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Spatial control of nucleoporin condensation by Fragile X-related proteins

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1st Editorial Decision 14th Apr 2020

Dear Izabela,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments below the referees find the analysis interesting, but also finds that further work is needed to support the reported conclusions. Should you be able to extend the findings then I would like to invite you to submit a revised manuscript.

I think it would be good to discuss the raised points further and that it would be helpful to do so via phone or skype. I will contact you in the next few days to discuss this further. I am also aware that with the current Covid-19 situation and lab closures that carrying out experimental revisions is not so straightforward. We can discuss this further in the call.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
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I thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further with you

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
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Referee #1:

In this manuscript, Sumara and colleagues describe a potential function of Fragile X-related proteins in the biogenesis of nuclear pore complexes.
Overall this paper contains a large number of results and reports interesting observations that should spark interest in the nuclear pore field and might contribute to our understanding of the Fragile X syndrome. Therefore, this paper should be published in EMBO J. However, on multiple occasions the results are overinterpreted and do not directly address the mechanism of FXR function. In my view, the authors cannot address these mechanistic questions (and therefore they should not address them at this stage) as this would go beyond the current scope of this paper. However, the manuscript needs to be revised to address some experimental shortcomings and the results need to be more carefully interpreted.

Specific points:
(1) The weakest part of the manuscript is the evidence for an interaction between FXR1 and various Nups. Some of the IP results shown in Figure 1 (pull-down followed by Western blot) show a modest enrichment at best (this also goes for Fig 5a). This figure should be quantified and some negative controls (with non-Nups) need to be included. For example, does the GFP-FXR1 'bind' to other cytoplasmic or nuclear proteins in this Western blot. Likewise, more details need to be provided on the mass-spec results. What controls were used and how were significant peptide hits identified? How does the Nup 'hits' compare to other proteins in the IP, and is their enrichment significant (also relative to protein length and overall protein abundance)?
(2) The authors check protein levels of multiple Nups upon FXR1 knock-down and find them unaltered. Yet, they cannot exclude that any other NupX (which might be specifically important for NPC assembly) is down-regulated. In general, since FXRs bind RNA cannot be excluded that gene

expression of some critical NPC biogenesis factor is affected, which then triggers the observed phenotypes. While experiments to address this (e.g., proteome-wide protein abundance analysis, or transcriptomics) are likely beyond the scope of the current manuscript, that needs to be discussed and the results need to be more cautiously interpreted.
(3) The authors state in the abstract: "in the cytoplasm, a large excess of soluble Nups exists". How do we know this? Very little is known about NPC biogenesis and its timing, and the size of potential soluble precursor pools have not been assessed to my knowledge. Some cells have large pools of annulate lamellae but they are lacking in other cell types, and ALs are not 'soluble' as they are membrane embedded.
(4) The authors state "our analysis demonstrates that FXR proteins can act as molecular chaperones". I would disagree. Even if they were to function directly (see also point 2), FXRs could function as NPC biogenesis factors. The authors are certainly free to discuss this but it is definitely not 'demonstrated' in the current manuscript.
(5) Line 312 typo: should be 'nucleocytoplasmic'.

Referee #2:

This study makes the unique observation that the Fragile X family of proteins, which contains FMRP, FXR1 and FXR2, form a complex that associates with nuclear porins (Nups), functioning as a molecular chaperone to facilitate dispersal of Nups and the reversal of cytoplasmic Nup assemblies. In the absence of any of the Fragile X family members, the percentage of cells containing these cytoplasmic Nup assemblies increases. It is interesting to note that these Cytoplasmic Nucleoporin Granules (CNGs), as the authors name the Nup assemblies, are normally present in a low percentage of cells but then that percentage significantly increases in in the absence of any of the Fragile X family members, dynein, or BICD2. The significance of these CNGs is important to establish, as loss of either FMRP or FXR2 is still viable in mice and humans, in the case of Fragile X syndrome for FMRP. Thus, cell division and function are not lethally impaired. The authors provide evidence that there is a transient defect in protein export from the nucleus during early G1 when FXR1 is reduced. This small decrease could affect cellular homeostasis and asymmetric cell division. In brain, this would only seem to be an issue in dividing neural precursor cells. In post-mitotic neurons, the CNGs could potentially exert cytotoxic effects by sequestering factors.

This story is impressively rendered with clear and compelling images-particularly the presence of the fragile X family members in the nuclear envelope and characterization of the CNGs formed.

Major points.
1. It is critical that the scoring of cells containing CNGs was done by an experimenter blinded to the treatment conditions-especially since control cells have a low level of CNGs. I may have missed it but I did not see this explicitly stated in the Materials and Methods.
2. If the Fragile X family of proteins are acting like chaperones for the Nups, wouldn't one expect to see them consistently present in the NCGs induced in the absence of dynein or BICD2 in Figure 5B or 5E? I realize that colocalization of FMRP with CNGs is shown in Figure 7G and FXR1 with CNGs in Fig. 1E but there does not appear to be colocalization of FXR1with CNGs in the experiments in Figure 5. Was this examined?
Minor points
1. In Figure 1, why wasn't Nup 188 confirmed in the co-IP?
2. In Figure 1D, why is there no Lamin A staining in the digitonin treatment but Lamin A is present in the triton/SDS?

