EMBO reports

Recognition of phylogenetically diverse pathogens through enzymatically amplified recruitment of RNF213

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1st Editorial Decision **26th Jul 2024**

Dear Dr. Randow,

Thank you for transferring your revised manuscript to EMBO reports. I now went through the manuscript, the referee reports from the assessment of the revised manuscript at the previous journal (outside EMBO press) and your point-by-point response. As you know, two referees are satisfied with the revisions and support publication of the study, whereas two referees remain critical, also indicating novelty concerns.

However, after looking through your point-by-point response, I have decided to invite a final revised manuscript that addresses the remaining referee points as indicated in your point-by-point response. Please also provide a detailed final point-by-point-response to these points.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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If n<5, please show single datapoints for diagrams. Please add to each legend (main, EV and Appendix figures, where applicable) a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales. See:

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

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13) All Materials and Methods need to be described in the main text using our 'Structured Methods' format, which is required for all research articles. According to this format, the Materials and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software, and relevant equipment and including their sources and relevant identifiers), uploaded as separate file, followed by a Methods and Protocols section in which we encourage the authors to

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15) Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Point-by-point reply

Reviewer #1 (Remarks to the Author):

The authors answered my questions thoroughly and I appreciate the work that they put into making a new Fig. 2.

We are pleased that Reviewer 1 is fully satisfied by how we addressed their questions.

Reviewer #2 (Remarks to the Author):

Despite the addition of some new structural data, the revised manuscript and the authors' responses have not addressed my primary concerns regarding data integrity, approach, and novelty claims.

The reviewer feels that we have not addressed his/her "… concerns regarding data integrity, approach, and novelty claims."

Our previous point-to-point reply tackled these concerns extensively. For example, regarding novelty we wrote:

"… Our manuscript provides the following novel insights:

- enzymatically driven cooperative recruitment of RNF213 enables sensing of evolutionarily distant pathogens
- a detailed kinetic analysis of RNF213 coat formation, providing time constants for coat initiation (T=27±1min) and coat expansion (5.0±2.4min) (new data in Fig.2C-F and supplementary). The difference in the time constant of coat initiation and the time required for coat expansion explains why coats on T. gondii vacuoles are initiated from a single focus; rarity of coat initiation and rapid coat completion limit the likelihood of more than one initiation event occurring per vacuole
- identification of a cluster of fast evolving residues near the N-terminus of RNF213, indicative of PRR function for RNF213
- a novel cryo-EM structure of human RNF213, revealing a carbohydrate-binding domain in the previously unresolved N-terminus that encompasses the aforementioned cluster of positively selected residues and strongly supports the proposed PRR function of RNF213 (new data in Fig.3)
- a cryo-EM structure and AlphaFold model of the RZ finger (new data in Fig.4C), supported by mutational analysis, offering mechanistic insight into the catalytic mechanism of the novel RZ-family class of E3 ligases."

Previous work had demonstrated that human RNF213 is essential for IFNgamma-activated cell-autonomous immunity to Toxoplasma in human cells. The previous version of this manuscript claimed that mouse Rnf213 was similarly important for host defense to Toxoplasma in mouse cells. These claims were based on the use of an Rnf213 KO cell line made in an immortalized mouse embryonic fibroblasts (MEFs) cell line. Due to the nature of Ko generation in immortalized MEFs, it is likely that the Rnf213 KO MEF cell line is clonal. It is well documented that clonal effects can result in artificial phenotypes. I pointed out in my original critique that the data presented by Crespillo Casado et al was surprising for the reasons briefly recaptured here: the original version of this paper claimed that Rnf213 KO MEFs were defective in controlling the replication of both type I and type II Toxoplasma strains. Importantly, type I Toxo is resistant to IFNgamma-mediated ubiquitination in mouse cells, and therefore the data presented by Crespillo Casado et al would have meant that Rnf213 would be exerting its anti-Toxoplasma activity independent of ubiquitination. In their rebuttal the authors now acknowledge that they cannot reproduce these findings in primary (non-clonal) Rnf213 KO MEFs and therefore it must be concluded that mouse Rnf213 – at the very least – is not essential for host defense to Toxoplasma – and mouse Rnf213 may very well not be sufficient either.

