nature portfolio

Peer Review File

Elaboration of molecular glues that target TRIM21 into TRIMTACs that degrade protein aggregates

Corresponding Author: Dr Drew Adams

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)
The discovery of molecular glues remains largely empirical. A phenotypic screening approach identified PRLX-93936 and BMS-214662 as small molecules whose cytotoxicity is mitigated by inhibition of the ubiquitin-activating enzyme UBA1, revealing their role as TRIM21-directed molecular glues. These compounds induce degradation of nucleoporins, disrupt nuclear trafficking, and exhibit cytotoxicity correlated with TRIM21 expression. PRLX-93936 and its analogs define a distinct structural class with enhanced potency and limited off-target effects, and have been elaborated into a TRIMTAC capable of degrading a multimeric target via wild-type TRIM21.

I truly enjoyed reading this manuscript. The study is significantly expanded relative to the preprint, and the addition of detailed structure–activity relationships that culminate in enhanced potency is particularly compelling. The development of PROTACs capable of degrading multimeric protein aggregates is a creative and impactful advance. The manuscript reads exceptionally well, and I would strongly support publication without the need for additional experiments.

Reviewer #2

(Remarks to the Author)
The authors identify several ligands that bind the E3 ligase TRIM21 and degrade Nup proteins as a molecular glue or, when synthesised as a heterobifunctional, a model BRD4-PML-GFP ligand. The manuscript includes the following findings:

Identification of PRLX-93936 and BMS-214662 as ligands that kill cells in a UPS dependent manner and sensitivity correlates with TRIM21 expression.
Cell killing is dependent upon TRIM21 expression using knockout cells.
Over-expression of TRIM21 increases sensitivity and is dependent upon catalytic activity.
Site of binding is PRYSPRY domain, identified by using ligands to prevent binding to IgG, which is recognised by the PRYSPRY domain of TRIM21.
Proteomic identification of nuclear pore proteins as targets that are degraded
Deleting autoproteolysis domain of Nup98 prevents PRLX-93936 activity
A model PROTAC made usng a JQ-1 ligand degrades BRD4 when fused to PML-GFP

There are 3 other existing studies which report similar data - Lu et al., Cell 2024, Li et al. bioRxiv 2024 and Cheng et al. bioRxiv 2024. In each case, these studies identify ligands that bind TRIM21, act as a molecular glue with Nup proteins and kill cells. In the case of Cheng et al. bioRxiv 2024, exactly the same compounds are used. The most problematic of these studies, in terms of novelty, is Lu et al., Cell 2024. This paper contains much more comprehensive data than the present manuscript and has already reported the novel findings - that TRIM21 can be used as a molecular glue, that ligands glue TRIM21 to Nup proteins like Nup98 and cause cell death and that a TRIM21 ligand can be turned into a heterobifunctional to degrade a target protein. The weakness of the present manuscript is therefore that it does not add any novel conclusions or fndings but shows the same things with a different ligand.

Main issue is that in most if not all graphs no statistics have been performed. In some cases this may mean an additional replicate needs to be performed to give N=3 and allow a proper assessment of the error.

Minor points:

1. Figure 1D&H, Figure 2C-G, 2D-H, Figure 3B&C, E&F, H&I, M. Is the data significant? Needs stats and another replicate (minimum of N=3 for proper stats).
2. Quantify the blots in Figure 3. Also please provide copies of the full blots in the Supplementary materials.
3. Line 185-189 - Figure 3L needs to be referenced here.
4. Liine 195-209. It's probably appropriate for Lu et al., Cell 2024 to be referenced here as this study had already identified Nup protein depletion by TRIM21 ligands. Thus the data here is supportive of this previous work but not surprising.
5. Figure 4F. What is the variability in fold-change in this experiment. It would be useful for the replicates to be plotted with errors.
6. Figure 4M. A larger and higher resolution image would be helpful, perhaps showing zoomed in views of single cells.

Reviewer #3

(Remarks to the Author)
In this manuscript from Adams and colleagues, the antiproliferative activity of PRLX-93936 is explained as a molecular glue degrader of nuclear pore proteins. Using a chemical genetic approach, the authors discover two structurally distinct molecules that arrest cancer cell line proliferation, and both emerge as TRIM21-dependent glue degraders. Paths to discovering glue degraders (or glues otherwise) are needed. The SAR study of PRLX derivatives is a highlight, culminating also in a bispecific degrader opportunity that will help the field. Overall, this is a fine paper that is well suited to publication in Nature Communications. I have only a few comments worth considering.