3. I am confused by the description of Figure 3 in the Results compared to what is shown in the Figure itself. In the Results, the GFP-Nup85 positive granules are described as detectable at 48 minutes after chromosome segregation and 30 minutes after decondensation (pg. 7 lines 163 and 164). But it is unclear how that corresponds to granules being present starting at 76 minutes, indicated by arrows in Figure 3A. Are these the granules the ones referred to in the Results? Put another way, what is the relationship between the 48 minutes and 30 minutes referred to in the Results and the 76 minutes shown in Figure 3A?
4. In the context of this study, it is unclear what process 1,6-Hexanediol is being used to disrupt. One or 2 sentences of introduction to this approach is needed on pg. 9 line 204. Similarly, if FXR1 has a low complexity domain that should be stated and referenced.
5. In the Figure legend for 8a, the arrow heads should be described after "a" in addition to after "g".
6. What is the difference between "blebbed" and "herniated" nuclei (line 570, pg.31)? Please also reference these characterizations.
7. Figure 8b.Why is the GFP-FXR1P band large and smeary? Presumably there are not multiple isoforms. Is this caused by post-translational modifications?
8. Figure 8e. Why does loss of FXR2 have less of an effect than the other 2 family members?
9. Figure 8g. Is the magnification the same between the control and the FXR1 siRNA treated cells? I realize that there is a scale bar in the top left panel but I do not see one in the first panel of the FXR1 knockdown series. The nuclei of the FXR1 siRNA-treated cells look bigger. It is important that the scale be the same because the nuclear problems are rather subtle in the FXR1 siRNA treatment so the control must be the same magnification to show that the nuclei are distinctly different under the two different conditions.
10. Please correct "IP" cells to "IPS" cells in line 356 pg. 15

Referee #3:

In the manuscript "Spatial control of nucleoporin condensation by Fragile X-related proteins" Agote-Aran et al show that Fragile X-related protein 1 (FXR-1) interacts with several nucleoporins, which are components of nuclear pore complexes (NPCs). Starting from this point they show that downregulation of FXR-1 and orthologues (FXR-2 and FMRP) induces in tissue culture cells cytoplasmic accumulations of various nucleoporins in dot-like structures and misshaped nuclei. Interestingly, downregulation of the FXR-1 interacting components of the dynactin complex induces a similar phenotype. The authors propose that FXR-1, FXR-2 and FMRP together with the dynactin complex is needed for microtubule mediated transport of cytoplasmic nucleoporins towards the nuclear envelope for NPC assembly in interphase.
The manuscript presents a huge body of data of very high quality including rescue experiments and cells derived from Fragile X-syndrome patients lacking functional FXR-1, which are all consistent. The main criticism is that the manuscript does not show that interphase NPC assembly is indeed affected. More specific assays would be required for this, e.g. counting NPC numbers (see e.g. Souquet et al, Cell Rep. 23:2443-2454) or cell fusion assays (Funakoshi et al, Mol Biol Cell 22:1058-69). Given the irregular shaped nuclei upon FXR-1-loss the relative mild loss of nucleoporin signal at the nuclear envelope (Fig. 2) does not prove a defect in NPC assembly and cannot assign the mode (mitotic vs. interphase) affected. Please note that the fact that ELYS (required for mitotic NPC assembly) is not localized to the cytoplasmic nucleoporins dots in contrast to pom121 (required for interphase NPC assembly) is not a strong argument for interphase NPC assembly affected by FXR-1 downregulation (line 150ff) as Nup153 (required for interphase NPC assembly) is also not found there.

Other specific points:
1.) It is not clear why the authors use different markers for cytoplasmic nucleoporin accumulations in the different experiments (mAB414, EGFP-Nup85, GFP-Nup107) in the different assays through the manuscript. Is the variability in the phenotypic strength observed upon FXR-1 downregulation caused by this (e.g. between Fig. 2 and 3). This change in readouts complicates the comparison of the different experiments also employing the dynactin complex.
2.) It is also not obvious why the mode of quantitation is changed in Figure 7a (from % of cells with cytoplasmic foci to cytoplasmic Nup intensity). Please avoid the normalization of the FSX-IPSC to 1 in figure 7f. Rather give % of cells with cytoplasmic foci as before.
3.) Lines 129 to 132: "as downregulated… telophase cells": This statement appears arguable since the images shown in figure S2 exhibit intranuclear foci of lamin A, emerin and Lap2B for interphase cells in the FRX1KD condition. Moreover, the nuclear rim staining of Lap2B seems also affected and the authors show a small, yet significant, difference between Lap2B signal in WT and KD FXR1 conditions. As it is an important point for the authors demonstration and should be made clearer. In general, I would recommend to introduce the misshaped nuclear envelope phenotype earlier in the manuscript.
4.) Line 374 to 376: "Downregulation… nuclear lamina": In line with the comment above, the pictures in figure S2 do not show a normal staining of lamin A and Lap2B.
5.) Line 395-397: "Our model… cell cycle": If FXR proteins act as molecular linker between cytoplasmic nucleoporins and the dyneien.BICD2 complex, one expects the nucleoporin granules in dynein KD containing FXR1, which is not the case in figure 5b. The authors should comment on this.
6.) Figure 6c: The data indicating the dependence of granule fusion on dynein needs to be substantiated by a quantitation.
7.) Lines 105 and Supp Table 1: Supp table 1 looks like a subset of data and not the entire set of Mass spec. If so, it should be stated in the legend.
8.) Lines 156-157: "FXR1… segregation.": Possibly break down the different mitotic phases in a supplementary figure to really prove that all mitotic phases are unaffected?
9.) Lines 162-164 and and Figure 3: "GFP-Nup85… decondensation": Given that about 20% cells show Nup85 granules in the Fig. 3b, the accumulation of granule in the control siRNA condition should be plotted in the figure 3c.
10.) Line 204 and Figure 4: The hexandiol treatment is approximately twice as long and twice more concentrated than the cited paper (5%, 30 sec max). Should we expect this blurring of GFP-Nup133 with hexandiol treatment as it belongs to the Y complex and it is not a FG-Nups? Is it possible to label other kind of protein aggregates (which would not be phase separation aggregates) as negative control?
11.) Line 240-241 and Figure 6: "we first… reversible way": Given that Nocodazole treatment in figure 6 and S9 appear the same in the Material and Method (10µM for 90 min), why are 80% of cells showing nucleoporin dots figure S9, while 50% of cells show such granules in the figure 6. Drugs concentration and incubation time could appear in the figure or in the figure legend for an easier understanding of the experiment.
12.) Line 249-250 and Figure 6: "GFP-Nup107… all conditions": Figure 5 shows interaction between FXR1 and Nup133 while figure 6 shows how Nup107 behave after Nocodazole release. Does Nup107 also bind FXR1? If so, it should be introduce in figure 5 along Nup133 binding.
13.) Line 266 and Fig. S10c: Short exposure (SE) and long exposure (LE) are not written in the legend.
14.) Line 280 and Figure 7g: "Re-expressed… Nup133": It is not clear to me why the authors show FMRP and Nup133 colocalization in such a complicated rescue setting. Can this be seen in HeLa/U2OS cells?
15.) Line 302-306: "Live video… cytoplasm": A time course graph similar to Fig. 3c would help