While I applaud the authors for removing their non-reproducible Toxoplasma restriction data from the revised manuscript, the revised manuscript continues to use these immortalized Rnf213 MEF KO cells, although the authors have now established that these cells are prone to generate artificial data – at least in Toxoplasma studies. In Fig.1F-G the paper continues to show data that would suggest that mouse Rnf213 promotes ubiquitination of the Toxoplasma parasitophorous vacuole (PV) in mouse cells. Again, these data – if reproducible – would be surprising because they would suggest that IFNgamma-induced PV ubiquitination does not lead to PV destruction. These data were again generated in the potentially clonal immortalized Rnf213 KO MEF cell line. As I stressed in my previous critique, it is essential to complement this single Rnf213 KO clonal cell line through ectopic expression of mouse Rnf213 to make sure that the phenotype is not an artifact. In their response the authors highlight the difficulties of working with the large sized ORF of Rnf213. While I appreciate the technical difficulties of these experiments, these experiments are doable and the authors themselves point out that they have done these types of experiments in the past when they complemented Rnf213 KO cells with ectopic RNF213 in Otten eta l. (2021). Alternatively, the authors could use independent primary KO MEFs (generated from different embryos as biological variables) and proper co-isogenic WT controls. These types of experiments are essential to support the paper's claim that Rnf213 promotes Toxoplasma PV ubiquitination in mouse cells.

The reviewer is concerned about potential species-specific differences in the ability of human and murine RNF213 to restrict Toxoplasma, specifically whether murine RNF213 promotes Toxoplasma PV ubiquitination in mouse cells. While this is a valid question, our manuscript aims to address how RNF213 senses phylogenetically distant pathogens, not whether murine RNF213 is deployed against Toxoplasma as was previously reported for human RNF213.

During the previous round of revision, we therefore removed data on the genetic interaction of Toxoplasma with murine RNF213 from the manuscript. Due to an oversight Fig.1F/G were left in the manuscript, which (understandably) triggered further questions from Reviewer 2. We would like to apologize for the mistake and have now removed the panels as originally intended. Importantly, our conclusion that RNF213 senses phylogenetically distant pathogens through enzymatically amplified RNF213 recruitment remains entirely unaffected.

The authors state in the rebuttal that because mouse and human RNF213 are quite homologous to each other, expression of human RNF213 in mouse cells is a sound approach. Yet, at the same time the authors claim (without any experimental evidence) that protein sequence variation in human RNF213 relative to other primate and monkey RNF213 orthologous is functionally important. I don't think you can have it both ways.

Because human RNF213 complements LPS ubiquitination in mouse Rnf213 KO cells (Otten et al.), the authors argue in their rebuttal that therefore "the evolutionary distance between humans and mice has not affected core RNF213 function." Of course, this logic is flawed. Just because LPS recognition is conserved between mouse and human RNF213 it does not many that every other function of this very large multi-functional protein is conserved, especially considering that some residues

in RNF213 are under strong positive selection which implies changes in function. As argued in my initial critique, there is no compelling reason to work with human RNF213 in mouse cells yet many reasons why this flawed approach may result in artificial results.

Notwithstanding positive selection in 59 of the 5241 aligned residues (Fig.3A), RNF213 is a well conserved protein, as is evident from the negative selection scores for most residues (Appendix Table EV1) and the striking structural similarities between human and murine RNF213 (Ahel eLife 2020, this study Fig.3). We have shown experimentally that human RNF213 complements the murine RNF213-/- MEFs used here regarding the ubiquitylation of Gram-negative bacteria (Otten Nature 2021) and Gram-positive bacteria (this study, Fig.1D, 4E).

Regarding the use of human RNF213 in murine cells, and as explained in our previous point-to-point reply, the enormous size of the RNF213 (with a cDNA larger than our mitochondrial genome!) poses significant practical challenges, which deterred us from generating murine copies of already existing human constructs. Expressing genes from phylogenetic distant species is a frequently used method to reveal functional conservation. In our case, expressing human RNF213 in murine cells is merely a practical approach to a technical difficulty.

To what degree the findings reported in this paper are novel is up to debate. However, I would argue that the novelty of the findings should be reflected in the title of the paper. The current title of the paper is ‘Recognition of phylogenetically diverse pathogens through enzymatically amplified recruitment of RNF213.’ The title therefore re-states what has already been reported by several previous publications: previous work had already reported that RNF213 can recognize phylogenetically diverse pathogens (Gram-negative and Gram-positive bacteria, protozoan pathogens, and viruses); and previous work has also already shown that recognition of these pathogens requires enzymatic activity of RNF213. The title should be changed and reflect (a) novel finding(s) of the study.

The reviewer incorrectly states that previous publication have shown that RNF213 “… recognizes phylogenetically distant pathogens”, while instead it was only reported that RNF213 restricts a multitude of pathogens. The question whether RNF213 directly recognizes phylogenetic different pathogens or merely acts downstream of specific receptors has not been asked, yet is at the center of this manuscript.

