THE EMBO JOURNAL

Arrayed Genome-Wide Perturbation Screen Identifies Hnrnpk As Rate-Limiting for Prion Propagation

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Review COMMONS

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1st Editorial Decision 6th Sep 2022

Dear Adriano,

Thank you for submitting your manuscript to The EMBO Journal. The submission is a transfer from Review Commons where the review process was carried out.

Your revised version has now been re-reviewed by the original referees and their comments are provided below. As you can see, the referees appreciate the introduced revisions and support publication here. I am therefore very pleased to let you know that we will accept the MS for publication here. Before sending you the formal acceptance letter there are some editorial issues we have to sort out. Please see the list below.

Also, we are piloting a new support service to guide authors through the process of selecting essential source data for the revised manuscript.

Our Source Data scientific coordinator, Daniele Viarisio (daniele.viarisio@embo.org CCed in) will be following up with you shortly to provide more details on what figures we need source data for.

I have provided the list below for the formatting issues that needs to be resolved.

I am still waiting to hear back from our publisher who are also doing their checks on the MS. I will send you the comments as soon as I receive them. Please wait to upload the revised version until you receive their checks from me.

That should be all! Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Revisions:

- we are missing 3-5 keywords

- we need a data availability section - this is the place to enter accession numbers. If no data needs to be deposited in a database please state: Data Availability: This study includes no data deposited in external repositories.

- Please add a Disclosure and competing interests statement

- The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y').

- Please provide an author checklist (see guide to authors)

- The FUNDING 670958 and 179040 is missing in ms file

- Supplemental Figures should be labelled as Apendix and with a ToC including page numbers. Figures should be be renamed to Appendix Figure S1-S4 - please also correct callout in text. The figure legends for appendix figures should also be added to the appendix and removed from the main MS file.

- Suppl.Materials file should be renamed Dataset EV1 and its legend should be removed from the manuscript and added to the excel file, in a separate tab. The callout in the manuscript will also need to be changed from "Suppl. Material" to "Dataset EV1".

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Can you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- I also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels).

- Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

- use this link below to submit the revision:

Link Not Available

Referee #1:

All my concerns have been adequately addressed.

Referee #2:

The authors have addressed all of my concerns and the revised manuscript is improved and I therefore recommend publication in its present form in EMBO Journal. I think that this screen approach and the findings will be broadly interesting to the scientific community.

Referee #3:

The authors addressed all my concerns satisfactorily.
The study is a very nice piece of work, a great conceptual and technological progress.
I have no more specific comments.

***
Rev_Com_number: RC-2022-01377
New_manu_number: EMBOJ-2022-112338
Corr_author: Aguzzi
Title: Arrayed Genome-Wide Perturbation Screen Identifies hnRNP K As Rate-Limiting for Prion Propagation

Full Revision

Manuscript number: RC-2022-01377
Corresponding author(s): Adriano, Aguzzi

[Please use this template only if the submitted manuscript should be considered by the affiliate journal as a full revision in response to the points raised by the reviewers.

If you wish to submit a preliminary revision with a revision plan, please use our "Revision Plan" template. ***It is important to use the appropriate template to clearly inform the editors of your intentions.****]*

1. General Statements [optional]

We are grateful for the reviewers' constructive comments and suggestions which contributed to improving our manuscript. In addition, we are very pleased to see that our method was described as a "great technological improvement" and "really ingenious new method" and that the findings have been described as "novel and very significant".

To address the remaining concerns, we performed several experiments and revised the manuscript as further detailed below in the point-by-point response in this letter.

2. Point-by-point description of the revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

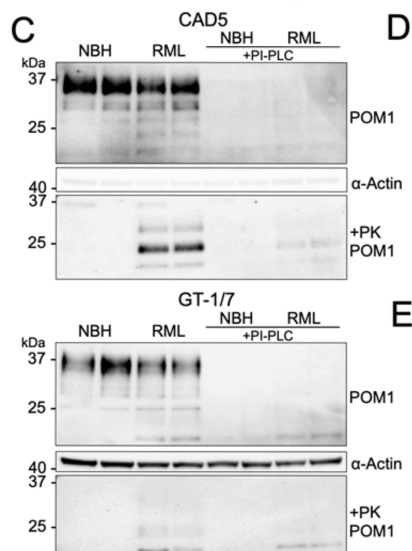
Avar et al report on the development of a high-throughput method to screen modifiers of prion replication in cell lines using a genome-wide siRNA library. They identified a number of hits and further studied one candidate, the ribonucleoprotein Hnrnpk. The authors convincingly show the interest of their method. However, the claims that the ribonucleoprotein Hnrnpk impact prion propagation need to be more quantitatively and statistically substantiated.

