, I cannot support the publication of this manuscript in Nature Communications.

Major comments:

1. A substantial portion of the manuscript (Fig 1, 2, and 4) is dedicated to reproducing findings from previous literatures. The primary advancement presented in this study is the development of compounds "2" and "6" for reducing bystander degradation of IKZF proteins in IMiD-based PROTACs. However, their characterization remains limited.
2. In Fig 6F and the corresponding results section on page 9, as well as in the discussion, the authors state that IMiDs rewire HSC differentiation through the degradation of Ikaros and Aiolos, which is avoided by using compound "2." However, the changes in the abundance of selected proteins shown in Fig 6F do not provide sufficient evidence to support this authors' claim. To clarify this point, colony formation assays and/or in vitro differentiation assays using IMiDs- and compound "2"-treated HSCs should be conducted.
3. In Fig 6 and the corresponding text on page 9, the authors do not exclude the possibility that compound "2" fails to bind to CRBN in NB-4 cells and HSPCs in ex vivo experiments. In in vivo experiments, they also did not exclude the possibility of reduced bioavailability of compound "2" compared to lenalidomide, in addition to the potential abrogated binding to CRBN. This requires careful examination, particularly because compound "6," which was derived from compound "2," still exhibited some degradation of Ikaros and Aiolos whereas compound "2" did not affect the abundance of Ikaros and Aiolos. To address this point, a pull-down assay should be conducted to confirm the direct interaction of compound "2" with CRBN, and a pharmacokinetic study to assess its bioavailability and stability.
4. Since compound "6" is a newly designed heterobifunctional PROTAC, comprehensive comparative studies against existing BRD4-targeting PROTAC (ZXH-3-26), and compound "2" should be performed. Specifically, proteomics analysis, evaluation of effects on hematopoietic cell survival/proliferation and differentiation, and in vivo experiments using Crbn-I391V mice are necessary.

Minor comments:

1. The number of replicates is not consistently reported across the manuscript (e.g., Fig 2, 3E, 4D, 4F, 6F, S1B, S3, and S6). To ensure clarity and reproducibility, the exact number of replicates should be stated for each experiment.
2. Page 3, Line 56: The phrase "thousands of children to be born with severe birth defects" is vague and lacks precise quantification. If a numerical estimate is being referenced, please provide a more accurate figure along with the source. Additionally, clarify what is meant by "severe birth defects" by specifying the types of conditions being referred to.
3. Page 3, Line 58: Trade names should be omitted to maintain consistency and adhere to scientific writing.
4. Page 3, Line 61: The expression "MM clinical activity" is unclear. Please specify what it indicates.
5. Page 4, Line 116 and throughout the manuscript: The compounds generated in this study are referred to only by numbers (e.g., "1" and "2"), which may cause confusion. To improve clarity and differentiation, consider assigning unique identifiers to each compound.
6. Page 4, Lines 123-124: The dosing regimen described in this section is unclear. Please rephrase to ensure clarity.
7. Fig S1B and Page 5, Lines 151-152: Why are the values for ZXH-3-26 fall below -100%?
8. For Fig 2, the color coding information is not clearly indicated, making it difficult to interpret the comparisons. Additionally, since the comparisons are not explicitly defined, it is unclear whether the use of a t-test is appropriate in this context. Please provide clearer labeling for the color scheme and specify the statistical comparisons to justify the chosen test.
9. The current data do not provide sufficient evidence to support the hypothesis that IMiDs act through a cytostatic rather than a cytotoxic mechanism. To clarify this, IMiDs should be added to the culture medium during the course of HSC expansion, and cell numbers should be assessed longitudinally to confirm that IMiD treatment does not induce cytotoxic effects. Additionally, in Fig 3C, 3D(ii), and 3E, the current y-axis labeling suggests that IMiD treatment leads to reduced viability. To better reflect the intended interpretation, the y-axis labels should be reconsidered, such as using absolute cell numbers

instead of viability percentages.

10. Several non-standard abbreviations were observed throughout the manuscript and should be clearly defined for clarity. For example: "min. 15k" (Page 4, Line 117), "mpk"*** (Fig 4C). Additionally, consider rephrasing the use of "downregulation" (Page 6, Line 172 and throughout the manuscript). Since the proteins were degraded rather than actively regulated, terms such as "reduction" or "decrease" would be more accurate.

11. The following points in the figures should be addressed for improved clarity:

- Fig 1B: Different color codes were applied to the same compounds in panels (i) and (ii-v). To enhance consistency and readability, consider either separating them into distinct figures (e.g., Fig 1B and 1C) or combining them into a single plot.
- Fig 4D: The color coding is unclear due to the use of different fill and border colors. Please adjust the color scheme to improve distinction and readability.
- Fig 3D: The legend is incorrectly assigned, with the labels for (i) and (ii) swapped.

12. Non-mandatory suggestions:

- Fig S2 may be omitted from the manuscript, as the authors do not discuss the differences between conditions, and the current description in the main text sufficiently represents the results shown in Fig S2.
- Fig 4A should be moved to the supplementary figures, as the generation of a CRISPR-mediated knock-in mouse strain is a widely used technique and is not central to the main focus of the study.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Overall authors have done a great job in improving the manuscript and bringing the key points about how the new molecule (compound 2 as MG and later compound 16 as protac) spared the key TFs such as Aiolos, Ikaros, etc and were specific in their activity. The authors have taken care of all the concerns raised by me.

Some minor comments:

1. For fig.2B, since the results are statistically not relevant, there is no need for the graph below the Western blot results. Instead the authors can draw a red box around the CRBN and Ikaros blot to highlight the effects of CRBN KO.
2. For fig.2C(i and ii), I know levels of ATP were measured as a surrogate for cell number, so should the y-axis for them be same as 2D(ii) where it is (ATP(pmol))?
3. For Fig. 3F and other similar figures, please label at the top as lenalidomide or compound 2 for the deep global proteomics results.

Reviewer #2

(Remarks to the Author)

Thank you for the revisions to the manuscript and for the answers to my questions. My concerns are resolved and I recommend the manuscript for publication.

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily responded to the points I raised with changes to the text that address the overarching concern. The changes to the manuscript also more effectively draw out the new insights in this work, which were not very clear in the prior version.

Reviewer #5

(Remarks to the Author)

In the revised manuscript, Rodrigo-Brenni, Moreau, and colleagues have made significant improvements to the manuscript's structure, figure presentation, and characterization of compounds 2 and 6. While it is unfortunate that compound 6 could not be tested in vivo, the data clearly support the potential of dihydrouracil (DHU)-based CRBN ligands in enhancing target specificity while minimizing bystander degradation. This reviewer has several minor points as outlined below:

1. Regarding the newly added Supplemental Figure 6 (flow cytometric analysis of HSPC differentiation), the condition treated with compound 2 should be included for comparison. Additionally, while the legend notes five technical replicates, the inclusion of two to three biological replicates would be necessary to ensure reproducibility.
2. For Figure 3D and the corresponding text on Page 12, Lines 7–13, the competition assay does not directly assess

compound permeability. Therefore, it seems inappropriate to emphasize permeability in this context. Please consider rephrasing the text or incorporating direct permeability assays to support this point.

3. In the newly added Figure 6D (ii), a reduction in MYC levels is observed in cells treated with compound 6. This observation should be discussed in the manuscript. Furthermore, MYC should be included in the immunoblot panel in Figure 6D (i).

4. In response to the previous comment suggesting rephrasing of the term “downregulation,” the authors noted that they revised the text accordingly. However, instances of “downregulation” remain (e.g., on Pages 9, 12, and 15). Please review and revise these to maintain consistency.

5. Several panels in Supplemental Figure 1 are not readable.

Reviewer #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 3:

Reviewer comments:

Reviewer #5

(Remarks to the Author)

Authors revised manuscript well, and I do not have any further comments/request. So I endorse the revised manuscript for publication in Nature Communication.