supporting this statement.
16.) Line 308-310: "Our data... early G1": This statement on impaired nuclear architecture after FRX1-dynein pathway disruption seems contradictory with the statement line 129-132 saying that the lamins are not affected by FXR KD.
17.) Line 450: It should be discussed how the nuclear area can be slightly, yet significantly, bigger in FXR1 KD cells (Fig. 8c) while the import and export mechanisms are overall not affected, Are the 30 min slower export in early G1 enough to explain this difference?
18.) For all figures: The statistical significance of the asterisks should be written.
19.) A few spelling mistakes: Change "reates" and "nucleaocytoplasmic (line 314), y- axis figure 9a
20.) Please be consistent in naming, use either GP210 or Nup210
21.) NPC scheme in figure 2: the inner ring is usually referred to as the NPC part formed by non-transmembrane nucleoporins sandwiched in between the two outer rings. The arrow rather points to what is sometimes referred to as "lumenal or membrane ring".

Referee #1:

In this manuscript, Sumara and colleagues describe a potential function of Fragile X-related proteins in the biogenesis of nuclear pore complexes.
Overall this paper contains a large number of results and reports interesting observations that should spark interest in the nuclear pore field and might contribute to our understanding of the Fragile X syndrome. Therefore, this paper should be published in EMBO J. However, on multiple occasions the results are overinterpreted and do not directly address the mechanism of FXR function. In my view, the authors cannot address these mechanistic questions (and therefore they should not address them at this stage) as this would go beyond the current scope of this paper. However, the manuscript needs to be revised to address some experimental shortcomings and the results need to be more carefully interpreted.

We thank reviewer 1 for his/her enthusiastic support of our study and for recognizing the significance and novelty of our findings to the nuclear pore field and its importance in the context of human disease. We also would like to thank for the helpful suggestions and comments, which clearly improved the manuscript.

We have extensively worked on the manuscript to address some experimental shortcomings and overinterpretation of our data as summarized below.

Specific points:
(1) The weakest part of the manuscript is the evidence for an interaction between FXR1 and various Nups. Some of the IP results shown in Figure 1 (pull-down followed by Western blot) show a modest enrichment at best (this also goes for Fig 5a). This figure should be quantified and some negative controls (with non-Nups) need to be included. For example, does the GFP-FXR1 'bind' to other cytoplasmic or nuclear proteins in this Western blot. Likewise, more details need to be provided on the mass-spec results. What controls were used and how were significant peptide hits identified? How does the Nup 'hits' compare to other proteins in the IP, and is their enrichment significant (also relative to protein length and overall protein abundance)?

We agree with reviewer 1 that the evidence for an interaction between FXR1 and various Nups could be improved. However, Nups are very “sticky” proteins and it is indeed very difficult to get rid of some small remaining background in the negative control IPs. Based on our data, we also predict that this interaction has a transient character and solely a small fraction of Nups interact with FXR1 at a given time point.

Nevertheless, we have meanwhile optimized the IP protocol (Fig 1 below) with various stringent washing conditions and this new data make us feel very confident about the interaction between FXR1 and Nups presented in the manuscript. Unfortunately, the figure is not yet of sufficient quality due to undetected bands in the inputs so that it can be included in the revised version of the manuscript and due to the current pandemic situation, we will not be able to repeat these experiments in the upcoming weeks.

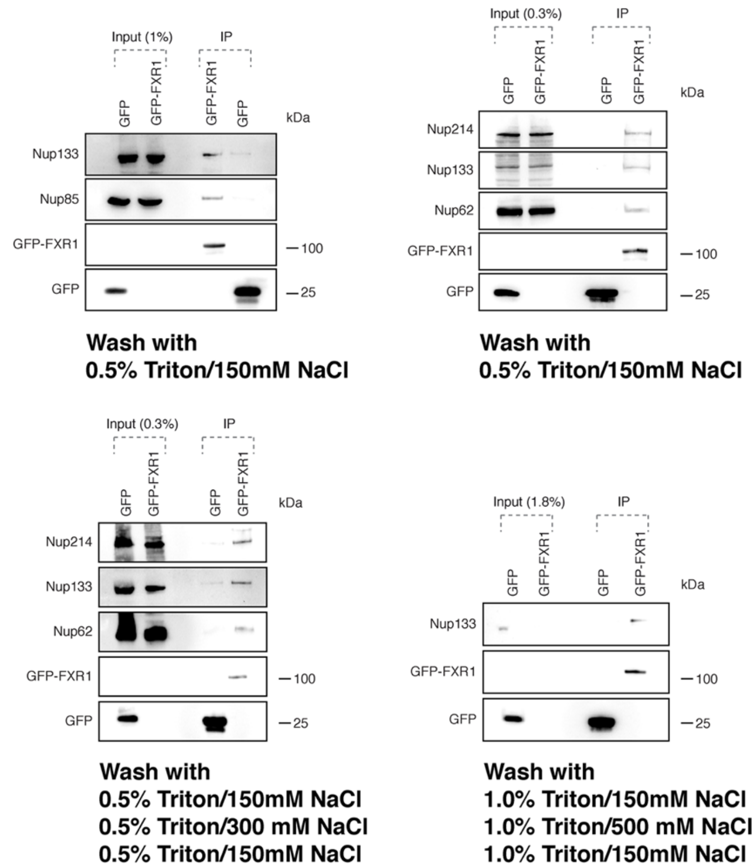

Figure 1 – Optimization of the GFP-FXR1 immunoprecipitation protocols. *Lysates of HeLa cells stably expressing GFP alone or GFP-FXR1 were subjected to immunoprecipitation using GFP-Trap beads (GFP-IP), analysed by Western blot. Examples of experiments using various washing conditions (as indicated) are shown.*

As suggested by reviewer, we have therefore quantified the data from the experiments already presented in the manuscript (3 replicates each) illustrated by the representative examples in the Figures 1 and 6 and the average intensities and statistical significance is indicated below each IP panel in the revised manuscript version.