The novelty of our manuscript therefore comes from addressing the question posed in the abstract “…How the E3 ubiquitin ligase RNF213 can respond to phylogenetically distant pathogens, including Gram-negative Salmonella, Gram-positive Listeria, and eukaryotic Toxoplasma, remains unknown.”

Along the same lines, the claim made in the rebuttal that “enzymatically driven cooperarative recruitment of RNF213 enables sensing of evolutionarily distant pathogens” is a novel finding is at the very least a vast exaggeration. First of all it has already been shown that RNF213 senses many phylogenetically diverse pathogens: the protozoan Toxoplasma (PMID: 38376248, PMID: 38147552, PMID: 36154443); Gram positive Listeria in the cytosol (PMID: 34599178, PMID:

34804992), gram-positive in a vacuole (PMID: 38147552), viruses (PMID: 33420513, PMID: 34599178, PMID: 36917666,), gram-negative in the cytosol (PMID: 34012115), Gram-negative in a vacuoles (PMID: 36084633).

The reviewer again claims that previous publication have shown RNF213 '…senses phylogenetically diverse pathogens.' This statement is not correct because previous publications have merely shown restriction by RNF213 without addressing how pathogens are detected, i.e. whether RNF213 is a pattern recognition receptor of unprecedentedly broad reactivity or acts as an effector molecule downstream of hypothetical pattern recognition receptors.

It has also already been shown that sensing requires RNF213 enzymatic activity in the case of Toxoplasma (PMID: 38147552) and gram-negatives (PMID: 34012115 – this group's own work). Time-lapse microscopy data (which is the only data underlying the claim of 'cooperative recruitment') similar to what is shown in Fig.2 of this manuscript has also already been reported (see figure 6 of PMID: 38147552). And time lapse microscopy for RNF213 binding to cytosolic Salmonella was also already published by this group and similar conclusions were already made – to quote from Otten et al (PMID: 34012115) "Instant structured illumination microscopy (iSIM) revealed that RNF213 recruitment started focally and subsequently spread around the bacterium, indicating cooperative behaviour (Video1, Fig.4c)." Just calling the same thing 'cooperarative recruitment' instead of ' cooperative behaviour' doesn't make it novel. Yes, the current study provides a more detailed analysis of the video microscopy data but does not reveal fundamentally novel insights.

In Otten et al (Nature 2021) we reported on the cooperative recruitment of RNF213 to Salmonella. Subsequently it became clear that other phylogenetically distant pathogens are also restricted by RNF213 (many references in our manuscript), suggesting that if RNF213 was a PRR, it would be of unprecedently broad reactivity. An urgent need therefore exists to investigate how phylogenetically distant pathogens might be recognized by one single receptor. Answering this question requires comparison of multiple pathogens side-by-side, which is what we have done in this study and which has never been done before. The reviewer's suggestion that we merely renamed 'cooperative behaviour' into 'cooperative recruitment' is therefore incorrect. Instead we propose that recognition of all pathogens under study by RNF213 occurs in two steps, a rate-limiting initiation step that is not diffusion controlled and subsequent cooperative incorporation of further RNF213 molecules for multi-directional growth of the coat, for which we provide a detailed quantitative analysis.

I am not saying that everything reported in the current manuscript is already known. However, as stated in my previous critique of the paper, the genuinely novel finding of the original version of paper – in my view - is the identification of signatures of positive selection in human RNF213. While this is an exciting starting point for further exploration, the paper lacks any experimental evidence demonstrating that any of these signatures are functionally important. This type of functional analysis is typically expected of a high impact paper in this field. The revisions do not provide any new data demonstrating functional relevance and therefore the computational analysis on its own is only an incremental advance.

We disagree with the reviewer's opinion on novelty. Although not all questions have been answered, progress has been made. While the reviewer claims that the identification of positive selection in RNF213 is the only novel finding, we think that the following points are novel (and worth reporting):

- enzymatically driven cooperative recruitment of RNF213 enables sensing of evolutionarily distant pathogens
- a detailed kinetic analysis of RNF213 coat formation, providing time constants for coat initiation (T=27±1min) and coat expansion (5.0±2.4min) (new data in Fig.2C-F and supplementary). The difference in the time constant of coat initiation and the time required for coat expansion explains why coats on T. gondii vacuoles are initiated from a single focus; rarity of coat initiation and rapid coat completion limit the likelihood of more than one initiation event occurring per vacuole
- identification of a cluster of fast evolving residues near the N-terminus of RNF213, indicative of PRR function for RNF213
- a novel cryo-EM structure of human RNF213, revealing a carbohydrate-binding domain in the previously unresolved N-terminus that encompasses the aforementioned cluster of positively selected residues and strongly supports the proposed PRR function of RNF213 (new data in Fig.3)
- a cryo-EM structure and AlphaFold model of the RZ finger (new data in Fig.4C), supported by mutational analysis, offering mechanistic insight into the catalytic mechanism of the novel RZ-family class of E3 ligases.