Reviewer #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source. The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Overall authors have done a great job in improving the manuscript and bringing the keys points about how the new molecule (compound 2 as MG and later compound 16 as protac) spared the key TFs such as Aiolos, Ikaros, etc and were specific in their activity. the authors have taken care of all the concerns raised by me.

Some minor comments:

1. For fig.2B, since the results are statistically not relevant, there is no need for the graph below the Western blot results. Instead the authors can draw a red box around the CRBN and Ikaros blot to highlight the effects of CRBN KO.

We have removed the graph and added a box around CRBN and Ikaros. See new Figure 2B.

2. For fig.2C(i and ii), I know levels of ATP was measured as a surrogate for cell number, so should the y-axis for them be same as 2D(ii) where it is (ATP(pmol))?

The data in figure 2C represents relative levels of luminescence to those found in the DMSO control, and the y-axis reflects this. For Figure 2D, an ATP standard curve was used to transform luminescence to amount of ATP, hence the difference in y-axis. To avoid confusion, we have changed the data in 2D to represent relative changes to DMSO and changed the y-axis accordingly

3. For Fig. 3F and other similar figures, please label at the top as lenalidomide or compound 2 for the deep global proteomics results.

We have added the label at the top of the panel as suggested. See new Figures 3F and 6D.

Reviewer #2 (Remarks to the Author):

Thank you for the revisions to the manuscript and for the answers to my questions. My concerns are resolved and I recommend the manuscript for publication.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily responded to the points I raised with changes to the text that address the overarching concern. The changes to the manuscript also more effectively draw out the new insights in this work, which were not very clear in the prior version.

Reviewer #5 (Remarks to the Author):

In the revised manuscript, Rodrigo-Brenni, Moreau, and colleagues have made significant improvements to the manuscript's structure, figure presentation, and characterization of compounds 2 and 6. While it is unfortunate that compound 6 could not be tested in vivo, the data

clearly support the potential of dihydrouracil (DHU)-based CRBN ligands in enhancing target specificity while minimizing bystander degradation. This reviewer has several minor points as outlined below:

1. Regarding the newly added Supplemental Figure 6 (flow cytometric analysis of HSPC differentiation), the condition treated with compound 2 should be included for comparison. Additionally, while the legend notes five technical replicates, the inclusion of two to three biological replicates would be necessary to ensure reproducibility.

The additional flow cytometry data examining HSPC differentiation represents a comprehensive and resource-intensive experiment, comparable in many respects to an in vivo study. To ensure robust statistical power, we performed multiple technical replicates (n = 5), and we are confident that this approach offers reliable results without the need for repeated experimentation, much like the standard practice in in vivo studies. Based on our findings that compound 2 does not degrade Ikaros in vitro or in vivo (Figures 3C, 3E, 3F, and Figure 5A), does not impact HSPC differentiation (Figure 3G), and does not induce significant changes to the proteome (Figures 5B, 5C), we did not consider it essential to further expand the flow cytometric analysis or revisit our conclusion. Our data collectively support that compound 2 provides a superior profile compared to IMiDs with respect to off-target degradation of IMiD neosubstrates and potential effects on HSPC biology.

2. For Figure 3D and the corresponding text on Page 12, Lines 7–13, the competition assay does not directly assess compound permeability. Therefore, it seems inappropriate to emphasize permeability in this context. Please consider rephrasing the text or incorporating direct permeability assays to support this point.

We have edited the text (see words in red) to nuance the interpretation of direct permeability. See new text (Page 12, line 7-13):

“To make sure that the lack of degradation was not due to a lack of cell permeability, we performed a competition experiment between compound 2 and the IMiD molecules and an IMiD-based PROTAC (dBRD9) to indirectly assess this. We observed dose-dependent inhibition of Aiolos degradation by compound 2, irrespective of the initial level of degradation elicited by each compound, confirming that compound 2 binds CRBN and that its apparent superior selectivity did not misleadingly originate from a lack of cell permeability (Figure 3D).”

3. In the newly added Figure 6D (ii), a reduction in MYC levels is observed in cells treated with compound 6. This observation should be discussed in the manuscript. Furthermore, MYC should be included in the immunoblot panel in Figure 6D (i).

We have added MYC level to the panel and would like to emphasize that the quantification is mass spectrometry based (see new Figure 6D).

We have also discussed the MYC reduction in the text (see page 15, line 27-33):

“When compound 6 was assessed in our high throughput proteomics assay, we demonstrated its increased selectivity against Ikaros and Aiolos compared to ZXH-3-26 and its overall selectivity with only few proteins showing a reduction including BRD2, BRD3 and MYC (Figure 6D and Supplementary Table 5). The reduction of MYC is consistent with the role that BRD4 plays in its transcriptional regulation and importantly showcases the benefits of unbiased proteomic analysis achieved in our high throughput assay.”

4. In response to the previous comment suggesting rephrasing of the term “downregulation,” the authors noted that they revised the text accordingly. However, instances of “downregulation” remain (e.g., on Pages 9, 12, and 15). Please review and revise these to maintain consistency. We apologise for the missing correction and have edited the text accordingly. Note that on page 13, we still use the word downregulation to discuss pathways.

5. Several panels in Supplemental Figure 1 are not readable.

We have edited the figure to make the text more visible. See new Supplementary Figure 1.

Reviewer #1 (Remarks to the Author):

Overall the paper is well written with a lot of cell based and proteomic studies. However, I got a feeling that paper was written in a hurry with little consideration to overall presentation of figures which could have been much better. Also, my main concern is that out of 7 main figures, 5 have been devoted to show that conventional IMiDs (lenalidomide, pomalidomide, thalidomide, etc) or PROTACs based on these can degrade Ikaros and Aiolos, which is a well known fact. there are a lot publications to show this :<https://pubmed.ncbi.nlm.nih.gov/24328678/>, <https://www.nature.com/articles/bcj201566>, etc. I could be very naive if I am missing something novel here and would really appreciate if the authors can enlighten me more about this point and bring this out in the intro- or discussion section. Figure 6 and 7 shows some novelty where they identified the hi-hydroxyrubicin CRBN binder that spares Ikaros and Aiolos whether as molecular glues or as PROTACs.

We would like to thank the reviewer for the feedback. It has helped us to reorganise our manuscript focusing on the novelty of our findings. In the revised manuscript, we have focused on the new molecular insights into the influence of IMiDs on primary human hematopoietic stem and progenitor cells by rewiring the differentiation process using high-throughput proteomics and flow cytometry. We have also extended the section showing the utility of an alternative CRBN ligand in mitigating IMiD safety liabilities, facilitating the development of safer degraders.

Below are few specific points added to the revised manuscript:

- a) Addition of HSPC viability data over 13 days using flow cytometry, confirming a cytostatic rather than cytotoxic effect of IMiDs (**new Supplementary Figure 3**).
- b) We present new flow cytometry data with IMiDs at the single-cell level, demonstrating orthogonally a rewiring effect on cell fate towards megakaryocytes, complementing very well the proteomics data (**new Supplementary Figure 6**).
- c) Together with the new flow cytometry data, we further explored the proteomics data at the pathway enrichment level, again confirming a rewiring stem cell differentiation mechanism through the induction of platelet activation pathways that is mitigated by compound 2 (**new Figures 4C & 5C**).
- d) Clarification of the rationale behind the improved safety profile of compound 2 over IMiDs using chemical insights and discussion of the literature around other strategies to mitigate IMiD safety liabilities (**see new discussion**).
- e) Addition of pharmacokinetic data for compound 2, demonstrating the superior exposure of compound 2 compared to IMiDs following oral administration (**new Figure 3B**).
- f) Address the permeability question with a competition experiment. The data indicate that compound 2 competes with PROTAC, underscoring its intracellular penetration and CRBN binding (**new Figure 3D**).
- g) Addition of proteomics data with compound 6 to complement the cellular HiBiT-based degradation assay, confirming selectivity of compound 6 sparing IMiD neosubstrates degradation (**new Figure 6D**).

1. For Fig. 1.B and similar figures subsequently, the authors should use a consistent color code for lenalidomide, Pomalidomide, etc. if iberdomide is green in one, then please do not use the same color for other IMiDs. it will be easy for the readers while reading.