1. A large part of the manuscript is dedicated to the validation of the high-throughput assay (called QUIPPER). QUIPPER is made in 384-plates and provides great technological improvement. It works with different prion-permissive cell lines and different prion strains. QUIPPER is an antibody-FRET-based assay that detects a specific population of PrPSc that resists phospholipase C (PIPLC) treatment. Historically, PIPLC has been shown to cleave cell surface PrPC while preserving PrPSc (which is endocytic or inaccessible). I would recommend that the authors quantify the proportion of PIPLC-resistant PrPSc (PrPPIPLC) versus total PrPSc in their different models. First, PrPPIPLC proportion may be cell and strain dependent. Second and most importantly, as siRNA effects are studied using PrPPIPLC as readout, it is crucial to know if this form is a bona fide surrogate of PrPSc and infectivity or only a specific, subcellular, potentially minor form of PrPSc. This is particularly important as the effects of Hnrnpk knock-down in QUIPPER and western blot sounds discordant; in

QUIPPER, the effects are strong (> 5-fold) while by western blot, the effects are much more modest (< 2-fold).

We addressed this issue in several ways; firstly, we quantified the proportion of PIPLC-resistant PrP (PrP^{PLC}) versus PrP^{Sc} in two different models (Fig. 1B and D). Secondly, we directly compared residual infectivity of cells treated with PK or PIPLC (Figure 1C), using the standard scrapie cell assay. The results show that infectivity is retained upon PIPLC treatment. In addition, we assessed the 161 hits obtained via QUIPPER using PrP^{Sc} as a readout (Fig. 3B).

To provide further data on the robustness of our PIPLC-based readout, we have performed western blotting of infected and uninfected cells upon PIPLC treatment and assessed the band patterns following PIPLC administration. This Figure is now incorporated in the manuscript as Supp. Fig. 1C and demonstrates that upon PIPLC digestion of NBH and RML infected CAD5 and GT-1/7 cells, PrP is barely detectable in the non-infected cells, while it is in the prion infected ones. The blots also show that the PIPLC-resistant PrP (PrP^{PLC}) is resistant to PK digestion. These new data, together with those provided in Fig. 1B and Figure 1C, show that PrP^{PLC} is equivalent to PrP^{Sc} in terms of PK resistance and infectivity.

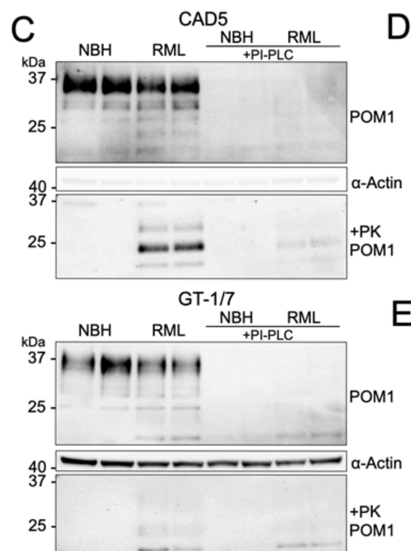


The reviewer pointed out a discordance between Western Blotting and QUIPPER. Although it is not clearly stated, we think the reviewer may be suggesting a discordance based on Fig. 3D. We would like to point out that Fig. 3D does not report fold changes as the reviewer is suggesting, but Z-scores, measured by standard deviations from the mean, not allowing to infer fold-changes. We quantified the effect of NT and HNRNPK targeting siRNAs on prion levels (Fig. 4A) and saw a three-fold change. We believe that the quantifications provided in the new version of the manuscript alleviate the concerns regarding any discordance.

Technically, this is quite easy as it necessitates, after PIPLC treatment, the quantification of PrPSc in the supernatant versus PrPSc in the cell pellet. In Fig. 1C, the authors show that PrPPIPLC is

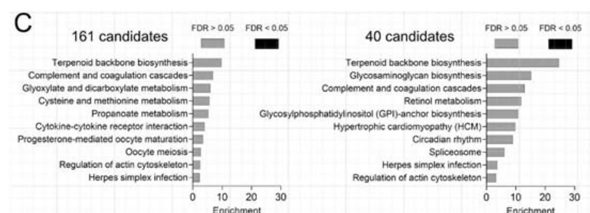
infectious in a cell-scrapie assay. Using this approach, they could also quantify the infectivity of these species relative to the total infectivity content.

We addressed this in Supplementary Fig. 1C as depicted above. Supplementary Fig. 1C shows the alikeness of the PrP species measured via the QUIPPER vs. the canonical PK digestion: upon digestion with PIPLC following a PK treatment, we detect PrP^{Sc}. Therefore, the experiment demonstrates that PrP^{PLC} is alike in nature to PrP^{Sc}. The difference between the PK digested (lanes 3&4) vs PIPLC treated then PK digested lanes (lanes 7&8) is the PrP^{Sc} that is released into the media following PIPLC digestion.



2. The authors identified a list of prion modifiers candidate. Surprisingly, the authors did not perform a pathways analysis to identify potential pathways that could impact prion propagation.

Despite extensive efforts, there were no pathways that were enriched in our 40 hits, which is mentioned in the discussion part of the manuscript. Two analyses (for the 161 candidates and 40 hits) are now added to Supplementary Fig. 3C and pasted below.