Similarly, new data incorporated into the revised manuscript reveals the presence of a domain with structural similarity to the CMB20 carbohydrate-binding module of a fungal glucosamylase. This is an intriguing observation. However, the paper does not validate that the CMB20-like domain in RNF213 has any carbohydrate-binding activity or identifies any ligand – microbial or otherwise. The conclusions drawn by the authors are not substantiated by any experimental follow-up studies

With the possible exception of LPS, no ligand for RNF213 has been identified so far in any study and for any pathogen. Identifying the CBM20 domain in RNF213 as a hotspot of positive selection (and therefore likely receptor domain) should hence be considered significant progress.

please provide the sequence of the Rnf213 KO allele in MEFs. What was the sgRNA used to make the KO? Is it possible splice variants are still being expressed that may have functional effects?

The MEFs used here carry frameshifts in exon 31. If exon skipping occurs, multiple exons (i.e. exons 31-35) would need to be skipped to maintain an open reading frame. We therefore consider the existence of functional splice variants unlikely.

An additional comments regarding the authors' response:
- The authors argue that they cannot monitor restriction of Toxoplasma by DOX-inducible RNF213 because the experiments last for 10 – 15 days and DOX over this timeframe causes toxicity. However, RNF213 mediated host defense can be assessed by quantifying parasites per vacuole by IF at 24 hours post infection (see PMID: 36154443). Therefore, using their DOX-inducible constructs to assess anti-parasitic effects is doable.

This comment refers back to old data no longer present in the manuscript.

Reviewer #3 (Remarks to the Author):

I very much appreciate the authors' efforts to address my comments experimentally. The novel data, in particular the quantitative data on RNF213 coat formation on T.gondii vacuoles and the cryoEM structure of human RNF213 with CBM20 domain, significantly strengthen the manuscript which can now be accepted for publication. As a final remark: you might want to mention the new cryoEM structure explicitly in the abstract. Congratulations on the outstanding work - Francis Impens.

We are delighted that Reviewer 3 supports publication of our manuscript, which he/she considers significantly strengthened through the inclusion of the newly solved cryo-EM structure of human RNF213 and the addition of quantitative data on RNF213 coat formation.

Reviewer #4 (Remarks to the Author):

The revised manuscript presents two major updates: 1) quantitative analysis of RNF213 targeting and amplifying on the Toxoplasma vacuole-containing member (new Figure 2 C-F); 2) cryo-EM data on human RNF213 revealing a CBM domain in the N-terminus.

We are happy that the newly added quantitative analysis of RNF213 coat formation and the newly added high-resolution cryo-EM structure of RNF213 leading to the discovery of the previously unknown CBM20 domain in RNF213 are recognized by Reviewer 4 as 'major updates'.

While the current version of the manuscript resolves some of my previous concerns, there are still major issues that must be addressed, rendering it unsuitable for publication in its present form. Detailed comments are listed below:

1. Although the authors have determined the structure of human RNF213 and identified the CBM domain in the previously unresolved N-terminus region, their argument that RNF213 acts as a bona fide PRR serving as a bona fide PRR because of the positive selection in the CBM domain is too speculated and requires experimental evidence.

We agree with the reviewer that strong circumstantial evidence from evolutionary analysis does not provide direct proof of RNF213 serving PRR function. We therefore avoided any overstatements in our manuscript by merely concluding that our data are '…consistent with' RNF213 serving as a PRR.

Is there any prior study that suggests CBM is involved in pathogen recognition?

We thank the reviewer for asking about the CBM20 domain. We now realize that we may not have sufficiently explained why strong positive selection in a carbohydrate binding domain adds to the likelihood of RNF213 serving PRR function: a) pathogen detection through carbohydrate binding domains is a well-established concept and of particular appeal in the otherwise carbohydrate-devoid cytosol and b) the notorious diversity of microbial carbohydrates fits well with the pronounced positive selection observed in the CBM20 domain of RNF213.

In addition, since the truncation of the entire N-terminus (N586) leads to aggregation, it suggests CBM domain may serve as a signal anchor for correct trafficking of RNF213 or maintaining the oligomerization status of RNF213 rather than substrate binding. To support the current conclusion, experiments with point mutations in the CBM domain need to be carried out.