Specific comments and critiques are below:
1. The authors cite a competing study of a TRIM21-dependent molecular glue degrader early in the Introduction. This previous work does not, in my opinion, detract from this study that explains two bioactive compounds that are advanced investigational agents. In fact, i am impressed by the early citation of this recent work early in the manuscript, as most authors would bury this work in the Discussion.

2. Figure 1B is interesting to me as the assay positives are not terribly far afield from the library, highlighting the subtlety of glue discovery.

3. The use of gene expression differences for target discovery is simple, and here effective in also guiding to a second TRIM21-dependent glue candidate.

4. In Figure 2, it would be helpful to know the impact of TRIM21 KO on cell viability (e.g impact on doubling rate, viability)

5. In Figure 3, it would be helpful to know the impact of TRIM21 overexpression on cell viability (e.g impact on doubling rate, viability)

6. Overexpression of catalytically dead TRIM21 was a very nice control.

7. A little more biochemical evidence of selective target engagement would be a welcome addition. In Figure 3, does the inactive BMS-225975 derivative displace TRIM21?

8. The focused PRLX chemistry was a nice addition, with methyl diastereomer 1 showing remarkable activity and the active/inactive atropisomeric pair emerging as fine mechanistic probes.

9. The only missing ingredient, in my opinion, is the delivery of a TRIM21-dependent chemical probe targeting nucleoporins. Surely one of the derivatives is such a molecule. This could be approached down the road, but at a minimum the generation of gene expression data or other phenotyping to reveal a general lack of off-target effects (recall these molecules were created without this mechanism in mind).

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Thank you for addressing each of my comments. In light of these I am happy to now recommend acceptance.

Reviewer #3

(Remarks to the Author)
The authors have experimentally or textually addressed all critical comments, and I support publication in the present form.

Response to Reviewers

We appreciate the constructive comments we received from each of the three reviewers which have significantly strengthened our revised manuscript. Our point-by-point responses are below in blue font.

Reviewer #1 (Remarks to the Author):

The discovery of molecular glues remains largely empirical. A phenotypic screening approach identified PRLX-93936 and BMS-214662 as small molecules whose cytotoxicity is mitigated by inhibition of the ubiquitin-activating enzyme UBA1, revealing their role as TRIM21-directed molecular glues. These compounds induce degradation of nucleoporins, disrupt nuclear trafficking, and exhibit cytotoxicity correlated with TRIM21 expression. PRLX-93936 and its analogs define a distinct structural class with enhanced potency and limited off-target effects, and have been elaborated into a TRIMTAC capable of degrading a multimeric target via wild-type TRIM21.

I truly enjoyed reading this manuscript. The study is significantly expanded relative to the preprint, and the addition of detailed structure–activity relationships that culminate in enhanced potency is particularly compelling. The development of PROTACs capable of degrading multimeric protein aggregates is a creative and impactful advance. The manuscript reads exceptionally well, and I would strongly support publication without the need for additional experiments.

We appreciate the reviewer's close read and strong support.

Reviewer #2 (Remarks to the Author):

The authors identify several ligands that bind the E3 ligase TRIM21 and degrade Nup proteins as a molecular glue or, when synthesised as a heterobifunctional, a model BRD4-PML-GFP ligand. The manuscript includes the following findings:

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Proteomic identification of nuclear pore proteins as targets that are degraded
Deleting autoproteolysis domain of Nup98 prevents PRLX-93936 activity
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There are 3 other existing studies which report similar data - Lu et al., Cell 2024, Li et al. bioRxiv 2024 and Cheng et al. bioRxiv 2024. In each case, these studies identify ligands that bind TRIM21, act as a molecular glue with Nup proteins and kill cells. In the

case of Cheng et al. bioRxiv 2024, exactly the same compounds are used. The most problematic of these studies, in terms of novelty, is Lu et al., Cell 2024. This paper contains much more comprehensive data than the present manuscript and has already reported the novel findings - that TRIM21 can be used as a molecular glue, that ligands glue TRIM21 to Nup proteins like Nup98 and cause cell death and that a TRIM21 ligand can be turned into a heterobifunctional to degrade a target protein. The weakness of the present manuscript is therefore that it does not add any novel conclusions or fndings but shows the same things with a different ligand.

We appreciate that others have recently reported their TRIM21-focused work, and we have cited these papers in the introduction, results, and discussion sections. We feel these studies reinforce central conclusions of our work; however, our work has its own innovative and impactful aspects, including using medicinal chemistry to generate a nucleoporin degrader with field-leading potency and elaboration of this lead to the first PROTAC that degrades an aggregated protein using wild-type TRIM21 (the noted *Cell* 2024 reference used a mutant TRIM21 due to the low affinity of their molecule for TRIM21). Our work goes beyond the published work by providing a road map for follow-on chemistry and biology studies that can further assess the utility of TRIM21 in targeted protein degradation, particularly of aggregated proteins.