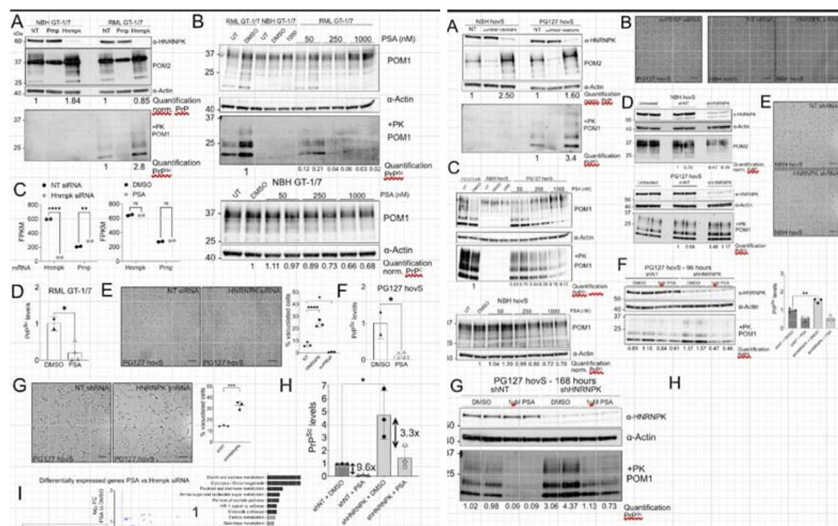


3. The authors then studied in more details one hit, the ribonucleoprotein Hnrnpk. They studied the impact of Hnrnpk knock-down on PrPC and PrPres levels in different cell lines. These data (Fig 4 and Fig S4) lack quantitative (on a higher number of wells) and statistical analyses. The western blot that are shown suggest that PrPC levels are slightly increased by the siRNA and that the increase in

PrPres levels is modest, barely significant given the western blot method. Same comment after PSA treatment, at least in PG127-infected hovS cells.

We performed a quantification on the western blots for all figures mentioned by the reviewers throughout the manuscript. These are incorporated to the manuscript for the figures: Fig. 4A, Fig. 4B, Supplementary Fig. 4A, Supplementary Fig. 4C, Supplementary Fig. 4D, Supplementary Fig. 4F, Supplementary Fig. 4G.

Additionally, statistical analyses have been incorporated into the manuscript in these figures: Fig. 4C, Fig. 4D, Fig. 4E, Fig, 4F, Fig, 4G, Fig, 4H, Supplementary Fig. 4F. The analyses and the quantitative data demonstrate the effect of Hnrnpk downregulation and PSA treatment on prion levels to be significant. Moreover, we also addressed the regulation of prions via HNRNPK using vacuoles as a read-out as well as with a different mode of regulating HNRNPK expression using shRNAs. All these results, point to HNRNPK as a true modulator of PrP^{Sc}.

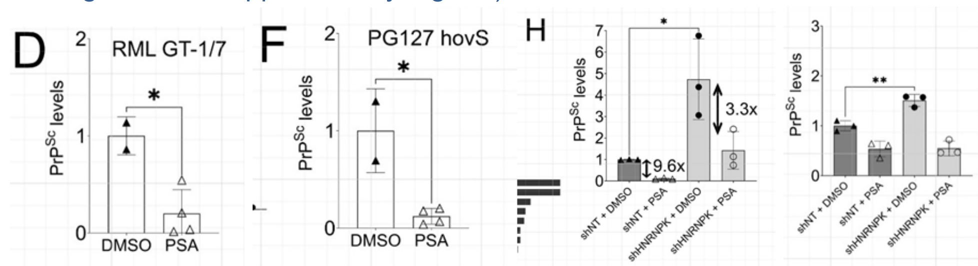


In Figure 4A and B, the use of POM1 and/or POM2 to detect PrPC / PrPres is confusing. POM2 is supposed to detect mostly full-length PrPC (Fig 4A top panel), but more than 3 glycoforms are detected. In Fig 4B, POM1 is used for PrPC but because it has a central epitope, it detects both PrPC and PrPSc.

Both antibodies are able to recognize both PrP^{C} and PrP^{Sc} as it has been shown in many publications from the Aguzzi lab as well as other labs in the field. https://pubmed.ncbi.nlm.nih.gov/19060956/

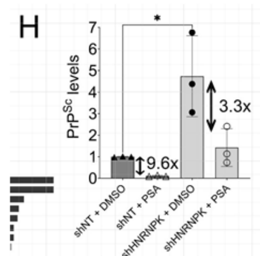
Note also in Fig 4B, that DMSO alone seems to impact PrPC levels in PG127-infected hovS cells. This advocates again for a more quantitative analysis.

We have quantified the western blots using the DMSO control as standard value. As DMSO was used to dilute PSA, this should take into account potential effects coming from DMSO (Fig. 4D, Fig. 4F, Fig. 4H and Supplementary Fig. 4F).