Additionally, Figure 6D shows the interaction of Nup133 and BICD2 with GFP-FXR1, where other cytoplasmic proteins (Mitosin and HOOK3) were used as negative controls and confirmed by the quantifications.

For the mass spectrometry data, HeLa Kyoto cells stably expressing GFP were used as negative controls of the GFP-FXR1 stable cell line. We have provided the complete mass spectrometry data set including the quantifications based on the following parameters: Σcoverage = total coverage of the indicated protein in all the experiment; Σ#proteins = the number of proteins that could correspond to the identified peptides; Σ#Unique peptide = the number of peptides that are found exclusively in the hit protein; Σ#peptides = the number of peptides found corresponding to the hit protein; Σ#PSM = the number of peptides detected for a hit protein; and the “score” representing an arbitrary quantification calculated by

integration of the coverage, the size of the protein and the PSM. The score was used to discriminate the hits in addition to the number of unique peptides and hits were considered significant if three or more peptides were unique for the GFP-FXR1 immunoprecipitations.

We have provided this detailed information as new Table EV1 and in the corresponding legend of the revised manuscript.

(2) The authors check protein levels of multiple Nups upon FXR1 knock-down and find them unaltered. Yet, they cannot exclude that any other NupX (which might be specifically important for NPC assembly) is down-regulated. In general, since FXRs bind RNA cannot be excluded that gene expression of some critical NPC biogenesis factor is affected, which then triggers the observed phenotypes. While experiments to address this (e.g., proteome-wide protein abundance analysis, or transcriptomics) are likely beyond the scope of the current manuscript, that needs to be discussed and the results need to be more cautiously interpreted.

We completely agree with this point. We cannot exclude the possibility that other Nups' expression is not changed by FXR1 silencing therefore affecting the Nup soluble balance and presumably NPC biogenesis.

We have discussed this point carefully and interpreted our results with caution throughout the revised manuscript.

(3) The authors state in the abstract: "in the cytoplasm, a large excess of soluble Nups exists". How do we know this? Very little is known about NPC biogenesis and its timing, and the size of potential soluble precursor pools have not been assessed to my knowledge. Some cells have large pools of annulate lamellae but they are lacking in other cell types, and ALs are not 'soluble' as they are membrane embedded.

Based on the study by Onischenko and colleagues (Onischenko et al. 2004) in which the cellular sub-fractionation was used to determine the abundance of different Nups in different cellular compartments in Drosophila embryos, the major fraction of Nups is soluble. Although we realize that the differentiated cells used in our study cannot be directly compared to fly embryos, the process of Nup translation in the cytoplasm must produce soluble Nup proteins before they assemble either into NPCs or to ALs in some cell types. We believe, that due to their intrinsic ability to undergo phase separation, Nups would be susceptible to aggregate before they reach their final destinations even if they are not in excess.

We have carefully rephrased the statements about the abundance of soluble Nup pool in the revised manuscript.

(4) The authors state "our analysis demonstrates that FXR proteins can act as molecular chaperones". I would disagree. Even if they were to function directly (see also point 2), FXRs

could function as NPC biogenesis factors. The authors are certainly free to discuss this but it is definitely not 'demonstrated' in the current manuscript.

We agree that based on the available data, it cannot be ruled out that FXR proteins regulate the translation of a specific Nup not yet identified in this study, which could alter the NPC biogenesis. We have broadened our discussion to better present different possible interpretations of our observations.

(5) Line 312 typo: should be 'nucleocytoplasmic'.

Thank you, we have corrected the typo.

Referee #2:

This study makes the unique observation that the Fragile X family of proteins, which contains FMRP, FXR1 and FXR2, form a complex that associates with nuclear porins (Nups), functioning as a molecular chaperone to facilitate dispersal of Nups and the reversal of cytoplasmic Nup assemblies. In the absence of any of the Fragile X family members, the percentage of cells containing these cytoplasmic Nup assemblies increases. It is interesting to note that these Cytoplasmic Nucleoporin Granules (CNGs), as the authors name the Nup assemblies, are normally present in a low percentage of cells but then that percentage significantly increases in in the absence of any of the Fragile X family members, dynein, or BICD2. The significance of these CNGs is important to establish, as loss of either FMRP or FXR2 is still viable in mice and humans, in the case of Fragile X syndrome for FMRP. Thus, cell division and function are not lethally impaired. The authors provide evidence that there is a transient defect in protein export from the nucleus during early G1 when FXR1 is reduced. This small decrease could affect cellular homeostasis and asymmetric cell division. In brain, this would only seem to be an issue in dividing neural precursor cells. In post-mitotic neurons, the CNGs could potentially exert cytotoxic effects by sequestering factors.

This story is impressively rendered with clear and compelling images-particularly the presence of the fragile X family members in the nuclear envelope and characterization of the CNGs formed.

We thank reviewer 2 for his/her appreciation of our study and quality of the data as well as enthusiastic support of our concept. We also would like to thank for the helpful suggestions and comments, which clearly improved the manuscript.

Major points.
1. It is critical that the scoring of cells containing CNGs was done by an experimenter blinded to the treatment conditions-especially since control cells have a low level of CNGs. I may have missed it but I did not see this explicitly stated in the Materials and Methods.

Three different experimenters involved in the study performed the quantifications of the CNGs (or other phenotypes) in a strictly double-blinded manner.