Main issue is that in most if not all graphs no statistics have been performed. In some cases this may mean an additional replicate needs to be performed to give N=3 and allow a proper assessment of the error.

Minor points:

1. Figure 1D&H, Figure 2C-G, 2D-H, Figure 3B&C, E&F, H&I, M. Is the data significant? Needs stats and another replicate (minimum of N=3 for proper stats).

While we recognize the utility of p-values and biostatistical analyses, there is not a standard method in use in chemical biology for evaluating whether two full dose-response curves (as in the panels noted) are statistically significantly different from each other. Given this limitation, we have pursued other approaches to demonstrating the robustness and rigor of our results:

In Figure 1, two independent scaffolds of small molecules are shown to be comparably rescued. Additionally, similar effects are seen with two independent methods of perturbing the ubiquitin/proteasome system.

In Figure 2, multiple unrelated small molecules are tested in two independent runs in two different cell lines with highly comparable results, providing an effective n of 4 for each of the two molecules. We have now expanded upon the existing data in Fig. 2c by adding new data to achieve n = 3 runs. Additionally, a Welch's t-test performed on the highest dose tested revealed a p-value of 0.008, indicating a statistically significant effect (Supp. Fig. 2a). Moreover, the shift in cell killing potency is not slight: TRIM21 KO shifts PRLX sensitivity more than 1,000-fold for PRLX (in both cell lines) and more

than 100-fold for BMS (both cell lines). Additionally, we provide three independent runs for nearly all of the 30 newly-synthesized analogs that have been evaluated (Supporting Table 2).

In Figure 3 both PRLX and BMS are shown to give comparable results in three independent cell lines, each tested in two independent runs, meaning that each molecule has in fact been tested 6 independent times in the WT and TRIM21 overexpression context. Additionally, we also used a catalytically inactive TRIM21 mutant to provide additional context to these studies, an approach Reviewer 3 called a 'very nice control'. Again, the shift in cell killing potency is stark following TRIM21 overexpression: C33A and HEK293T cells go from fully resistant to PRLX and BMS to killed with an EC50 of 10-50 nM following TRIM21 expression.

In short, by using multiple unrelated small molecules, multiple cell lines, multiple independent experiments for each dose-response curve shown (including n = 3 for many compounds (Fig. 2 and Supp. Table 2), and the evaluation of both loss-of-function and gain-of-function genetic manipulations, we have established with extraordinary rigor that TRIM21 loss robustly blocks the cytotoxic effects of these glues while TRIM21 overexpression drives strong sensitivity.

2. Quantify the blots in Figure 3. Also please provide copies of the full blots in the Supplementary materials.

We have quantified the blots in Fig. 3 as requested (Supporting Fig. 2d-f). Full blots are available in the source data section per journal policy.

3. Line 185-189 - Figure 3L needs to be referenced here.

Thank you for this catch, we have added the reference.

4. Line 195-209. It's probably appropriate for Lu et al., Cell 2024 to be referenced here as this study had already identified Nup protein depletion by TRIM21 ligands. Thus the data here is supportive of this previous work but not surprising.

We appreciate this comment and understand the need to present our work in the context of other recent papers reporting TRIM21 glues, including the recent paper that reported PRLX-93936 and BMS-214662, which emerged while our paper was under consideration. After the presentation of our own proteomics data showing NUP degradation and nuclear export defects, we have modified the text to read:

"Recent studies, including two published during review, have also noted TRIM21-mediated degradation of nucleoporins by small molecules including hydroxy-acepromazine, HGC652, PRLX-93936, and BMS-214662.[29,43,44] These studies also provided genetic evidence that hydroxy-acepromazine induced degradation of multiple nucleoporins by recruiting TRIM21 specifically to NUP98….."

We feel this revision fairly balances giving us a chance to present our work while being transparent that recent publications have reported similar findings regarding nucleoporin degradation.

5. Figure 4F. What is the variability in fold-change in this experiment. It would be useful for the replicates to be plotted with errors.

We have now added the raw LFQ intensity plots (n = 3) for four of the NUPs shown in Fig. 4f (NUP214, NUP88, NUP188, NUP155; Supporting Fig. 3e).

6. Figure 4M. A larger and higher resolution image would be helpful, perhaps showing zoomed in views of single cells.

We have reorganized Fig. 4 to create room for larger images that we hope capture the nuclear localization of RANBP1 that ensues from PRLX-93936 treatment only when catalytically active TRIM21 is expressed. We have also included a second version (Supporting Fig. 3h) that includes both RANBP1 and a nuclear stain, which may provide additional clarity regarding the overlap of RANBP1 with the nuclear stain.