4. Psammaplysene A (PSA) is a pharmacological Hnrnpk binder. The authors used this molecule to further demonstrate that Hnrnpk is involved in prion propagation. I disagree with the author's conclusion that "PSA effect does seem to be limited when HNRNPK shRNAs are applied". In Fig S4D, 1µM PSA seems do decrease PrPres levels at similar levels whether the shRNA is applied or not. Again quantification and statistical analyses from several independent experiments would help supporting the authors conclusions.

We assessed this point carefully by quantification of the western blots (Fig. 4H) and providing statistical data (Student's t-test) from three experiments. As we see a threefold lower decrease of prions with and without Hnrnpk regulation when PSA is present, we concluded that the effect we see from PSA should be arising through Hnrnpk. However, we cannot conclusively delineate the effect of PSA, because Hnrnpk ablation is not possible due to essentiality of Hnnrpk. This has now been added to the discussion portion of our manuscript.



5. The authors finally tested PSA on organotypic brain slices (in that case, they provide statistical results) and on flies infected with ovine PG137 prions. PSA administration significantly reduced the locomotor deficits prion-infected flies. The authors quantified the effects of PSA on prion accumulation in flies. Because the overall levels were not detectable by immunoblot, they used a cell-free assay termed RT-QuIC to address prion seeding activity in fly heads. I have specific comments about these experiments:
- Maybe I missed it, but I could not find which recombinant PrP is used in RT-QuIC assay.
This information is provided in the M&M section of the manuscript at hand. The relevant section on P25 reads, where HaPrP23-231 refers to hamster PrP:

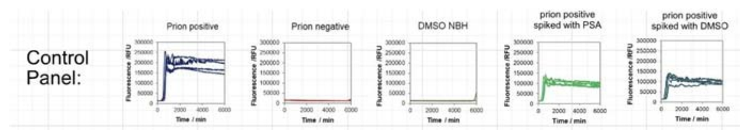
The reaction buffer of the RT-QuIC consisted of 1 mM EDTA (Life Technologies), 10 µM thioflavin T, 170 mM NaCl, and 1× PBS (incl. 130 mM NaCl) and HaPrP23-231 filtered using 100-kD centrifugal filters (Pall Nanosep OD100C34) at a concentration of 0.1 mg/ml.

In addition, we added this information to the main text as well.

- This is important as recombinant PrP self-polymerize after a period of time and here the authors have left the RT-QuIC assay running for unusually long period of times (RT-QuIC are stopped after 24h-48h).

For prions, long RT-QuIC experiments are often performed (also see: https://pubmed.ncbi.nlm.nih.gov/32598380/, https://journals.asm.org/doi/10.1128/mBio.02451-14, https://www.nature.com/articles/s41598-021-84527-9, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3458796/ and others).

In addition, this is controlled for in all experiments performed in the lab, as the prion-negative sample containing the same RT-QuIC substrate does not become positive after the entire duration of the assay (Fig. 5D).

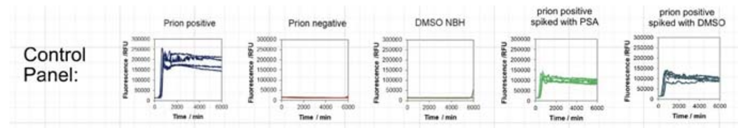


- Instead of titrating prion seeding activity by endpoint titration, the authors quantified PSA activity by measuring the effect on another parameter of the RT-QuIC, the length of the lag phase before the conversion reaction is visible. While this is an interesting criterion, reduction of seeding activity must be shown to unequivocally demonstrate that PSA has delayed prion pathogenesis in flies.

Based on the data presented in the manuscript, we assessed prion pathogenesis in flies using a well-established climbing assay, demonstrating that treatment with PSA significantly improves locomotor behavior, which has been shown to be directly linked to prion levels and is known to have even greater sensitivity then the traditional mouse bioassay (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5998032/, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6113635/, https://link.springer.com/article/10.1007/s00441-022-03586-0).. The RT-QuIC represented here represents itself as a secondary read-out to the climbing assay, for which Lag-time quantification is used routinely (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3893511/, https://www.nature.com/articles/s41598-017-10922-w, https://journals.asm.org/doi/10.1128/mBio.02451-14, https://www.nature.com/articles/s41598-021-87295-8). Our results effectively highlight the overlap between the complementary read-outs.

- Can the authors exclude any interfering effect of PSA on the RT-QuIC reaction, given the amount of material used to seed the reaction (1:20 diluted head homogenates)?

We do not know how much PSA has reached the Drosophila brain, therefore, the experiment suggested by the reviewer cannot be tied to a 1:20 dilution. However, the concern of the reviewer is valid, and we therefore performed a spiking experiment of a prion positive sample using 1uM PSA (the highest amount used to treat cells, for which we saw a strong prion-reducing effect). We did not see an interference in the RT-QuIC signal due to PSA in the reaction. This has been incorporated into Figure 5D.

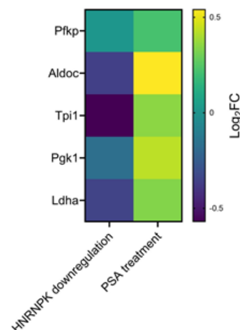


6. could the authors comment on the fact that HNRNPK knock-out is not possible and that their siRNA and shRNA are not affecting the cell viability?