Not relevant to the revised manuscript (data removed)

2. Please use contrasting colors to make out the differences between the compounds for all HiBiT degradation assays. also, please clearly state what does the Y-axis for all the HiBiT degradation assays refer to. although normalized to DMSO control but putting a label "% of DMSO" in Y-axis with scale ranging from 0 to negative 100 does not make sense. it should be the protein levels or related readings on the y-axis. please use appropriate label.

Many graphs previously shown have been removed in the revised manuscript, but the changes have been made to the graphs in the **new Figures 3 and 6**. Colours are contrasting as requested, and consistent with other graphs in the manuscript. The y-axis has been changed from "% of DMSO" to "normalized" to reflect the normalization protocol. The normalization protocol is detailed in the methods section of the manuscript.

3. For fig.2, please make the scale showing logFC bigger as its very small compared to the actual figure.

We have adjusted the font, see **new figures 1C, 3F, 4B, 5B and 6D**.

4. Please make better use of the real estate in Fig.5. The whole figure panel has been devoted to two chemical structures. Its a suggestion and not a critic.

The figure describing the DHU CRBN ligand has been extended (see **new Figure 3**), adding safety, PK and competition data to emphasize the improved properties of compound 2 over traditional IMiDs.

5. For Fig.6, the authors try to show that compound 2 does not cause any degradation of Ikaros or Aiolos and no off-targets were detected in deep global proteomics experiment. although, compound 2 and thalidomide are not much different physiochemically (table 2), have the authors done cell permeability assay to assess the compound 2 concentration in the cell, just to be sure that the lack of degradation is not because of compound 2 being less permeable as compared to thalidomide.

We have added a new competition experiment between IMiDs and compound 2, showing that compound 2 can reduce IMiDs induced Aiolos degradation in a dose dependent manner, demonstrating its cell permeability and binding to CRBN (see **new Figure 3D**). The data are discussed in the revised text (see **page 12, line 7-13**):

"To make sure that the lack of degradation was not due to a lack of permeability, we performed a competition assay between 2 and the IMiD molecules and an IMiD-based PROTAC (dBRD9). We observed dose-dependent inhibition of Aiolos degradation by 2, irrespective of the initial level of degradation elicited by each compound, confirming that 2 binds CRBN and its selectivity did not come from a lack of permeability."

6. For fig.6F, please make the LogFC scale bigger for visibility.

We have adjusted the scale bar, see **new Figure 5A**.

7. For fig.7A, more labeling is needed. Please label the residues that are shown as sticks. Label the PDB code and which structure is what, etc. although some of the info is given in the legends, its would be better for the readers if the figure is labeled as much as possible. also the corresponding text (line 314-318) explaining about the structure should be well written. for e.g."with the DHU mimicking thalidomide's glutarimide ring, forming the same hydrogen bonds to the backbone carbonyl and NH groups as seen in the equivalent thalidomide crystal structure", it is not clear what residues/chemical group we are talking about as nothing is mentioned/labeled in the Fig.7A. since the authors have taken so much effort to obtain the crystal for the complex, they should take time to explain some of the structural observations in the text.

We have edited the figure with CRBN bound by compound 2 (green and blue carbons) overlaid with the crystal structure of CRBN bound by thalidomide (white carbons). Hydrogen bonds from the DHU group of 2 to CRBN are shown as black dashes (see new Figure 6A).

8. For Fig. 7C, clearly the protac 6 does not degrade Aiolos and SALL4 as compared to ZXH-3-26. However, the authors should mention around line 352-359, that protac 6 is less efficient as compared to ZXH-3-26 by a factor of atleast more than 10 in degrading BRD4 if we compare Fig.7C (i) and (ii).

A sentence has been added to the revised text (see page 15, line 16-18):

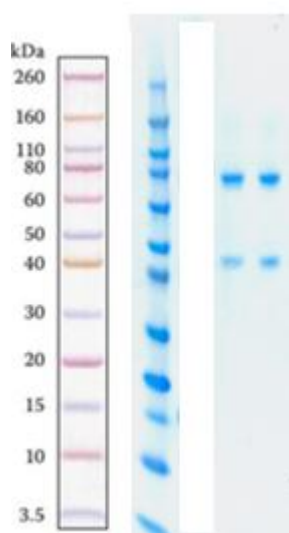
"Intriguingly, the shortest of the PROTACs compound 6 is the most potent PROTAC from this set with a $DC_{50} = 172$ nM and a $D_{max} = 95\%$, although less potent than the optimised ZXH-3-26 by an order of magnitude (Figure 6C).

9. For supplementary fig.5, more labeling is needed. Please mention the ITC parameters obtained here, rather than putting it in the methods section. Could the authors comment on the nature of the graph in Supp Fig.A, where its positive at the beginning and becomes negative later on. maybe change in enthalpy? Also, how to infer $n=0.6$? are there more than 1 binding sites?

The ITC parameters have been added directly to the figures (see new Supplementary Figure 5). The binding is endothermic, a similar thermodynamic profile has been observed before for Lenalidomide binding to CRBN/DDB1 (DOI: 10.1074/jbc.M117.816868). Regarding the quantification of stoichiometry " n " in ITC, it is dependent on several factors. A differ from $n=1$ could possibly be due to heterogeneities in the samples.

10. In relation to point 9 above, could the authors provide the gel of a final purified product of DDB1/CRBN? Could the authors mention whether S75 or S200 was used? Also, I am curious to know whether authors got a stoichiometric DDB1/CRBN after doing only gel filtration because there is always an IEX step associated to obtain a pure stoichiometric DDB1/CRBN after gel filtration step.

This is the gel with the final purified product of DDB1/CRBN. We used a S200 column due to the size of the complex. No IEX step was performed after size exclusion chromatography.



Reviewer #2 (Remarks to the Author):

The authors of this manuscript address a critical concern regarding the safety of IMiD-based PROTACs, which have increasing numbers entering clinical trials. IMiDs target Cereblon and lead to degradation of IMiD neosubstrates, such as Ikaros, Aiolos, and SALL4, which causes adverse effects; heterobifunctional IMiD-based PROTACs may, therefore, also be expected to have potential safety issues given their ability to engage CRBN through the same IMiD substructure as well as the intended protein of interest. This manuscript describes the author's work to:

1-Develop new in vitro assays in hematopoietic stem and progenitor cells and a CRBN knock-in transgenic mouse model which could form the basis of a screening cascade to profile potential safety concerns with emerging IMiD-based PROTACs during pre-clinical development

2-Identify an alternative CRBN ligand which can avoid the safety liabilities associated with the IMiD substructures.

It is not in my area of expertise to address any technical aspects of the in vitro assays or the in vivo model. However, I appreciate the authors' methodical approach to compare a panel of reported IMiD-based PROTACs with known IMiDs (e.g. thalidomide, lenalinomide, and pomalidinomide) and their ability to degrade neosubstrates – first via a nanoluciferase/HiBIT system and then via a high-throughput (mass spec) Proteomics approach. They move on to a proliferation assay in primary HSPCs (controlled by a cell line where CRBN is genetically deleted by CRISPR) and then a I391V transgenic mouse in which IMiDs are pharmacologically active, so they can observe neosubstrate degradation and lymphopenia. Throughout these studies, the authors note various selectivities in behaviors of the reported PROTACs, underlining the value of the proposed screening cascade to identify where off-target degradation of neosubstrates would be of concern, which cannot be easily predicted.

I was particularly interested in the authors' identification of new CRBN ligands. They first conducted a virtual screen based on reported uracil-containing CRBN ligands, shown to bind CRBN at the same site as thalidomide, and then an HTRF-based screen of a focused DHU motif library, upon which they identified Compound 2. Compound 2 has an IC₅₀ of 155 nM by HTRF (or is it 183 nM? Please see requested changes below) and K_d of 216 nM by ITC. It also has reduced neosubstrate degradation and reduced changes in hematopoietic stem and progenitor cell differentiation. The authors then used Compound 2 as a new CRBN-handle to replace the glutarimide IMiD-based

PROTACs to compare its behavior. They also solved a high-resolution X-ray structure showing that it binds similarly to CRBN compared to thalidomide. They then advise that the indole C3 position is favorable for growth when designing new PROTACs and test this theory by modifying a known BRD4 PROTAC to use the DHU motif instead of an IMiD handle. This new compound (6) demonstrated a better safety profile than the original PROTAC with the IMiD.