To address the reviewers request, we mutated residues in the CBM20 domain likely involved in carbohydrate recognition (F391, F401, W413, H421, Y422, Y462). However, we observed that mutant proteins were still able to detect pathogens (new data, Appendix Fig.S1), an ambiguous result that, unfortunately, neither proves nor refutes a role of the CBM20 domain in pathogen recognition. Further studies on the role of the CBM20 domain will be needed to establish its precise function.

2. Based on the authors' responses to all reviewers, it appears that the focus of this manuscript is on RNF213-mediated pathogen recognition, which has led to the omission of the Toxoplasma plaque assay data. Nonetheless, if the authors assert that RNF213 is a bona fide PRR, it remains unclear how exactly RNF213 recognizes the evolutionally distinct pathogens. Moreover, new Figures 2C to 2F indicate that the initiation of RNF213 recruitment and the amplification of the coating signal are two separate molecular events. Since the experiments examining RNF213 mutations were all conducted at a single time point, it is difficult to determine if these mutants have defects in pathogen recognition or in the amplification of RNF213 coating signal.

We thank the reviewer for the above succinct summary. Initiation of RNF213 recruitment and the enzymatic amplification of the RNF213 coat are indeed two separate events – the former relies on rate-limiting initiation events, while the latter requires the cooperative incorporation of further RNF213 molecules. However, as can be concluded from the kinetic analysis of RNF213 recruitment to Toxoplasma (Fig.2), defects in either pathogen recognition or subsequent incorporation of RNF213 monomers into the coat will result in the absence of RNF213 coats. We therefore think that the reviewer's suggestion of analyzing multiple time points with established methodology is unlikely to provide further mechanistic insights

Minor comments:
1. I have come to realize that the cells used in this study express RNF213 under doxycycline-induced conditions. Given this, performing all experiments on IFNγ-stimulated cells is misleading. Since IFNγ is known to induce the expression of RNF213 in human cells, as shown in PMID 36154443 and PMID38147552, the effect of IFNγ induction is not evident in this study (although the current work mainly carried out in murine fibroblasts). So, I suggest removing all data related to IFNγ-stimulated cells from this manuscript.

IFNg is known to affect cells in many aspects, not only by controling RNF213 levels. Regarding the results reported here, there seems to be a misunderstanding – most experiments were not performed in the presence of IFNg (for example, those in Fig.2, 3, 4, or 5), while experiments involving IFNg (Fig.1B, E) also contain conditions without cytokine treatment to enable direct comparison.

2. Figure 2D, please clarify whether a synchronized invasion assay was performed (see PMID 20435700 as an example). If not, the observed difference in RNF213 coat formation among individual parasites could be attributed to variations in the timing of parasite invasion. The time frame for Toxoplasma to invade host cells can range from 10 minutes to 2 hours after the parasites are introduced to the cells.

Synchronized spin infections were performed. To further clarify the method, the section "Life-cell microscopy" in Materials & Methods has been updated. We thank the reviewer for pointing out the ambiguity in the previous version of our manuscript.

2. Figure 3A and 3B appear identical, with the only difference being the CBM labeled in Figure 3B. It would be nice to combine them into one figure.
3.

We deliberately created two cartoons as the panels have distinct purposes. Fig3A displays sites of positive selection, mapped onto the domain structure of RNF213 as it was known before our study, thus highlighting the need for further structural analysis. Fig3B is supposed to guide the reader in understanding Fig.3C-G.

Dear Dr. Randow,

Thank you for the submission of your further revised manuscript to our editorial offices. I now went through your final p-b-p-response and the revised manuscript and consider the remaining points of referees #2 and #4 as adequately addressed.

Before we can proceed with formal acceptance, I have these final editorial requests:

Moreover, I have these editorial requests:

- Please add the word 'Abstract' above the abstract.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams. Could statistics also be provided for the diagrams in panels 1B and 1E? Moreover:
- lease show the stats for all diagrams as in panel 1B (with a line indicating which datasets have been tested).
- Please provide exact p values in the legends of figures 1d; 3h-j; 4a-b, d-f.
- Please note that in figure 3i; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
- Please note that information related to n is missing in the legend of figure 2e.
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Best,

Achim Breiling
Senior Editor
EMBO Reports

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision **20th Sep 2024**

Dr. Felix Randow
MRC Laboratory of Molecular Biology
Division of Protein and Nucleic Acid Chemistry
Francis Crick Avenue
Cambridge CB2 0QH
United Kingdom

Dear Dr. Randow,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Yours sincerely,

Achim Breiling
Senior Editor
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The data shown in figures should satisfy the following conditions:
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