To select hits during the screen process, we apply a viability filter, excluding siRNAs that reduce viability by more than 50% when compared to the non-targeting control siRNA (Supplementary Fig. 1F). For GT-1/7 cells we do not see any effect on viability of siRNA treatment after 96h. However, as downregulation of HNRNPK worsens the cytopathological vacuolation in the hovS model, as shown in Supp. Fig 4A, we do see an effect on cell fitness using both siRNA as well as shRNA. In addition, as knocking down HNRNPK will not lead to its complete loss, the remaining levels might be enough to sustain viability. Moreover, the longest knockdown experiment we performed is 7 days, we cannot exclude that longer exposure would have an impact on viability, but this question is not in the scope of the paper.

7. In the discussion the authors do not discuss how Hnrnpk could impact prion propagation. This may deserve a comment as this protein is present in the nucleus. As PrPC has been also identified in this compartment, can this specific form be involved in prion pathogenesis?

We additionally elaborated on potential ways of how Hnrnpk might impact prion propagation in the discussion, which includes potential nuclear PrP^{Sc} as well as with regards to our data obtained from the sequencing efforts shown in Fig. 4I. In addition, we investigated some functional targets of Hnrnpk how they are affected by PSA, which is now added to Supp. Fig 4G.



Reviewer #1 (Significance (Required)):

The QUIPPER method is a great conceptual and technological approach that could be applied to genome-wide analyses and screening for therapeutic molecules.

The study will interest a general audience interested in neurodegenerative diseases linked to protein misfolding. There are commonalities in pathways and modifiers of the conversion. Further PrP has emerged as a receptor for alpha-synuclein (Parkinson disease) and A-beta peptides (Alzheimer's disease).

Expertise key words: prion diseases - prion pathogenesis in cell models

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Prions are protein-based infectious agents that underlie neurodegenerative disease. For prion diseases (e.g., mad cow disease), the infectious agent is the cellular prion protein (PrPc). It exists in a normal conformation and carries out its normal cellular function. However, when it becomes misfolded and aggregates it can adopt an altered conformation, referred to as the prion conformation, or PrPSc. PrPSc aggregates can template the conversion of other PrPc molecules into the PrPSc form. In this way the prions can propagate from one cell to the next and throughout an organism. Prion diseases are truly devastating and identifying ways of stopping prion propagation is of great interest. In this manuscript by Aguzzi and colleagues, the authors designed a way to screen for prion propagation modifiers in mammalian cells. They built a highly sensitive readout of PrPSc propagation and adapted it to a 384-well plate format in adherent cells. They then used this to perform a genomewide siRNA screen, looking for genes that increased or decreased PrPSc propagation when knocked down.

They identified nearly 1,200 modulators of prion propagation and then subjected them to various validations and filtering to focus on only those hits that affected PrPSc but not PrPc (though hits that affect levels of PrPc could certainly be interesting). All this led to 40 genes (20 that increased and 20 that decreased prion propagation.

Among these 40, the authors focused on one hit, hnRNPK, an essential RNA-binding protein with diverse cellular functions. They provide evidence that reducing levels of hnRNPK leads to increase prion levels.

They next move to a marine compound called Psammaplysene A (PSA), which had previously been shown to have some neuroprotective properties and to be able to bind to hnRNPK. Because of the latter observation, the authors test if PSA can affect prion levels. They show that indeed treatment of their cell line prion infection model, or an organotypic slice model, or a fly model with PSA is sufficient to decrease prion levels.

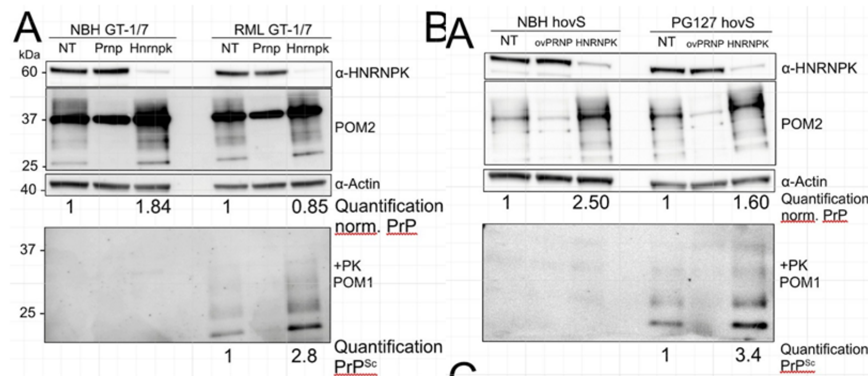
The authors propose that PSA works to reduce prion levels by increasing the activity of hnRNPK and that this also implies a role of RNA (because hnRNPK is an RNA-binding protein) in prion