The manuscript and experiments are well done and I recommend it for publication, if the following questions and/or small edit requests can be addressed:

1. Please correct the discrepancy of IC₅₀ = 155nM for compound 2 as reported in text line 256 vs Table 2 and Figure 5, with 183 nM.

The value has been corrected in the revised text (see page 11, line 23-25):

"The screening of this library led to the discovery of compound 2 which has an IC₅₀ of 183 nM in the HTRF assay (Figure 3A (i) and Supplementary Figure 5C) and a K_d of 216 nM as determined by isothermal titration calorimetry (ITC) (Supplementary Figure 5)."

2. Add color notations to the caption for Figure 7A (X-ray structure) to clearly indicate compound 2 vs. thalidomide (blue/gray) in the figure.

This has now been done and the labels have also been added to the new Figure 6A.

3. Although the CRBN protein from the published thalidomide co-ligand structure has been removed from the figure, can the authors please comment in the main text as to whether there were any rearrangements of protein backbone with the two ligands (thalidomide vs. Cmpd 2)? i.e. Does the binding pocket change to accommodate the new ligand or are they identical? This is perhaps relevant to rationalize why the molecular glue MoA of thalidomide leading to neosubstrate degradation is not applicable for Compound 2.

There are some slight backbone shifts comparing the CRBN-2 and CRBN-thalidomide xray structures, however, we do not consider these to be meaningful, particularly in light of the slight sequence differences between the two CRBN protein constructs used for the crystal structures. We have added a line in the main text to clarify this matter. As for the difference in neosubstrate recruitment comparing thalidomide versus compound 2, we hypothesise that this is due to the differing molecular surfaces that the CRBN-2 and CRBN-thalidomide complexes present to the solvent for neosubstrate recruitment (see new Figure 6A).

Do the authors have a biophysical rationale as to why compound 2 would not induce the same effects, leading to a safer compound? They even remark (lines 393-96) that the DHU motif alone is not responsible for the selectivity. Is the selectivity just due to the N-1 motif?

The selectivity indeed stems from the pendant functionality which results in effectively modifying – or not – the surface of CRBN to accommodate a neosubstrate. This is in fact accepted for CRBN ligands where you can get neosubstrate selectivity by modifying the pendant group (<https://pubs.acs.org/doi/10.1021/acs.jmedchem.3c01872>). Directly comparing e.g. thalidomide with compound 2, the indole is a significant change away from the phthalimide group of thalidomide which is how we've rationalised the selectivity.

Text modified to the following (see page 16, line 29-35):

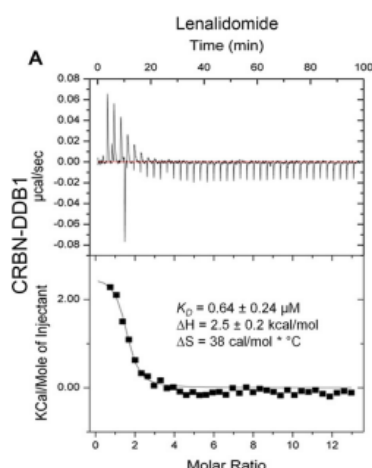
"As observed with glutarimide-based ligands, where neosubstrate selectivity is influenced by appended functional groups rather than the glutarimide ring itself, it is important to recognize that the

DHU scaffold alone does not inherently confer selectivity with regard to molecular glue polypharmacology.⁵⁸ Indeed, previous studies have shown that DHU-based CRBN ligands, bearing alternative N-1 position substituents, can themselves function as molecular glue degraders for various neosubstrates.^{40,59}

4. The ITC data in supplementary Figure 5 demonstrates that the interaction of compound 2 with CRBN is endothermic, which is unusual. Do the authors have a reasoning for this? Is it disrupting the DDB1 complex? Did they try using a truncated CRBN-only construct similar to the one used for X-ray for comparison? Or, perhaps, this relates to my comment #3—a change in the CRBN backbone structure compared to apo-CRBN that is less favorable thermodynamically? 5. If #4 cannot be rationalized, could the authors conduct the ITC experiment with thalidomide for comparison to see if it is also endothermic?

We do not have a strong explanation for the endothermic behaviour. However, a similar thermodynamic profile has been observed before for Lenalidomide binding to CRBN/DDB1 (Fig 4 A in [Ligand-mediated protein degradation reveals functional conservation among sequence variants of the CUL4-type E3 ligase substrate receptor cereblon - Journal of Biological Chemistry](#)). Here the authors have commented on the endothermic reaction in the following way:

"Moreover full length CRBN and CRBN-TBD have endothermic and exothermic reactions, respectively. Therefore, residues in the full-length protein appear to augment protein ligand interactions in the binding pocket."



6. Please include the HTRF curves for IC₅₀ determination of Compound 2 in the supplemental data.

The relevant data has been added to **new Supplementary Figure 5C**.

7. For the ITC data (supplementary Figure 5), can the authors please adjust the text in the title to indicate that CRBN/DDB1 is used?

CRBN/DDB1 has been added to the title of the **new Supplementary Figure 5**.

8. Can they also add language in the Supplementary methods (Page 18, Expression and purification for ITC, lines 12-13) to clarify whether or not the protein is a co-expression of two proteins to obtain the CRBN/DDB1 complex or whether it is a fusion construct? The :: notation would suggest a fusion construct, but there are two different purification tags and cleavages suggesting the former.

It is a co-expression of two proteins indeed. We have amended the text in the [supplementary information \(page 16, line 35-36\)](#):

“In the ITC setup, 40 μ M CRBN/DDB1 construct (co-expressed using different vectors) was put in the ITC cell and 200 μ M of the compound was in the syringe.”

Reviewer #3 (Remarks to the Author):

Rodrigo-Brenni et al report the investigation of adverse effects from canonical IMiDs (lenalidomide, pomalidomide) in vitro and in vivo due to degradation of neosubstrates IKZF1/3 that represent safety liabilities, demonstrate that these same neosubstrates are degraded by certain PROTACs, and use that motivate studies to substitute thalidomide derivatives with an uracil derivative in a representative PROTAC. The most novel component of this work is the identification of a dihydrouracil derivative 2 that binds to CRBN at now submicromolar dissociation constants unlike previous dihydrouracil ligands, which opens the opportunity for this scaffold to be a more effective replacement of traditional glutarimide ligands. Although unfortunately, derivatization of the dihydrouracil derivatives elevates the dissociation constant, the ligand is demonstrated to be robust when directed against BRD4 and avoid degradation of canonical neosubstrates of thalidomide derivatives. These efforts echo prior art in the field directed at developing safer alternatives to traditional thalidomide derivatives, although does not acknowledge these efforts and their solutions and carefully selects PROTACs as examples that do have off-targets from molecular glues. There is still a place for the dihydrouracil derivative reported here due to metabolic liabilities that other glutarimide ligands possess, although its generalizability would need to be demonstrated beyond BRD4. Otherwise, the experimental data in this manuscript appear robust and confirm many of other efforts in the field that have demonstrated that degradation of IKZF1/3 have adverse effects on white blood cells and that certain PROTACs also degrade these same neosubstrates, which supports the important need for designing safer versions of thalidomide ligands for use in the clinic.

Major comments to support the authors as they revise this work:

1. The DHU moiety would need to be demonstrated for utility beyond BRD4 with similar safety profiles for it to be explored further as equivalent to other ligands used in the field.