Reviewer #3 (Remarks to the Author):

In this manuscript from Adams and colleagues, the antiproliferative activity of PRLX-93936 is explained as a molecular glue degrader of nuclear pore proteins. Using a chemical genetic approach, the authors discover two structurally distinct molecules that arrest cancer cell line proliferation, and both emerge as TRIM21-dependent glue degraders. Paths to discovering glue degraders (or glues otherwise) are needed. The SAR study of PRLX derivatives is a highlight, culminating also in a bispecific degrader opportunity that will help the field. Overall, this is a fine paper that is well suited to publication in Nature Communications. I have only a few comments worth considering.

Specific comments and critiques are below:
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We appreciate this comment and support for our work. We agree our work retains significant novelty and impact. However, during review, two additional papers were published reporting additional TRIM21-targeting glues. We now note these new studies in revised text in the introduction and in the results section on page 11: “Recent studies, including two published during review, have also noted TRIM21-mediated degradation of nucleoporins by small molecules including hydroxy-acepromazine, HGC652, PRLX-93936, and BMS-214662.” We feel this comment allows us to tell our story while also acknowledging these very recent reports.

2. Figure 1B is interesting to me as the assay positives are not terribly far afield from the library, highlighting the subtlety of glue discovery.

Yes, and as we note other molecules comparably positive in the primary screen did not confirm in our retest studies. It will be interesting to apply this screening strategy with additional modulators of the ubiquitin system to see if other glues can be discovered.

3. The use of gene expression differences for target discovery is simple, and here effective in also guiding to a second TRIM21-dependent glue candidate.

We appreciate this comment and agree that characterizing BMS-214662 strengthens the story by documenting a second unrelated chemical series of TRIM21-targeting glue.

4. In Figure 2, it would be helpful to know the impact of TRIM21 KO on cell viability (e.g impact on doubling rate, viability)

5. In Figure 3, it would be helpful to know the impact of TRIM21 overexpression on cell viability (e.g impact on doubling rate, viability)

These are great points. TRIM21 KO cells and TRIM21 overexpressing cells derived from multiple cell types proliferate normally. The high viability of TRIM21 KO cells is supported by unbiased data (www.depmap.org) which shows that TRIM21 is not a dependency across ca. 1,000 cancer cell lines.

We have added new data showing equivalent CellTiterGlo signal for TRIM21KO cells and NTC cells using OCI-AML-3 and Jurkat cells. We have also added new data showing equivalent CellTiterGlo signal for TRIM21-overexpressing cells and EGFP-expressing cells using OCI-AML-3 and C33A cells. These are included in Supporting Figure 2b,c. Our data are also consistent with past work, which we cite, showing TRIM21 overexpression is well-tolerated in the context of the genetic knockdown strategy TRIM-AWAY.

6. Overexpression of catalytically dead TRIM21 was a very nice control.

Thank you.

7. A little more biochemical evidence of selective target engagement would be a welcome addition. In Figure 3, does the inactive BMS-225975 derivative displace TRIM21?

We have added new data showing that BMS-214662 also competes TRIM21 from beads in our pulldown assay, providing additional evidence that both series of molecules we describe engage TRIM21 at its PRYSPRY domain (Supporting Fig. 2g). Further characterization of our set of analogs using a broader set of biochemical assays is ongoing.

8. The focused PRLX chemistry was a nice addition, with methyl diastereomer 1 showing remarkable activity and the active/inactive atropisomeric pair emerging as fine mechanistic probes.

Thank you.

9. The only missing ingredient, in my opinion, is the delivery of a TRIM21-dependent chemical probe targeting nucleoporins. Surely one of the derivatives is such a molecule. This could be approached down the road, but at a minimum the generation of gene expression data or other phenotyping to reveal a general lack of off-target effects (recall these molecules were created without this mechanism in mind).

Thanks for this comment. We agree that these molecules were created without this mechanism in mind, and it's true that we have not used transcriptomics analysis to characterize our small molecules.

However, we think multiple lines of evidence support our analog **1** as the desired TRIM21-dependent probe targeting nucleoporins. This analog is significantly more potent than all reported TRIM21-based nucleoporin degraders identified to date (more than 10x more cytotoxic than PRLX-93936), and all of its cytotoxic effects are dependent on TRIM21 (Fig. 5a). Also we confirmed that molecules in this series to not induce ferroptosis, despite being structurally related to the ferroptosis-inducer erastin (Fig. 2). Thus **1** represents an excellent tool compound for other researchers to use to probe TRIM21-mediated nucleoporin degradation and cytotoxicity that is unequivocally TRIM21-dependent and unrelated to ferroptosis.