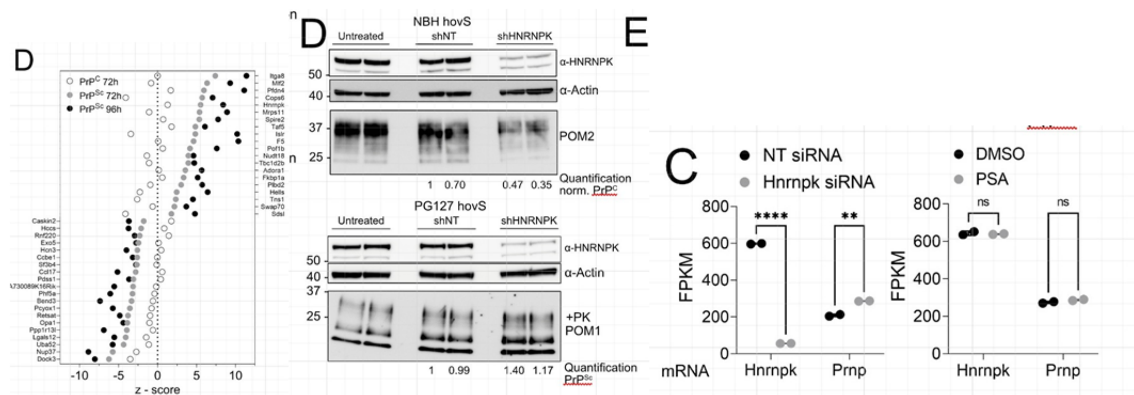
propagation.

In a nutshell, in my opinion the design and execution of this genomewide screen is ingenious and has yielded a treasure trove of potential prion modifiers. The ability to distinguish between modifiers of Prpc and PrpSc is super powerful. However, the follow-up and focus on hnRNPK and its connections (which seem tenuous) to the marine compound PSA are incomplete and raise more questions than answers. In its present form, it is hard to assess the potential significance of hnRNPK in prion propagation. I have some comments and suggestions for the authors to consider.

1.To my eye, Fig. 4A looks like Hnrnpk siRNA leads to slightly increased levels of PrPc (detected with POM2 antibody) and this could explain the increase in PrPSc levels. Can the authors assess Prnp RNA levels and the effects of their siRNAs on Prnp expression? It would also be useful to provide quantification of immunoblots if possible.

We quantified the western blots as mentioned in our response to reviewer 1. The quantifications are now provided for figures: Fig. 4A and Supplementary Fig. 4A, showing that the increase in prion levels is much stronger than that of PrP^{C}. These confirm the results from the screen as seen in Fig. 3D. In addition, we would again like to point out that the use of shRNAs to knockdown HNRNPK did not yield the increase in PrP^{C} levels aforementioned, as evident by Supplementary Fig. 4D which demonstrates a decrease of PrP^{C}, despite increasing PrP^{Sc} levels. Moreover, we show quantification of RNA levels upon downregulation of Hnrnpk and with PSA, which show that downregulation of Hnrnpk via siRNAs indeed increases Prnp mRNA levels and that PSA does not change RNA levels of neither Hnrnpk nor Prnp (Fig. 4C).





2. In Supplemental Fig. 4B it also looks like knocking down Hnrnpk results in decreased PrPc levels in this experiment and its not clear how robust the increase in PrPSc levels are. Quantification of these experiments, if possible, would be helpful.

Please see response above. We now provide quantification to all western blots.

3. The authors treat with PSA, which is supposed to bind to Hnrnpk. They state that this treatment does not affect PrPc levels but to my eye Supplemental Fig. 4C looks like highest doses of PSA cause a decrease in PrPc levels. Quantification of the immunoblots would also be useful here.

Please see response above. We now provide quantification to all western blots and added a sentence to the manuscript.

4. The authors use Hnrnpk knockdown along with PSA to test if the effects of PSA depend on Hnrnpk. They see PSA decreases PrPSc levels and that this is, to my eye, only slightly attenuated by Hnrnpk reduction. I interpret these results slightly different than the authors. To me, it seems that this result indicates that PSA's effects are (mostly) independent of Hnrnpk.

Addressed in point 4 from reviewer one.

5. In the original paper identifying PSA and hnRNPK physical interaction, RNA-binding was important. In the authors' assays, does Hnrnpk's effect on prions depend on RNA-binding? Specific mutations to the RNA-binding domains can be made to assess this.

This is a very interesting point. We did try to obtain data to support this claim, however, due to the essentiality as well as tight control of Hnrnpk expression, we were not able to express different forms of Hnrnpk and acquire conclusive data. Therefore, it is currently being pursued how Hnrnpk might affect prion propagation in the scope of another publication.

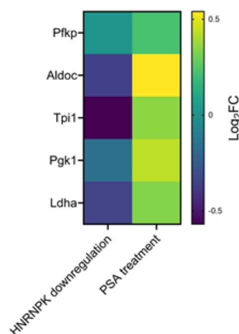
6. The genetic interaction in the vacuolation phenotype between Prnp and Hnrnpk that the authors report is very interesting (Supplemental Fig. 4A). It seems like this system and phenotype could be useful for the authors in exploring mechanisms by which HnrnpK is functioning.