[Regarding the demonstration of using compound 2 for targeting diverse targets for degradation, despite facing publishing limitations on live assets as a large pharmaceutical company, we can guarantee that compound 2 is well-suited for designing degraders across various targets and will help others designing safer PROTACs. We have emphasized our patent describing the use of DHU-based degraders for efficient androgen receptor degradation. We have also discussed other reports using DHUs of varying compositions and how this is becoming a recognized warhead for PROTAC design. The demonstration of a DHU scaffold in the clinic by C4 Therapeutics \(CFT-1946\) attests to this group's utility and will hopefully clarify this to the reader. See discussion page 16, line 17-41:](#)

“One strategy to mitigate the safety risks associated with the use of IMiDs in PROTACs is to reduce, or ideally eliminate, the degradation of IMiD neosubstrates such as Ikaros. Approaches to address this have included optimizing the linker attachment point on the IMiD scaffold, employing benzylamide glutarimide derivatives, developing bump-and-hole thalidomide analogues, and utilizing peptidic cyclimid ligands for cereblon engagement. 54-57 However, it remains uncertain whether these strategies can fully prevent the degradation of other, perhaps as-yet-unidentified, IMiD

neosubstrates during molecule optimization. Thus, the use of alternative CRBN ligands represents a preferable route, particularly as the PROTAC modality expands beyond oncology indications.

In this work, we identified alternative CRBN binders featuring a DHU core rather than a glutarimide motif. Notably, a representative CRBN binder—compound 2—did not induce degradation of the established IMiD neosubstrates Ikaros and Aiolos, nor did it reduce levels of other proteins, as determined by proteomic analysis in the NB-4 leukemia cell line. As observed with glutarimide-based ligands, where neosubstrate selectivity is influenced by appended functional groups rather than the glutarimide ring itself, it is important to recognize that the DHU scaffold alone does not inherently confer selectivity with regard to molecular glue polypharmacology.⁵⁸ Indeed, previous studies have shown that DHU-based CRBN ligands, bearing alternative N-1 position substituents, can themselves function as molecular glue degraders for various neosubstrates.^{40,59}

Although compound 6, as described in this manuscript, lacked the requisite properties for advancement into in vivo studies, our results—together with the successful design of DHU-containing active degraders for other targets within the AstraZeneca portfolio—underscore the potential of this new CRBN ligand.^{46,47} We are confident that this scaffold can facilitate the development of chemically and configurationally stable, potent, and selective PROTACs, ultimately contributing to improved safety profiles for future therapeutics.”

2. A rationale for why the dihydrouracil indoline scaffold 2 has tighter binding capacity for cereblon by an order of magnitude, as compared to prior dihydrouracil scaffold 1 is not apparent from the text or structure. Description of the structure–activity relationship studies or additional insight here would be interesting.

We chose not to speculate on this in the manuscript as we are unsure of the reason. It could be the electronics of the indole aromatic ring in compound 2 vs the tolyl group in compound 1. Furthermore, there is a water-mediated interaction compound 2 makes with H56 and which compound 1 would not be able to recapitulate.

(see new Supplemental Figure 7) and text (page 13, line 40 and page 14, line 1-8):

“From these experiments a 1.39 Å crystal structure was determined, revealing 2 to adopt a binding mode for CRBN highly similar to that of thalidomide,³⁷ with no significant conformational changes observed between the two complexes (Figure 5A). The DHU group mimics thalidomide’s glutarimide ring, forming the same hydrogen bonds (shown as black dashes in Figure 5A) to the backbone carbonyl and NH groups of residues H77 and W79 as seen in the equivalent thalidomide crystal structure (Figure 5A). Notably, the NH of the indole makes a water-mediated interaction with H56 (Supplemental Figure 7), which may rationalize the LLE improvement seen when compared to compound 1.”

3. Alternate solutions in other reports need to be incorporated to the introduction and this work placed in their context. These include examination of alternate linkers, benzylamide glutarimide derivatives, bump hole derivatives of thalidomide for reducing molecular glue targets, and peptidic cyclimid ligands for cereblon.

We have added these alternative strategies to the discussion of our revised manuscript (see page 16, line 17-25):

“One strategy to mitigate the safety risks associated with the use of IMiDs in PROTACs is to reduce, or ideally eliminate, the degradation of IMiD neosubstrates such as Ikaros. Approaches to address this have included optimizing the linker attachment point on the IMiD scaffold, employing

benzylamide glutarimide derivatives, developing bump-and-hole thalidomide analogues, and utilizing peptidic cyclimid ligands for cereblon engagement. 54-57 However, it remains uncertain whether these strategies can fully prevent the degradation of other, perhaps as-yet-unidentified, IMiD neosubstrates during molecule optimization. Thus, the use of alternative CRBN ligands represents a preferable route, particularly as the PROTAC modality expands beyond oncology indications.”

4. The IC50 values of thalidomide and compound 2 represented in Table 2 do not match those reported in the text.

The value has been corrected in the revised text (see page 11, line 23-25):

“The screening of this library led to the discovery of compound 2 which has an IC50 of 183 nM in the HTRF assay (Figure 3A (i) and Supplementary Figure 5C) and a Kd of 216 nM as determined by isothermal titration calorimetry (ITC) (Supplementary Figure 5).”

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5 (Remarks to the Author):

In this manuscript, Rodrigo-Brenni et al. developed a novel proteolysis-targeting chimera (PROTAC) utilizing immunomodulatory drug (IMiD) scaffolds. While several PROTACs have been designed using IMiD-based structures to recruit Cereblon (CRBN) for targeted protein degradation, a major limitation of these PROTACs is the bystander degradation of CRBN neosubstrates, such as Ikaros and Aiolos. To mitigate these safety liabilities, this study identifies a novel dihydrouracil (DHU)-based CRBN ligand, which binds to CRBN effectively while sparing the degradation of off-target neosubstrates. When incorporated into PROTACs, exemplified by compounds targeting BRD4, the DHU scaffold enabled selective BRD4 degradation without affecting Ikaros and Aiolos. This work presents a promising strategy for developing safer and more selective CRBN-recruiting PROTACs. However, a major limitation of this study is insufficient characterization of the newly designed BRD4-targeting PROTACs in in vitro HSC models and in vivo mouse models.

The in vivo experiments using Crbn-I391V mice primarily reproduced findings from a previous report, with the exception of validating that compound "2" does not degrade Ikaros and Aiolos. As noted in Major Comment #3, the authors have not ruled out the possibility that compound "2" failed to bind to Crbn-I391V or that it exhibited different pharmacokinetics compared to existing IMiDs. Also, as detailed in Major Comment #4, it is essential to validate the efficacy of target protein degradation and the substrate specificity of compound "6" (the PROTAC developed in this study) in the in vivo model. Thus, I cannot support the publication of this manuscript in Nature Communications.

We would like to thank the reviewer for the feedback on our manuscript. Regarding Reviewer 5's suggestion to include compound 6 in in vivo studies, it is regrettably not feasible due to compound 6's lack of the necessary pharmacokinetic properties and its design limitations for in vivo optimization. Our objective with compound 6 was to demonstrate the feasibility of designing efficient degraders

using compound 2 as a CRBN binder. Also, since compound 6 targets BRD4 and BRD4 inhibition is detrimental to HSPCs, differentiating effects mediated by BRD4 from those involving Ikaros/Aiolos degradation is problematic.

In the revised manuscript, we have focused on the new molecular insights into the influence of IMiDs on primary human hematopoietic stem and progenitor cells by rewiring the differentiation process using high-throughput proteomics and flow cytometry. We have also extended the part showing the utility of an alternative CRBN ligand (compound 2) in mitigating IMiD safety liabilities, facilitating the development of safer degraders.