We apologize for not having included this note in the Materials and Methods section and we have corrected this error in the revised manuscript.

2. If the Fragile X family of proteins are acting like chaperones for the Nups, wouldn't one expect to see them consistently present in the NCGs induced in the absence of dynein or BICD2 in Figure 5B or 5E? I realize that colocalization of FMRP with CNGs is shown in Figure 7G and FXR1 with CNGs in Fig. 1E but there does not appear to be colocalization of FXR1with CNGs in the experiments in Figure 5. Was this examined?

We thank reviewer for this comment. Indeed, the co-localization of FXRPs and Nups is difficult to observe in the cytoplasm except for the iPSCs where their co-localization was clear especially in the surroundings of the nucleus probably due to the lower levels of FMRP protein re-expression in the rescue system (Xie et al. 2016) which could result in a slowdown of the process. As illustrated in the Figure 6B, E, we unfortunately did not observe an increase in co-localization of the double complex (FXR-Nups) in the absence of dynein/BICD2. We predict that either all three components are needed to form the transport complexes in the cytoplasm or that the formation of the FXRP-Nup complex is very transient and is needed for the transport of soluble Nups which are harder to visualize in the cytoplasm. The CNGs that we observe would be the result of the absence of this transport mechanism and the consequent local increase of Nups leading to aberrant formation of bigger (easy to visualize) Nup granules that do not necessarily contain FXR1.

We have carefully discussed this data and possible interpretations in the revised version of the manuscript.

Minor points
1. In Figure 1, why wasn't Nup 188 confirmed in the co-IP?

Unfortunately, we were unable obtain a working antibody for detection of Nup188 by Western blotting.

2. In Figure 1D, why is there no Lamin A staining in the digitonin treatment but Lamin A is present in the triton/SDS?

We apologize if we did not explain this panel with sufficient precision which could have led to a misunderstanding. We have therefore re-written this part of the text so that this result is presented properly.

The aim of the experiment shown in Figure 1D was to further characterize the FXR1 localization at the NE and to understand if it localized to the nuclear or to the cytoplasmic side of the NE. For this purpose, we compared two different permeabilization methods. The triton/SDS can permeabilize the plasma membrane and the nuclear envelope allowing for the antibodies to reach the epitope in any of these two compartments (FXR1 and Lamin A). The second permeabilization protocol uses low concentration of digitonin which can only permeabilize the plasma membrane but not the nuclear envelope. In this case, if the epitope of the FXR1 antibody is located exclusively on the nuclear side of the nuclear envelope (as it is the case for Lamin A), the antibody will not be able to reach it and the signal will be lost. Thus, the Lamin A signal is absent as expected under these experimental conditions. We observed the nuclear envelope enrichment of FXR1 using the digitonin permeabilization method, suggesting that FXR1 can localize to the cytoplasmic side or alternatively to both sides of the nuclear envelope.

3. I am confused by the description of Figure 3 in the Results compared to what is shown in the Figure itself. In the Results, the GFP-Nup85 positive granules are described as detectable at 48 minutes after chromosome segregation and 30 minutes after decondensation (pg. 7 lines 163 and 164). But it is unclear how that corresponds to granules being present starting at 76 minutes, indicated by arrows in Figure 3A. Are these the granules the ones referred to in the Results? Put another way, what is the relationship between the 48 minutes and 30 minutes referred to in the Results and the 76 minutes shown in Figure 3A?

We thank reviewer for noticing this error. We have modified the entire figure (now Figure 4) and included new data of a better quality obtained with the HeLa cells stably expressing GFP-Nup107 and with the corresponding quantifications. Better quality of the movies allowed us to more precisely quantify the appearance of CNGs in the FXR1-deficient cells (35 min after anaphase onset) but also in the control cells (44 min after anaphase onset) (Figure 4D). The timing of first CNGs observed strongly correlates with the nuclear blebbing seen in FXR1-downregulated cells (31 min after anaphase onset) (revised Figure 3J). This data fully support and extend our previous conclusions.

4. In the context of this study, it is unclear what process 1,6-Hexanediol is being used to disrupt. One or 2 sentences of introduction to this approach is needed on pg. 9 line 204. Similarly, if FXR1 has a low complexity domain that should be stated and referenced.

We have added the requested background information and the references to the revised manuscript as requested by reviewer.

Briefly: aliphatic alcohols like hexanediols are good solvents for FG-Nup hydrogels probably due to their ability to compete with the hydrophobic interactions between FG-repeats (Patel et al. 2007). In this study it was used to understand the nature of the CNGs and to understand if they could be disrupted by hexanediol as FG-Nup hydrogels are. The structure of a

recombinant peptide containing RGG domain of FMRP was studied using circular dichroism and NMR techniques. The study confirmed that the region containing the RGG box has a low-complexity sequence composition and is unfolded and flexible (Ramos 2003).

5. In the Figure legend for 8a, the arrow heads should be described after "a" in addition to after "g".

We appreciate the careful reading and we have changed the legend of the old Figure 8 (now presented as new Figure 3 as requested by reviewer 3) accordingly.

6. What is the difference between "blebbed" and "herniated" nuclei (line 570, pg.31)? Please also reference these characterizations.

Nuclear envelope herniations are understood as protrusions that extend into the cytosol, ranging in size from nanometer to micrometer scale. It is a broad term that does not indicate the specific morphology nor the causing underlying molecular mechanism. When these herniations are also visible by light microscopy they are often termed “blebs” (Thaller and Patrick Lusk 2018).

We have replaced all “herniation” terms in the revised manuscript with “bleb” to avoid reader’s confusion, since we can observe them by light microscopy.

7. Figure 8b.Why is the GFP-FXR1P band large and smeary? Presumably there are not multiple isoforms. Is this caused by post-translational modifications?

This slightly smeary pattern is most likely due to the quality variation of this particular SDS gel or blot transfer as it cannot be seen in other experiments. However, we believe that it does not dramatically change the main message of this experiment.