Review COMMONS

We absolutely agree to the reviewer's comment. As mentioned above a second publication is under way to investigate the mechanisms of Hnrnpk's antiprion function, which is not in the scope of this study.

7. The authors propose that PSA increases activity of Hnrnpk but does it change any Hnrnpk RNA targets from their RNA sequencing? Some functional readout of Hnrnpk function would be useful here to test this hypothesis.

Although we do suspect RNA binding has an important role in the anti-prion function of Hnrnpk, we cannot exclude other modalities which Hnrnpk might be function through, such as DNA binding and protein-protein interactions. Therefore, to answer this question, a considerable effort that explores each of the potential of these modalities with regards to the anti-prion function of Hnrnpk would be needed. This extensive effort, however, is out of the scope of the manuscript at hand. However, we investigated the effect of PSA on some known functional targets of Hnrnpk (as suggested by the reviewer) from our sequencing efforts and added this analysis as Supplementary Fig. 4H to the manuscript. These results suggest that PSA leads to an increase of the expression of DNA targets of Hnrnpk, potentially suggesting a modality of action. Moreover, we amended the discussion with regards to potential pathways that might be yielding the effect seen as evidenced by the RNAseq data.



8. In the Introduction, the authors mention two yeast papers in introducing the concept of using unicellular model organisms to perform modifier screens. The first paper (Outeiro and Lindquist, 2003) is a classic but does not contain a yeast screen. The other one does include a loss of function screen in yeast (for polyQ toxicity modifiers) but those results seems to be due to loss of the [RNQ+] prion from certain deletion strains instead of from specific roles of modifier genes, so that paper might not be the best exemplar of yeast modifier screens.

We sincerely thank the reviewer for their careful readthrough of the manuscript, the portion that refers to the manuscripts as screens was amended and two new citations for appropriate yeast screens were added to the manuscript.

9. The authors asked if any of their hits from their screen had human genetics connections to neurodegeneration. They mention one of their hits Dock3 right after saying that no hit reached statistical significance after multiple testing corrections. This seems a bit misleading since any time one makes a list of anything there will always be, by definition, one at the top of the list.

We amended the wording to improve clarity of the manuscript.

10. The authors perform RNA sequencing on prion infected cells that either had Hnrnpk siRNA or PSA and since these two treatments had opposite effects they looked for genes that went in the corresponding directions. They didn't find anything significant when looking for genes downregulated by Hnrnpk siRNA and upregulated by PSA. They did find glucose metabolism genes when looking in the opposite direction. The significance of this finding is unclear and the authors do not expand on it.

Addressed in point 7 of reviewers 1 and 2, we expanded the discussion portion of the manuscript with regards to these results.

11. To me, the data with PSA seem more robust than the Hnrnpk data and it seems that the authors are trying to perhaps over-fit them together. It is possible that PSA affects prion levels independent of Hnrnpk function. This would not dampen my enthusiasm at all for this finding and could be of interest to those in the prion field, in which the search for anti-prion compounds is of great interest.

Upon statistical analysis of the result in Fig 4H, we see a three-fold decrease of PSA activity upon HNRNPK downregulation, suggesting PSA activity might be linked to HNRNPK. However, the reviewers point is well taken and we emphasized the value of understanding the function of PSA or mimicry of its effect as potential therapy in the future.

Cross-commenting:

All three reviewers seem to appreciate the novelty and impact of the new QUIPPER method the authors have developed to discover modifiers of prion propagation. All three reviewers also seem to be somewhat less convinced by the connection to hnRNPK, including how the compound PSA's anti-prion effects involve hnRNPK (or not).

In my opinion, this manuscript presents important and novel work and a really ingenious new method to study prion propagation, which will be broadly useful to the prion field. I feel that the hnRNPK data could be strengthened, especially with more quantitative analyses. The PSA treatment data are compelling but it seems that the effects might be independent of hnRNPK and that the authors are trying to force a connection which might not be there.

Reviewer #2 (Significance (Required)):

*** Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate. ****

I have expertise in neurodegenerative disease, protein misfolding, yeast modifier screens, CRISPR modifier screens in human cells, and RNA-binding proteins. I have general knowledge about prions, including PrP, but I am not a prion expert.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

The authors conducted an arrayed RNAi-based genome-wide high-throughput screening of all protein-coding modifier genes that affect prion propagation in cultured cells (murine and human cell lines) using a novel quantitative high throughput QUIPPER assay that they developed. They identified 1191 genes, of which 40 selectively affect PrPSc. Half of the 40 genes seem to inhibit PrPSc (limiter) whereas the other half do the opposite (stabilizers). One of the strong limiters is Hnrnpk, is an essential small heterogeneous nuclear ribonucleoprotein that has been implicated in a few protein misfolding diseases. The biological relevance of the findings is demonstrated by the detection of previously reported modifier genes as well as thorough verification of Hnrnpk as an effective prion limiter that seems to be independent of the two prion strains or host species (mouse and human cell lines as well as Drosophila).