Below are few specific points added the revised manuscript:

- a) Addition of HSPC viability data over 13 days using flow cytometry, confirming a cytostatic rather than cytotoxic effect of IMiDs (**new Supplementary Figure 3**).
- b) We present new flow cytometry data with IMiDs at the single-cell level, demonstrating orthogonally a rewiring effect on cell fate towards megakaryocytes, complementing very well the proteomics data (**new Supplementary Figure 6**).
- c) Together with the new flow cytometry data, we further explored the proteomics data at the pathway enrichment level, again confirming a rewiring stem cell differentiation mechanism through the induction of platelet activation pathways that is mitigated by compound 2 (**new Figure 4C and 5C**).
- d) Clarification of the rationale behind the improved safety profile of compound 2 over IMiDs using chemical insights and discussion of the literature around other strategies to mitigate IMiD safety liabilities (**in new discussion**).
- e) Addition of pharmacokinetic data for compound 2, demonstrating the superior exposure of compound 2 compared to IMiDs following oral administration (**new Figure 3B**).
- f) Address the permeability question with a competition experiment. The data indicate that compound 2 competes with PROTAC, underscoring its intracellular penetration and CRBN binding (**new Figure 3D**).
- g) Addition of proteomics data with compound 6 to complement the cellular HiBiT-based degradation assay, confirming selectivity of compound 6 sparing IMiD neosubstrates degradation (**new Figure 6D**).

Major comments:

1. A substantial portion of the manuscript (Fig 1, 2, and 4) is dedicated to reproducing findings from previous literatures. The primary advancement presented in this study is the development of compounds "2" and "6" for reducing bystander degradation of IKZF proteins in IMiD-based PROTACs. However, their characterization remains limited.

We have reorganised our revised manuscript focusing on the novelty of the findings, see comments above.

2. In Fig 6F and the corresponding results section on page 9, as well as in the discussion, the authors state that IMiDs rewire HSC differentiation through the degradation of Ikaros and Aiolos, which is avoided by using compound "2." However, the changes in the abundance of selected proteins

shown in Fig 6F do not provide sufficient evidence to support this authors' claim. To clarify this point, colony formation assays and/or in vitro differentiation assays using IMiDs- and compound "2"-treated HSCs should be conducted.

We have added new flow cytometry data with IMiDs at the single-cell level generated on primary human hematopoietic stem and progenitor cells (HSPCs) during in vitro differentiation, demonstrating orthogonally a rewiring effect on cell fate towards megakaryocytes, complementing very well the proteomics data (**new Supplementary Figure 6**). Together with the new flow cytometry data, we further explored the proteomics data, also generated on HSPCs during differentiation at the pathway enrichment level, again confirming a rewiring stem cell differentiation mechanism through the induction of platelet activation pathways that is mitigated by compound 2 (**new Figure 5**).

3. In Fig 6 and the corresponding text on page 9, the authors do not exclude the possibility that compound "2" fails to bind to CRBN in NB-4 cells and HSPCs in ex vivo experiments. In in vivo experiments, they also did not exclude the possibility of reduced bioavailability of compound "2" compared to lenalidomide, in addition to the potential abrogated binding to CRBN. This requires careful examination, particularly because compound "6," which was derived from compound "2," still exhibited some degradation of Ikaros and Aiolos whereas compound "2" did not affect the abundance of Ikaros and Aiolos. To address this point, a pull-down assay should be conducted to confirm the direct interaction of compound "2" with CRBN, and a pharmacokinetic study to assess its bioavailability and stability.

We have added in vivo pharmacokinetic data for compound 2, demonstrating the superior exposure of compound 2 compared to IMiDs following oral administration (**new Figure 3B**). The data are discussed in the revised text (**see page 11, line 35-38**):

"When dosed in mice for 24 h at 10, 30 and 100 mg/kg, compound 2 showed dose increased plasma Cmax and AUC, with levels higher than those observed with lenalidomide at the same concentrations, demonstrating good properties for oral exposure (Figure 3B)."

To address the question on compound 2 binding to CRBN inside the cells, we have added a new competition experiment between IMiDs and compound 2, showing that compound 2 can reduce IMiDs induced Aiolos degradation in a dose dependent manner, demonstrating its cell permeability and binding to CRBN (**see new Figure 3D**). The data are discussed in the revised text (**see page 12, line 7-13**):

"To make sure that the lack of degradation was not due to a lack of permeability, we performed a competition assay between compound 2 and the IMiD molecules and an IMiD-based PROTAC (dBRD9). We observed dose-dependent inhibition of Aiolos degradation by compound 2, irrespective of the initial level of degradation elicited by each compound, confirming that compound 2 binds CRBN and its selectivity did not come from a lack of permeability (Figure 3D)."

4. Since compound "6" is a newly designed heterobifunctional PROTAC, comprehensive comparative studies against existing BRD4-targeting PROTAC (ZXH-3-26), and compound "2" should be performed. Specifically, proteomics analysis, evaluation of effects on hematopoietic cell survival/proliferation and differentiation, and in vivo experiments using Crbn-I391V mice are necessary.

We have profiled compound 6 in vitro using global proteomics. The data show compound 6 selectivity against IMiD neosubstrates like Ikaros and Aiolos, in contrast to ZXH-3-26 and an overall "clean" profile where the top downregulated proteins are BRD4, BRD2 and BRD3 (**see new Figure 6D**).

As discussed above, compound 6 does not have the right properties for in vivo studies. Furthermore, as discussed above, as compound 6 targets BRD4 and BRD4 is cytotoxic to HSPC, we did not trigger further work on HSPC differentiation as the data would not be interpretable.

To make it clear to the reader that compound 6 is not an in vivo tool, we have added a sentence to the discussion (see page 16, line 36-41):

“Although compound 6, as described in this manuscript, lacked the requisite properties for advancement into in vivo studies, our results—together with the successful design of DHU-containing active degraders for other targets within the AstraZeneca portfolio—underscore the potential of this new CRBN ligand.^{46,47} We are confident that this scaffold can facilitate the development of chemically and configurationally stable, potent, and selective PROTACs, ultimately contributing to improved safety profiles for future therapeutics.”

Minor comments:

1. The number of replicates is not consistently reported across the manuscript (e.g., Fig 2, 3E, 4D, 4F, 6F, S1B, S3, and S6). To ensure clarity and reproducibility, the exact number of replicates should be stated for each experiment.

We have added the number of replicates used through the experiments.

2. Page 3, Line 56: The phrase "thousands of children to be born with severe birth defects" is vague and lacks precise quantification. If a numerical estimate is being referenced, please provide a more accurate figure along with the source. Additionally, clarify what is meant by "severe birth defects" by specifying the types of conditions being referred to.

We have edited the text to be more descriptive, see Page 3, line 4-6:

“Initially launched in multiple countries to treat morning sickness in pregnant women, thalidomide caused more than 10,000 of children to be born with severe birth defects such as phocomelia, as well as thousands of miscarriages.”

3. Page 3, Line 58: Trade names should be omitted to maintain consistency and adhere to scientific writing.

We have removed the trade names. See page 3, line 6-8:

“Interestingly, thalidomide was later repurposed and shown to be efficacious in the treatment of multiple myeloma with the related IMiDs lenalidomide and pomalidomide now being the standard of care in this disease.”

4. Page 3, Line 61: The expression "MM clinical activity" is unclear. Please specify what it indicates.

We have removed MM and used multiple myeloma. See page 3, line 6-8:

“Interestingly, thalidomide was later repurposed and shown to be efficacious in the treatment of multiple myeloma with the related IMiDs lenalidomide and pomalidomide now being the standard of care in this disease.”

5. Page 4, Line 116 and throughout the manuscript: The compounds generated in this study are referred to only by numbers (e.g., “1” and “2”), which may cause confusion. To improve clarity and differentiation, consider assigning unique identifiers to each compound.

We would be happy to change if required by the journal. We have kept it this way in the revised manuscript.

6. Page 4, Lines 123-124: The dosing regimen described in this section is unclear. Please rephrase to ensure clarity.

We have edited the text, [see page 5, line 27-30 and page 6, line 1-23](#):

“HSPCs were cultured in expansion medium (StemSpan™ SFEMII, 09655, STEMCELL Technologies) supplemented with Stem Span™ CD34+ Expansion Supplement (02691, STEMCELL Technologies). For in vitro differentiation to the erythroid and myeloid lineages the HSPCs were resuspended in either BFUE-1 (SEC-BFU1-40H), or GM-CFC (SEC-GM1-40H) CellExpand™ Suspension Expansion Culture (SEC, Preferred Cell Systems) for 5 days. Treatment with either DMSO, lenalidomide, iberdomide or 2 using 1 & 10 µM for five days was performed during the differentiation phase. For flow cytometric analysis the cells were seeded 5000 cells per well of 24 plate and cultured for 5 days in differentiation media.

At the end of incubation cells were washed once (300 g, 5 min, 4 °C), resuspended in PBS-0.5% BSA and placed on ice. Next, the cells were incubated for 30 min at 4 °C in the dark with the antibody cocktail containing APC-Cy7-conjugated mouse anti-human CD34 (BioLegend), PE-conjugated mouse anti-human CD38 (BioLegend), BV421-conjugated mouse anti-human CD36 (BioLegend), BV786-conjugated mouse anti-human CD235a (BD Bioscience), APC-conjugated mouse anti-human CD41a (BioLegend), BUV395-conjugated mouse anti-human CD71 (BD Bioscience), PerCP-EF710-conjugated mouse anti-human CD16 (ThermoFisher), BUV737-conjugated mouse anti-human CD13. To assess viability of the cells, cells were stained with the primary amine-reactive Live/Dead Fixable yellow dead cell stain kit (ThermoFisher L34959). Alongside test samples, compensation controls using ArC Amine Reactive Compensation bead kit (ThermoFisher, for viability dye) and CompBeads (BD Bioscience, for antibodies) and fluorescence minus one (FMO) controls. Samples and FMO controls were washed twice and resuspended in PBS-0.5% BSA. Cells were fixed by addition of equal volume of 4% PFA (ThermoFisher) for 15 min at RT. Cells were washed twice and resuspended in PBS-1% BSA before acquisition on the BD LSR Fortessa (BD Biosciences). See diagram below for gating strategy. Data were analysed using FloJo 10 (FlowJo LLC).

Samples for high throughput proteomics (method below) were generated in parallel to the flow cytometry samples by seeding HSPCs +/- Ikaros KO cells for each condition in 96 well plates to obtain min. 15k cells per well at harvesting time..”

7. Fig S1B and Page 5, Lines 151-152: Why are the values for ZXH-3-26 fall below -100%?

We have removed the HiBiT degradation data to focus our revised manuscript on the novel findings. For information, the % can go beyond 100% due to normalisation to a positive control.

8. For Fig 2, the color coding information is not clearly indicated, making it difficult to interpret the comparisons. Additionally, since the comparisons are not explicitly defined, it is unclear whether the use of a t-test is appropriate in this context. Please provide clearer labeling for the color scheme and specify the statistical comparisons to justify the chosen test.

We have increased the size of the scale bar to better visualise the log2 fold change in protein expression. * indicates statistical significance (moderated t-test, $p < 0.05$) versus DMSO control. See new Figure 1.

9. The current data do not provide sufficient evidence to support the hypothesis that IMiDs act through a cytostatic rather than a cytotoxic mechanism. To clarify this, IMiDs should be added to the culture medium during the course of HSC expansion, and cell numbers should be assessed longitudinally to confirm that IMiD treatment does not induce cytotoxic effects. Additionally, in Fig 3C, 3D(ii), and 3E, the current y-axis labeling suggests that IMiD treatment leads to reduced viability. To better reflect the intended interpretation, the y-axis labels should be reconsidered, such as using absolute cell numbers instead of viability percentages.

h) We have added new HSPC viability data over 13 days using flow cytometry, confirming a cytostatic rather than cytotoxic effect of IMiDs (new Supplementary Figure 6).

10. Several non-standard abbreviations were observed throughout the manuscript and should be clearly defined for clarity. For example: "min. 15k" (Page 4, Line 117), "mpk" (Fig 4C). Additionally, consider rephrasing the use of "downregulation" (Page 6, Line 172 and throughout the manuscript). Since the proteins were degraded rather than actively regulated, terms such as "reduction" or "decrease" would be more accurate.

We have edited through the text to define abbreviations and change downregulation by reduction.

11. The following points in the figures should be addressed for improved clarity:

- Fig 1B: Different color codes were applied to the same compounds in panels (i) and (ii-v). To enhance consistency and readability, consider either separating them into distinct figures (e.g., Fig 1B and 1C) or combining them into a single plot.

Not applicable in the revised manuscript (removed).

- Fig 4D: The color coding is unclear due to the use of different fill and border colors. Please adjust the color scheme to improve distinction and readability.

We have reorganised our manuscript and moved this figure to the supplementary information.

- Fig 3D: The legend is incorrectly assigned, with the labels for (i) and (ii) swapped.

We have corrected the mistake, see page 18, line 3-5:

*"(i) Ikaros and CRBN CRISPR targeting efficiencies are represented in the SDS-PAGE analyses.
(ii) HSPC proliferation assay under pomalidomide treatment after 5 days of dosing in mock, CRBN or Ikaros knock-out cells"*

Response to reviewer 5

Dear reviewer,

Thank you again for your feedback on our revised manuscript. You requested additional evidence to strengthen confidence in the robustness of our bone-marrow flow cytometry assay (via a biological replicate) and further profiling of compound 2 to confirm its optimized profile versus IMiDs such as iberdomide.

We have now completed a new study that directly addresses these points. Specifically, we repeated the bone-marrow differentiation flow cytometry assay using a new human donor, five technical replicates, and a different flow cytometer due to availability with adjusted gating still capturing the same populations as in previous experiment. The study included Ikaros knockout, iberdomide alongside compound 2 head-to-head.

First, we confirmed assay reproducibility: both Ikaros knockout and iberdomide produced a similar increase in megakaryocyte populations and a decrease in erythroid populations, consistent with our prior findings (Supplementary Fig. 6, also added below called experiment 2).

Second, compound 2 showed no measurable effect in the same differentiation assay (Supplementary Fig. 6, see below). This result aligns with our existing data showing that compound 2 does not degrade Ikaros or other known IMiD neosubstrates (Fig. 3C–3F), does not impair proliferation of human hematopoietic stem and progenitor cells (Fig. 3G), and does not induce detectable phenotypic changes in our high throughput proteomics assay (Fig. 5).

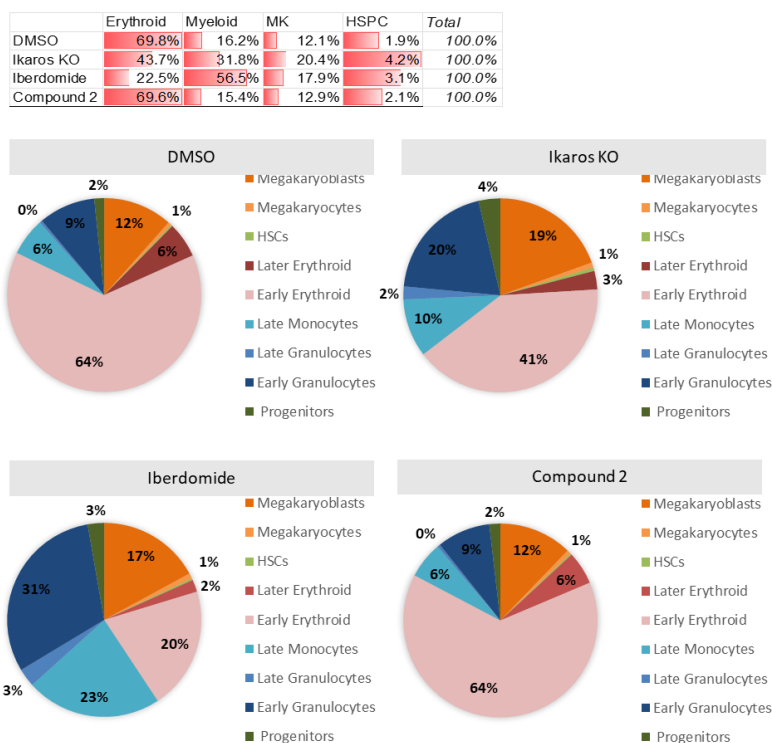
Together, these new data provide additional evidence that compound 2 has been rationally optimized to mitigate IMiD-associated safety liabilities. We believe this work will be valuable to the degrader community, particularly for advancing PROTACs toward clinical use in non-life-threatening indications where safety margins are paramount.

Before sending this response to you, we have had a discussion with the editor regarding the use of a new cytometer and different gating and whether this represents a true biological replicate. From our side, whether it is a “true” biological replicate or not does not fundamentally change the conclusion of the experiment. The key difference between the two cytometers used is that Cytex collects a full emission spectrum for each cell, allowing for the resolution of highly overlapping dyes and complex panels, while Fortessa uses bandpass filters to capture limited portions of the spectrum. This does not change the population being analyzed (see below). Across both experiments, we assessed the same cell population within a primary human cell background; the only biological difference was the donor.

The different cytometer should not introduce ambiguity. On the contrary, it strengthens confidence in the dataset by demonstrating robustness and reproducibility. This directly addresses your previous concern: the findings are consistent across instruments and donors, akin to independent reproduction of results—an important marker of scientific integrity. We have added below the comparison between the 2 experiments for full transparency.

Experiment 2:

Supplementary Figure 6: Ikaros knock-out, Iberdomide and compound 2 treatment on hematopoietic stem and progenitor cell differentiation



Supplementary Figure 6. Ikaros knock-out, Iberdomide and compound 2 treatment on hematopoietic stem and progenitor cell differentiation

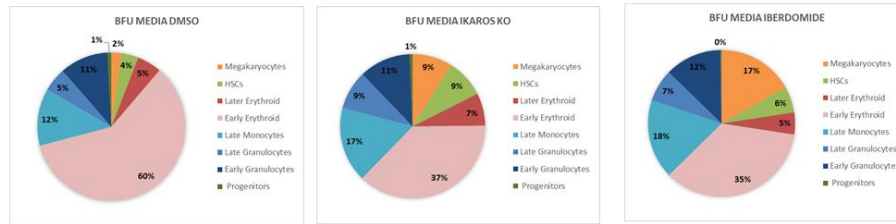
Table and pie charts showing distribution of bone-marrow populations of cells upon differentiation into the erythroid lineages in the absence of Ikaros (Ikaros KO), in the presence of 10 μ M Iberdomide or 10 μ M of compound 2. n=5 technical replicates pooled for the analysis. MK: megakaryocytes, HSPC: hematopoietic stem and progenitor cells.

Related to Figure 4.

Staining procedure: cells were washed once (300 g, 5 min, 4 °C), resuspended in cold PBS-0.5% BSA and maintained cold. Cellular expression of surface antigens was determined using flow cytometry. Reference controls for spectral unmixing were prepared alongside the samples using UltraComp eBeads™ Plus Compensation Beads (ThermoFisher 01-3333-42) for the CD markers and cells for the Viability and EdU reference controls. Group-specific unstained reference controls were prepared to allow spectral unmixing incorporating condition-specific autofluorescence. Independent FMO controls were prepared alongside the samples and reference controls for BFU-E media- and GM media-cultured cells to aid gating accuracy. Antibodies and Viability dye listed in table below were dispensed using the Dragonfly® Discovery (sptlabtech) liquid dispenser into a 96-well plate, into which the staining was carried out. The cells were incubated for 30 min at 4 °C in the dark with the antibody cocktail. Samples were washed twice and resuspended in PBS-0.5% BSA. Samples were subsequently washed with wash buffer containing DPBS (Gibco) with 0.5% BSA (Sigma), 5 mM EDTA (ThermoFisher) and 25 mM HEPES (Sigma) and fixed with 2%. Cells were fixed PFA (ThermoFisher) for 20 min at RT. Samples were then washed and resuspended in acquisition buffer containing DPBS (Gibco) with 0.5% BSA(Sigma), 5 mM EDTA (ThermoFisher) and 25 mM HEPES (Sigma) before acquisition. All plate washes were performed using the Curiox HT2000™ (Curiox BioSystems; settings: 9 washes at 5uL/sec performed after staining and fixation). Samples were analysed using the **Cytek Aurora™ 5L (Cytek Biosciences)** spectral flow cytometer, spectral unmixing was performed on the SpectroFlo software (Cytek Biosciences) and analysis was carried out using OMIQ™ (Dotmatics). See diagram below for list of antibodies and gating strategy.

Experiment 1:

Supplemental Figure 6: Ikaros knock-out and Iberdomide treatment affect hematopoietic stem and progenitor cell differentiation



Supplementary Figure 6. Ikaros knock-out and Iberdomide treatment affect hematopoietic stem and progenitor cell differentiation

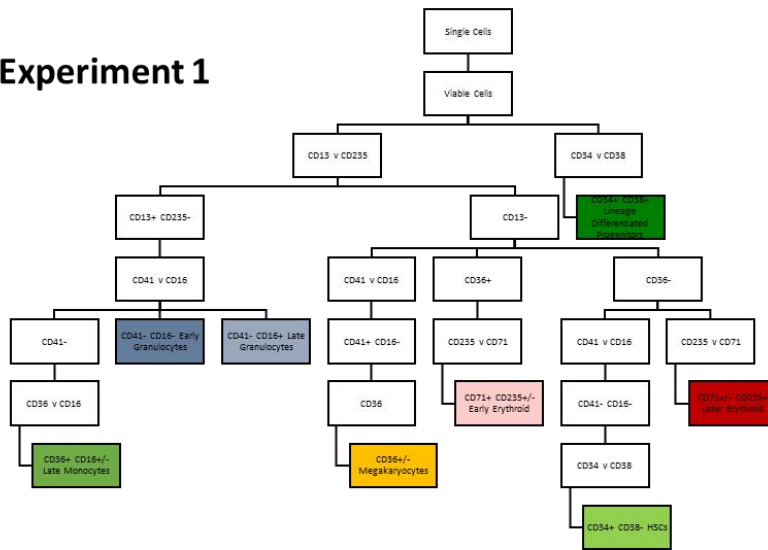
Pie charts showing distribution of 8 populations of cells upon differentiation into either the erythroid (BFU media) or myeloid (GM) lineages in the absence of Ikaros (Ikaros KO) or in the presence of 0.1 μ M Iberdomide (Iberdomide). n=5 technical replicates pooled for the analysis. HSC: hematopoietic stem cells

Related to Figure 4.

Staining procedure: cells were washed once (300 g, 5 min, 4 °C), resuspended in PBS-0.5% BSA and placed on ice. Cellular expression of surface antigens was determined using flow cytometry. Alongside test samples, compensation controls using Arc Amine Reactive Compensation bead kit (ThermoFisher, for viability dye) and CompBeads (BD Bioscience, for antibodies) and fluorescence minus one (FMO) controls. The cells were incubated for 30 min at 4 °C in the dark with the antibody cocktail containing APC-Cy7-conjugated mouse anti-human CD34 (BioLegend), PE-conjugated mouse anti-human CD38 (BioLegend), BV421-conjugated mouse anti-human CD36 (BioLegend), BV786-conjugated mouse anti-human CD235a (BD Bioscience), APC-conjugated mouse anti-human CD41a (BioLegend), BUV395-conjugated mouse anti-human CD71 (BD Bioscience), PerCP-EF710-conjugated mouse anti-human CD16 (Thermofisher), BUV737-conjugated mouse anti-human CD13. To assess viability of the cells, cells were stained with the primary amine-reactive Live/Dead Fixable yellow dead cell stain kit (Thermofisher L34959). Samples and FMO controls were washed twice and resuspended in PBS-0.5% BSA. Cells were fixed by addition of equal volume of 4% PFA (Thermofisher) for 15 min at RT. Cells were washed twice and resuspended in PBS-1% BSA before acquisition on the **BD LSR Fortessa (BD Biosciences)**. See diagram below for gating strategy. Data were analysed using FloJo 10 (FlowJo LLC).

Gating strategy between experiment 1 and 2 leads to same population identification

Experiment 1



Experiment 2