8. Figure 8e. Why does loss of FXR2 have less of an effect than the other 2 family members?

Thank you for this comment. We do not know why this is the case. Interestingly, it is known that FMRP is more similar to FXR1 than to FXR2 (86% identity between FMRP and FXR1P, and 70% identity between FMRP and FXR2P in the amino terminal region) (Hoogeveen, Willemsen, and Oostra 2002), which could suggest that they share more common functions.

We have briefly discussed this issue in the revised version of the manuscript.

9. Figure 8g. Is the magnification the same between the control and the FXR1 siRNA treated cells? I realize that there is a scale bar in the top left panel but I do not see one in the first

panel of the FXR1 knockdown series. The nuclei of the FXR1 siRNA-treated cells look bigger. It is important that the scale be the same because the nuclear problems are rather subtle in the FXR1 siRNA treatment so the control must be the same magnification to show that the nuclei are distinctly different under the two different conditions.

We have added the scale bar to the FXR1 series in the old Figure 8 (now 3) and we confirm that the same magnification is shown in both cases.

10. Please correct "IP" cells to "IPS" cells in line 356 pg. 15

We have corrected the typo, thank you.

Referee #3:

In the manuscript "Spatial control of nucleoporin condensation by Fragile X-related proteins" Agote-Aran et al show that Fragile X-related protein 1 (FXR-1) interacts with several nucleoporins, which are components of nuclear pore complexes (NPCs). Starting from this point they show that downregulation of FXR-1 and orthologues (FXR-2 and FMRP) induces in tissue culture cells cytoplasmic accumulations of various nucleoporins in dot-like structures and misshaped nuclei. Interestingly, downregulation of the FXR-1 interacting components of the dynactin complex induces a similar phenotype. The authors propose that FXR-1, FXR-2 and FMRP together with the dynactin complex is needed for microtubule mediated transport of cytoplasmic nucleoporins towards the nuclear envelope for NPC assembly in interphase.
The manuscript presents a huge body of data of very high quality including rescue experiments and cells derived from Fragile X-syndrome patients lacking functional FXR-1, which are all consistent. The main criticism is that the manuscript does not show that interphase NPC assembly is indeed affected. More specific assays would be required for this, e.g. counting NPC numbers (see e.g. Souquet et al, Cell Rep. 23:2443-2454) or cell fusion assays (Funakoshi et al, Mol Biol Cell 22:1058-69). Given the irregular shaped nuclei upon FXR-1-loss the relative mild loss of nucleoporin signal at the nuclear envelope (Fig. 2) does not prove a defect in NPC assembly and cannot assign the mode (mitotic vs. interphase) affected. Please note that the fact that ELYS (required for mitotic NPC assembly) is not localized to the cytoplasmic nucleoporins dots in contrast to pom121 (required for interphase NPC assembly) is not a strong argument for interphase NPC assembly affected by FXR-1 downregulation (line 150ff) as Nup153 (required for interphase NPC assembly) is also not found there.

We are grateful to reviewer 3 for his/her appreciation of a very high quality of our data. We also would like to thank for the helpful suggestions and comments, which have clearly improved the manuscript.

We agree with this reviewer that we did not formally demonstrated that the NPC assembly at NE is indeed affected by downregulation of FXR1 and what is the temporal classification of this pathway. Given our existing evidence that the NE Nup signal is only very moderately affected in the FXR1-deficient cells, we would not expect to observe huge differences in the numbers of NPCs using the suggested assays.

Therefore, we have tuned down our conclusions about NPC biogenesis and stated throughout the manuscript that our pathway may solely contribute to the existing NPC assembly pathways. We have additionally described the timing of the observed events (appearance of CNGs) without classifying the pathway as "interphase" assembly mode.

Other specific points:
1.) It is not clear why the authors use different markers for cytoplasmic nucleoporin accumulations in the different experiments (mAB414, EGFP-Nup85, GFP-Nup107) in the different assays through the manuscript. Is the variability in the phenotypic strength observed upon FXR-1 downregulation caused by this (e.g. between Fig. 2 and 3). This change in readouts complicates the comparison of the different experiments also employing the dynactin complex.

We used several antibodies to observe the Nup phenotypes at the endogenous levels whenever possible, which we thought would be more meaningful than using overexpressed GFP-Nup systems such as the GFP-Nup85, GFP-Nup133 or GFP-Nup107 cell lines, which were used for the live video experiments.

We have also used many different markers to observe co-localization of different Nups belonging to distinct NPC subcomplexes and sometimes combinations of the GFP-Nup expressing cells and endogenous Nups to validate the phenotype in the cell lines used for the live video experiments (please see Fig EV2F, EV7B, EV8). In addition to live video experiments, the GFP-Nup-107 cell line was also used for the mRNA FISH experiments due to compatibility issues with the fixation protocol which is not suitable for the endogenous Nup staining.

We believe that usage of all these markers showing overall the same phenotype strengthens the conclusions of our study.

2.) It is also not obvious why the mode of quantitation is changed in Figure 7a (from % of cells with cytoplasmic foci to cytoplasmic Nup intensity). Please avoid the normalization of the FSX-IPSC to 1 in figure 7f. Rather give % of cells with cytoplasmic foci as before.

We have presented the data in a more homogeneous manner as requested by reviewer. Old Figure 7C now new Figure 8C as well as 8F (iPSCs) show the percentage of cells with CNGs.

3.) Lines 129 to 132: "as downregulated... telophase cells": This statement appears arguable since the images shown in figure S2 exhibit intranuclear foci of lamin A, emerin and Lap2B for interphase cells in the FRX1KD condition. Moreover, the nuclear rim staining of Lap2B

seems also affected and the authors show a small, yet significant, difference between Lap2B signal in WT and KD FXR1 conditions. As it is an important point for the authors demonstration and should be made clearer. In general, I would recommend to introduce the misshaped nuclear envelope phenotype earlier in the manuscript.