The manuscript is very well written, the approach is novel, very well verified, and effective, the data are solid, and the main conclusions convincing.

Two issues need to be discussed.

Major comments:

First, some genes encoding proteins involved in PrP processing, such as ADAM10 and ADAM8, are known to affect PrPC levels, but they are not among the modifier genes identified. Based on Table 2, ADAM8 expression is very low in the GT-1/7 cells. This points to one of the caveats of the RNAi screening approach in that potential roles of low expressing genes in the cell lines used could be missed. Although it is beyond the scope of this manuscript, it would be helpful to add discussions on complimentary screening enhancing gene expression and the use of more cell lines that will allow identification of more modifiers.

We thank the reviewer for their concern. The point regarding the screen being less sensitive for genes that are low-expressed in the cell line in question is valid. Upon advancing of the CRISPR-based technologies and the improvement of these technologies to be used in combination with prions, we see their value. We added a sentence to the discussion, talking about gene activation as a future alternative to perform a complimentary screen.

Second, the statement that PSA's anti-prion effect potentially arises through enhancing the activity of HNRNPK makes sense, but it is also possible that PSA can directly inhibit prion replication as well. It would be helpful to calculate the percentage of reduction in PrPSc by PSA treatment and the percentages compared between shNT and shHNRNK cells.

We thank the reviewer for the careful read through of the manuscript. The point was addressed for reviewer 1 point 4. In addition, if PSA is added to the RT-QuIC, it does not prevent aggregate formation, indicating that PSA is unlikely to directly inhibit prion replication, but rather depends on a cellular host-intrinsic molecule for its activity. However, we also elaborate more on the possibility of potential other mechanisms for Hnrnpk and PSA’s function on regulating prion levels in the

discussion section of our manuscript.

Minor comments:

First, Figure 1C shows that the relative intensity for RML CAD5 cell lysate infected cells is less than with PIPLC treated or PK treated, which seems to be the opposite of what is expected, because PIPLC or PK treatment should not increase infectivity. Please explain.

We agree with the reviewer that the results were surprising. For the practicality of the screen, we wanted to show that the treatment does not eliminate the infectious species, which we were able to demonstrate. However, the increase of infectivity could stem from many different factors, e.g. the amount of duration of PK treatment might not harm but instead rather expose the infectious species, or PIPLC might remove cell surface molecules that could prevent infection of cells. However, as there are a plethora of possible scenarios and it was not relevant for the study at hand, we did not go into further detail.

Second, in Fig S1 e, the labels are too small to read. In Fig 3D, it would be easier to match the stabilizer or limiter genes with the corresponding Z score dots if the genes with a negative Z scores are labelled on the left side while genes with positive Z scores be labelled on the right side.

We amended the figures as per the reviewer's suggestion.

Third, The following sentence on page 11 is confusing: "20 out of these 40 candidates reduce prion propagation upon silencing, and 20 candidates enhanced prion propagation, and henceforward are called stabilizers or limiters, respectively (Fig. 3D-E, Supplementary Table 1)." Did the author mean to say "....and 20 candidates enhanced prion propagation upon silencing, and hence..."?

We reworded the sentence according to the reviewer's comment.

Fourth, In the subheading "Hnrnpk expression limits of prion propagation in mouse and human cells", "of" should be deleted.

We addressed this in the main manuscript file.

Cross-commenting:

I agree with Reviewer #2's assessment that more quantification will be helpful and the link between the effect of PSA treatment and hnRNPK can be strengthened. I want to stress that the knockdown data clearly shows the involvement of hnRNPK as a prion limiter in cultured cells. The question on PSA does affect the interpretation of the ex vivo and in vivo data.

The blot in Fig. S4c seems to show some decrease in PrPC levels in NBH-treated GT-1/7 cells. This blot needs to be quantified to confirm whether the PrPC level is changed by PSA treatments. Whether PSA directly inhibits prion replication can be relatively easily assessed in RT-QuIC reactions. Alternative to the use of PSA, RNAi-mediated hnRNPK knockdown can also be done on

cultured tissue slices or in brain, but this will require a lot more time and efforts and may be too much to ask for in this manuscript.

Quantifications for blots were added throughout the manuscript and the text was amended accordingly, and all the points mentioned have been addressed throughout this response letter.

Reviewer #3 (Significance (Required)):

The findings are novel and very significant. They identified a large number of modifier genes, and established a solid foundation for future studies on prion modifier genes to study prion replication and pathogenesis and for novel therapies against prions and potentially some other protein misfolding diseases. HNRNPK seems to be good target for therapeutic intervention and PSA may be a good candidate for prion treatment. The novel QUIPPER assay can be used to screen for anti-prion compounds and potentially adapted to study other misfolding proteins associated with cells.

1st Revision - Editorial Decision 22nd Sep 2022

Dear Adriano,

Thank you for submitting your revised manuscript to The EMBO Journal.

I have now had a chance to look at it and all looks good! I am therefore very pleased to accept the MS for publication here.

Congratulations on a nice study!

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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