We thank reviewer for this comment and we have now stated that the distribution of these NE markers is altered in the absence of FXR1 even though the recruitment per se appears mostly unchanged (with exception of Lap2B). We have also introduced the nuclear shape phenotype (old Figure 8) earlier as a new Figure 3 as requested. We believe that this change improved the flow of the manuscript.

4.) Line 374 to 376: "Downregulation... nuclear lamina": In line with the comment above, the pictures in figure S2 do not show a normal staining of lamin A and Lap2B.

We have corrected the text as explained above in response to the point 3 and stated that the nuclear lamina shape is changed along with the small but significant increase in the nuclear shape, now presented together with the lamina markers pictures in the new Figure EV3H).

5.) Line 395-397: "Our model... cell cycle": If FXR proteins act as molecular linker between cytoplasmic nucleoporins and the dyneien.BICD2 complex, one expects the nucleoporin granules in dynein KD containing FXR1, which is not the case in figure 5b. The authors should comment on this.

Please see the response to the major point 2 of reviewer 2.

We have carefully discussed this data and possible interpretations in the revised version of the manuscript.

6.) Figure 6c: The data indicating the dependence of granule fusion on dynein needs to be substantiated by a quantitation.

We have quantified this phenotype upon different conditions as requested by reviewer. The quantifications of the percentage of cells with the fusion/fission events is shown in the new Figure 7D and the quantifications of the numbers of fusion/fission events per cell is shown in Figure 7E.

7.) Lines 105 and Supp Table 1: Supp table 1 looks like a subset of data and not the entire set of Mass spec. If so, it should be stated in the legend.

Please see the detailed response to the specific point 1 of reviewer 1.

We have provided this detailed information and entire set of mass spectrometry data in the revised manuscript.

8.) Lines 156-157: "FXR1... segregation.": Possibly break down the different mitotic phases in a supplementary figure to really prove that all mitotic phases are unaffected?

We have quantified the duration of different mitotic phases as requested by reviewer and the data are shown in Figure 3D-H.

9.) Lines 162-164 and and Figure 3: "GFP-Nup85... decondensation": Given that about 20% cells show Nup85 granules in the Fig. 3b, the accumulation of granule in the control siRNA condition should be plotted in the figure 3c.

Please see the same response to the minor point 3 of reviewer 2.

We have modified the entire figure (now Figure 4) and included new data of a better quality obtained with the HeLa cells stably expressing GFP-Nup107 and with the corresponding quantifications. Better quality of the movies allowed us to more precisely quantify the appearance of CNGs in the FXR1-deficient cells (35 min after anaphase onset) but also in the control cells (44 min after anaphase onset) (Figure 4D). The timing of first CNGs observed strongly correlates with the nuclear blebbing seen in FXR1-downregulated cells (31 min after anaphase onset) (revised Figure 3J). This data fully support and extend our previous conclusions.

Additionally, we included the examples of the original movies in the revised version of the manuscript (Movies EV1-5).

10.) Line 204 and Figure 4: The hexandiol treatment is approximately twice as long and twice more concentrated than the cited paper (5%, 30 sec max). Should we expect this blurring of GFP-Nup133 with hexandiol treatment as it belongs to the Y complex and it is not a FG-Nups? Is it possible to label other kind of protein aggregates (which would not be phase separation aggregates) as negative control?

We expect the blurring of GFP-Nup133 upon hexanediol treatment as also shown by Martin Beck's team in the Supplementary Figure S2 of their recent study (Hampoelz et al. 2019). The authors use 5% 1,6 hexanediol for 10 minutes in Drosophila embryos expressing RFP-Nup107 (belonging to the Y-complex) and GFP-Nup358. The authors observed that both markers disperse under 1,6 hexanediol treatment. We have added this citation and commented on the Beck's findings in the revised manuscript.

Unfortunately, we do not have access to the cellular models of the pathological non-liquid or non-gel like aggregates (for instance amyloids, which should be resistant to treatment with 1,6 hexanediol) but we have meanwhile analysed the effect of 1,6 hexanediol on the stress granules and as predicted this treatment disassembled stress granules validating our experimental approach. We have include this new data in the revised manuscript in Figure 5A).

11.) Line 240-241 and Figure 6: "we first... reversible way": Given that Nocodazole treatment in figure 6 and S9 appear the same in the Material and Method (10µM for 90 min), why are 80% of cells showing nucleoporin dots figure S9, while 50% of cells show such granules in the figure 6. Drugs concentration and incubation time could appear in the figure or in the figure legend for an easier understanding of the experiment.

The cells shown in Fig. S9 were not transfected with siRNAs in contrast to cells shown in the old Figure 6 (now 7). We predict that this could improve the synchronization in G1 cell cycle stage and resulted in a more robust phenotype.

We have added the drugs concentrations and the incubation time to the new Figure 7.

12.) Line 249-250 and Figure 6: "GFP-Nup107... all conditions": Figure 5 shows interaction between FXR1 and Nup133 while figure 6 shows how Nup107 behave after Nocodazole release. Does Nup107 also bind FXR1? If so, it should be introduce in figure 5 along Nup133 binding.

Unfortunately, we were unable to obtain good antibodies recognizing endogenous Nup107 and we did not test the interaction of GFP-Nup107 with the endogenous FXR1.

13.) Line 266 and Fig. S10c: Short exposure (SE) and long exposure (LE) are not written in the legend.

Thank you, we have included these abbreviations in the revised legend.

14.) Line 280 and Figure 7g: "Re-expressed... Nup133": It is not clear to me why the authors show FMRP and Nup133 colocalization in such a complicated rescue setting. Can this be seen in HeLa/U2OS cells?

Please see the response to the major point 2 of reviewer 2.

We have already demonstrated a moderate co-localization of Nups with GFP-FXR1 in the cytoplasm of HeLa cells in Figure 1e.

15.) Line 302-306: "Live video... cytoplasm": A time course graph similar to Fig. 3c would help supporting this statement.

We have quantified the nuclear blebbing phenotype upon FXR1 downregulation in the new Figure 3J as requested by reviewer.

16.) Line 308-310: "Our data... early G1": This statement on impaired nuclear architecture after FRX1-dynein pathway disruption seems contradictory with the statement line 129-132 saying that the lamins are not affected by FXR KD.

Please see the response to the specific point 3 above.

We have rephrased our statements regarding Lamin functionality and only refer to the recruitment.

17.) Line 450: It should be discussed how the nuclear area can be slightly, yet significantly, bigger in FXR1 KD cells (Fig. 8c) while the import and export mechanisms are overall not affected, Are the 30 min slower export in early G1 enough to explain this difference?

We believe that a moderate delay in the export during G1 could (through an unknown mechanism) lead to a small yet significant difference in the nuclear area. Alternatively, the nuclear size and shape could be related to the established structural roles of Nups independent of their function in protein and RNA transport.

We have briefly discussed this point in the revised manuscript.

18.) For all figures: The statistical significance of the asterisks should be written.

We have added this information in all legends of the revised manuscript.

19.) A few spelling mistakes: Change "reates" and "nucleaocytoplasmic (line 314), y- axis figure 9a

Thank you, we have corrected these mistakes.

20.) Please be consistent in naming, use either GP210 or Nup210

Thank you, we have maintained Nup210 term throughout the manuscript.

21.) NPC scheme in figure 2: the inner ring is usually referred to as the NPC part formed by non- transmembrane nucleoporins sandwiched in between the two outer rings. The arrow rather points to what is sometimes referred to as "lumenal or membrane ring".

We have adapted the scheme shown in Figure 2 as requested.

<u>*References:*</u>

Hampoelz, Bernhard, Andre Schwarz, Paolo Ronchi, Helena Bragulat-Teixidor, Christian Tischer, Imre Gaspar, Anne Ephrussi, Yannick Schwab, and Martin Beck. 2019. "Nuclear Pores Assemble from Nucleoporin Condensates During Oogenesis." Cell 179 (3): 671-686.e17. https://doi.org/10.1016/j.cell.2019.09.022.

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Thaller, David J., and C. Patrick Lusk. 2018. "Fantastic Nuclear Envelope Herniations and Where to Find Them." Biochemical Society Transactions 46 (4): 877–89. https://doi.org/10.1042/BST20170442.

Xie, Nina, He Gong, Joshua A. Suhl, Pankaj Chopra, Tao Wang, and Stephen T. Warren. 2016. "Reactivation of FMR1 by CRISPR/Cas9-Mediated Deletion of the Expanded CGG-Repeat of the Fragile X Chromosome." Edited by Barbara Bardoni. PLOS ONE 11 (10): e0165499. https://doi.org/10.1371/journal.pone.0165499.

1st Revision - Editorial Decision 27th May 2020

Dear Izabela,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referees #2 and 3 and their comments are provided below.

As you can see from the comments the referees appreciate the introduced changes and support publication here. They have a few minor comments to address in a revised version.

When you submit the revised manuscript please also take care of the following points:

- You can only have 5 EV figures - the rest would have to be added to an appendix. Please see guide to authors also regarding nomenclature. The appendix needs a ToC

- Please zip each movie file with its legend

- Please check figures and make sure that there are scale bars

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels). You can also use something from the figures if that is easier.

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

That should be all - congratulations on a nice study! Let me know if you have any further questions

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a word file of the manuscript text.
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- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Referee #2:

I am satisfied with the authors' response to my concerns

Referee #3:

The authors have sufficiently addressed the points raised by the reviewers. Three minor points remain which can be easily fixed:
1.) line 173: "emerin beta" should read "emerin"
2.) line 392: "analized"
3.) Fig. 3H: time is not indicated in the panel as described in the figure legend (line 1103)

Remaining comments of the reviewers:

Referee #2:

I am satisfied with the authors' response to my concerns

We thank reviewer 2 for his/her appreciation of our study.

Referee #3:

The authors have sufficiently addressed the points raised by the reviewers. Three minor points remain which can be easily fixed:
1.) line 173: "emerin beta" should read "emerin"
2.) line 392: "analized"
3.) Fig. 3H: time is not indicated in the panel as described in the figure legend (line 1103)

We are grateful to reviewer 3 for his/her appreciation of our work. We have corrected the remaining three errors in the manuscript and in the Figure 3H.

List of other changes made to the manuscript:

1. We have adapted then number of the Expanded view figures to 5, the remaining supplementary figures are presented in the appendix
2. All movies have been zipped with the corresponding legends
3. All figures have been checked and size bars were added
4. All figures have been adapted to fit the size requested in the figure guidelines
5. The reference Davis and Blobel 1987 has been added (line 111)
6. The lines on funding information from Pacini laboratory have been added in the acknowledgment section
7. Our submission includes the source data of all western blots presented in the manuscript
8. Our submission includes a synopsis and a synopsis figure

2nd Revision - Editorial Decision 22nd Jun 2020

Dear Izabela,

Thanks for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Izabela Sumara
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

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Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size per repeat varied between experiments and are indicated in the figure legends. Sample size was based on standard practise in cell biological assays and not specifically pre-estimated.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion criteria were used and all collected data were used for statistical analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Sample treatments were not randomized or blinded.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Image quantifications were carried out in a blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical tests are described in the figure legends and in the material and methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of data was verified using Shapiro-Wilk test in order to apply the appropritate parametric or non-parametric statistical tests.
Is there an estimate of variation within each group of data?	Variation within each group was not estimated.

Is the variance similar between the groups that are being statistically compared?	This was not assessed.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used are detailed in the materials and methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The origin of each cell line is indicated in the materials and method section. All cell lines were maintained by the IGBMC cell culture facility and tested for mycoplasma infection.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Proteomic data have not been deposited in a public repository. We provide the dataset of our proteomic data in the manuscript as an Extended table (EV1).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We provide the dataset of our proteomic data in the manuscript as an Extended table (EV1).
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA