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Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Atypical nuclear envelope condensates linked to neurological disorders reveal nucleoporin-directed chaperone activities
Corresponding author name(s): Christian Schlieker

Reviewer Comments & Decisions:

Decision Letter, initial version:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Christian,

Thank you for submitting your manuscript, "MLF2 modulates phase separated nuclear envelope condensates that provoke dual proteotoxicity", to Nature Cell Biology and thank you again for your patience with the peer review process. The manuscript has now been seen by 3 referees, who are experts in torsin/NPC (referee 1); chaperones (referee 2); and NPC/phase separation (referee 3). As you will see from their comments (attached below), they found this work of potential interest but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

As per standard journal practice, we have discussed the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority to strengthen the current conclusions, as opposed to requests that are beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further. Our typical revision period is six months; please do feel free to reach out if you anticipate any delay or issues addressing the points below.

We found the reviewers' points significant -- in particular Reviewers #2 and #3's comments about DNAJB6's role and the phase separation model. These are valid concerns from experts in these areas that would need to be addressed thoroughly with experiments and data, and reconsideration of the study for this journal and re-engagement of referees would depend on strength of these revisions.

In our view, efforts should be dedicated in revision to:

A. bolster the analyses linking MLF2 to Nup phase separation and droplet dynamics along the lines

suggested by Rev#3, considering Rev#1's major comment as well. More evidence and additional approaches would seem important to support these claims.

B. address Rev#2's questions about the function of DNAJB6 (#1-2-5) and address Rev#2's point #3, echoed by Rev#3.

C. All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes, should also be addressed.

D. Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

Lastly, although we agree with Reviewer #2 that addressing questions around the impact of MLF2 on polyQ aggregates (point #4) would provide valuable insights, we consider this point to be beyond the scope of the present study. Thus, while we encourage you to address it, we do not feel that addressing it experimentally will be strictly necessary for reconsideration of the manuscript at this journal.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and https://www.nature.com/nature/for-authors).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here https://www.nature.com/documents/nr-reporting-summary.pdf). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

When submitting the revised version of your manuscript, please pay close attention to our

href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope that you will find our referees' comments and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss. Thank you for considering NCB for your work.

Best wishes,

Melina

Melina Casadio, PhD

Senior Editor, Nature Cell Biology
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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This is an impressive study that aims at further characterizing nuclear envelope blebs that are the hallmark of Torsin deletion in vertebrates, and likely essential for understanding the molecular mechanism of the neuromuscular disorder dystonia.
Here, the authors use a proteomics approach to detail the make-up of the blebs and find a enrichment in a number of specific chaperones and the FG-Nup Nup98. Using a clever approach involving the viral ORF10 protein or an N-terminal deletion it is shown that the blebs unexpectedly stabilize an otherwise short-lived protein, which may be a generalizable property of these blebs. The study further reveals that MLF2 is an essential component of these blebs and that FG-condensation has a critical role as well.
The study utilizes a wide range of experimental techniques to fundamentally advance the understanding of these NE blebs, with wide ranging implications for NPC assembly, Dystonia etiology, and beyond. I am enthusiastically in favor of publication, expect for one major concern and a few minor ones.

Major concern:

Starting in line 203, the authors describe experiments to identify properties of MLF2 that make it localize to blebs. In one experiment, all (?) methionines of MLF2 are replaced with alanine. As a result, the protein does not localize to blebs anymore. Using the Alphafold model of MLF2, it is obvious that mutating all methionines to alanine almost certainly will result in misfolded protein, as several methionines are, expectedly, in the hydrophobic core. Thus, the experiment is not very well designed. In order to sensibly show which properties make MLF2 immerse into the bleb phase one has to use surface mutations that do not affect the protein structure. Given the preliminary nature of the results shown in Figs 7a,b, and the considerable effort it would take to make this more meaningful, I recommend leaving these experiments out. They are not necessary to convey the main message of this paper. Lines 306-312 in the discussion should be removed as well.

Minor revisions:

1.
Line 63 – missing word ‘of’ after word ‘property’

2.
Figure 5e – change labeling to be consistent with the other figures (‘Minus MLF2-HA’ ‘Plus MLF2-HA’ not consistent with general style)

3.
In lines 174-175 the authors write:
‘Together, we conclude that the FG-Nup Nup98 is required for the formation of an unusual NE

compartment consisting of K48-Ub, MLF2, specific FG-Nups, and chaperones'. Is there are reason to say 'unusual NE compartments' rather than 'NE blebs'. If not, 'NE blebs' should be used to avoid confusion.

4.)
Line 295: add 'the' after 'prevent'.

5.)
Line 745: please explain 'brSUMO'. What does br stand for?

Reviewer #2:
Remarks to the Author:
Summary

In this manuscript, Prophet et al study defects in nuclear pore complex (NPC) biogenesis that can manifest in diseases such as DYT1 dystonia. They could demonstrate that FG-rich Nups phase-separate in "blebs" that are the result of defective NPC biogenesis. They identified MLF2 as direct modulator of phase separation of Nup98 and they could further show that MLF2 can interact with various members of Hsp70-JDPs chaperone family. These chaperones can redistribute in an MLF2-dependent manner between their cytosolic protein substrates and the "blebs". Based on these data, the authors propose a dual toxicity mechanism that contributes to the DYT1 dystonia: 1st an impaired nuclear-cytosolic transport in TorsinKO cells. 2nd a sequestration of the chaperones in the "blebs". This could be tolerated if the protein homeostasis system of the cell/organism is still intact and could buffer a transient redistribution. However, it could be pathogenic in pre-sensitized cells or cells with excessive protein damage. This work adds to our growing knowledge of inter-compartmental regulation of protein homeostasis (here: cytosol and nuclear envelope) and provides mechanistic insight into the manifestation of movement disorders such as dystonia.

The conclusions of this study are based on very well-designed and -executed experiments throughout the manuscript. The obtained data and data presentation are of high standard with adequate statistical analyses and suitable controls. This study connects two research areas, the biogenesis and regulation of NPCs and the cytosolic protein homeostasis network and provides a solid foundation for future studies addressing the crosstalk of the compartmental surveillance systems in the context of diseases.

Major comments:

1. The observations that only class B JDPs are enriched in the "blebs" together with different members of Hsp70s is interesting and should be discussed. Does the prolonged G/F rich region of class B JDPs contribute to the interaction with Nups or MLF2? What exactly is the role of Hsp70? And how are the chaperones targeted to the "blebs"?

2. Do the JDPs and Hsp70 chaperones act independently on Nups or do they cooperate? In the reconstituted system, the authors could address this question by using a HPD-mutant or simply starting by testing the effect of DNAJB6 +/-Hsp70+/-ATP or using a DNAJB6 variant lacking the J-domain.

3. The immunoprecipitation of HSPA1A in WT and TorsinKO cells (Fig. 5b) shows an enrichment of several proteins. How did the authors assign the corresponding protein bands? Of course it is an educated guess to predict that some of the bands represent the known interaction partners, MLF2 and DNAJB6 – yet an assignment based on the MW alone is not sufficient. In addition they noticed a new interaction with a so far unknown protein (black arrow in Fig 5b) that should be characterized. Ideally the IP should be repeated and combined with an MS-analysis. The identification of the additional interaction partner of HSPA1A in TorsinKO cells only could further strengthen this analysis. Notably this interaction appears to be gone upon depletion of MLF2. This could suggest that this protein is bound via MLF2 or that it is a PTM-form of MLF2 itself that migrates faster in the SDS-PAGE.

4. The observation that MLF2 expression can cause a cellular redistribution of DNAJB6 from protein aggregates to "blebs" is very interesting (Fig. 5e). Would such an MLF2 expression in turn lead to a higher polyQ aggregation and toxicity caused by polyQ (e.g. sequestration of endogenous proteins into polyQ aggregates, intercalation and disruption of membranes etc)? And, would an overexpression of DNAJB6 be a fruitful strategy to ameliorate not just the polyQ aggregation but also the defects at the NE?

5. In figure 5d, the authors depict a trimeric complex of HSPA1A, MLF2 and DNAJB6. The way this cartoon is depicted suggests that MLF2 can interact with DNAJB6 and HSPA1A. What is the evidence for a direct interaction (i) and that they form a stable trimeric complex (ii) as opposed to two independent interactions? If they form a trimeric complex, than certainly all reconstituted assays should take this into account and should be performed with these 3 proteins + the substrate (Nup98 or Nup116) in Fig. 7

Minor comments:

1. The „bleb" enrichment analysis revealed a high abundance of molecular chaperones: e.g. Hsp70s (HSPA1A, Hsc70, HSPA2, HSPA4) and JDPs (DNAJB2, DNAJB6, DNAJB12). The authors test most of these hits by co-localization studies as well as knockdown/ectopic expression. Yet they jump quickly on DNAJB6: line 89: "First, we validated that delta133ORF stably interacts with DNAJB6 exclusively in TorsinKO cells….". This sentence is followed by a summarizing statement: line 91: "These data suggest that beyond K-48-Ub, MLF2, and FG-Nups, blebs harbor specific members of the Hsp70 and Hsp40 families". At this point, the authors have not yet validated the localization of any other chaperone apart from DNAJB6 in these "blebs". Only in the next paragraph with a separate header starting at line 94 they validate the location of the other chaperones. This section needs rephrasing / sorting.

2. How defined and characterized are „blebs". This term is frequently used throughout the manuscript and this reviewer wonders if "blebs" are unique structures with the same content as well as morphological and physical parameters in this work and the literature? This is relevant as the authors have analysed enriched proteins in these "blebs" by proximity labelling and "bleb"-specific probe, delta133ORF10. The authors should comment if similar data sets exist and how their proteomic data compare to potential other data sets.

Reviewer #3:
Remarks to the Author:
The paper by Prophet et al studies the role of Torsin in NE function. Torsin dysfunction leads to defects in NPC biogenesis and NE blebbing. The present paper studies the compositon and role of the blebs. As I detail below, I like to separate my discussion into Figure 1-5 and Figure 6-8.
In figure 1-5 they describe the development of a viral model substrate to study and define the bleb proteome. Their new tools enables them to find that herniations selectively sequester a specific chaperone network. The results range from proteomics to imagining and studies on primary neurons. I find the data shown in the first four figures convincing and solid and can follow the logic. This part in itself is certainly pubishable, but probabl;y more suited for a specialized jorunal.

However from figure 5 on to figure 6,7 they jump substantially, and I struggle with lots of the data and the arguing in the last three figures (6,7,8). In those figures an interesting story is generated that links Nup98 function to sequestration into granules. If true, the work would potentially be interesting for the audience of NCB, as it would link chaperone activity to biogenesis of the NPC permeability barrier. Right now, this evidence is very week and alternative explanations are not considered, and some experiments do not seem to be designed in such a way that they would sufficiently support their claims.

In figure 3 they report on DNAJB6 immunogold labelling inside the bleb lumen. The data lacks quantitation.

In figure 5 the correlation between the blots in panelb and c is weak, as the DNAJB6 bands in b are hardly recognizable. A more direct validation would be helpful

With nevertheless little criticism to Figure 1 to 5, I will now focus on my major concerns in figure 5 and 6 and the corresponding supplements.

Figure 6
In figure 6a to c, the authors follow up their APEX data which suggest also the presence of various Nups near MLF2. The data reveals large micrometer sized aggregates starining positive for Nups, MLF2, DNAJB6 and K48 in TorsinKO cells and knock downs of Nup98. Nup98 is a vital component of the NPC, and downregulating it can have severe effects on whole cell physiology and function and is certainly a stress signal for the cell.
One of the key claims of the paper is, that Nup98 forms a phase separated compartment inside the blebs. The only experimental evidence in that direction is coming from hexanediol experiments. Hexandiol experiments are widely used to interfere with hydrophobic interactions, but they are always just supportive, since adding 5% of an bi-alcohol can have all types of effects, including the most known effect to permeabilize the entire nuclear envelope. Seeing suddenly a fuzzy looking cell instead of dotty pattern is interesting but is in my eyes not sufficient evidence to built an entire biological mechanism on this, i.e. Nup98 is condensated inside the blebs and is suddenly released.
Figure 7 aims to provide additional in vitro data to support the claims made in Figure 6. Here I feel that I spotted a few misinterpretations in experimental design to study permeability function in vitro.

i) Since MLF2 forms a bight ring to the Nup98 droplets, it can bind Nup98 very tightly. The discussion

on relevance of glycosylation is potentially misleading. Droplets can be liquid, gel or amyloid rich. They do not classify their droplets. Metazoan Nups are glycosylated,. Depending on experimental conditions different outcomes with different permeability barrier properties can be achieved. Nup116 was not one of their APEX hits, and is certainly not identical to Nup98. As they try to identify a specific role of Nup98, swapping it against a different Nup without knowing what element might be key makes the sudden SWAP to get around an experimental difficulty hard to interpretate.

ii) Panel e: There is an alternative interpretation to Figure e that needs to be considered. Most likely, their droplets are solid, that is either gel or amyloid like. Thus, most proteins will behave as clients in the gel/solid, and not change the phase separated state itself. MLF2 can bind to the gel/solid and will also occupy space in the gel/solid. Same for JB6. Thus, due to an excluded volume effect or simply limited binding sites less GFP can enrich. This explains their observed drops in fluorescence signal. Consequently their interpretation that permeability properties are changed are most likely wrong, as what they see is simply a competitio and or excluded volume effect.

iii) Panel g) I feel there is a misconception here. If only thermodynamics area at play, which is what would be the case if all effects can be accounted for only towards phase separation, then we would only see one big droplets after long time, as phase separation always leads to one single droplet (i.e. two phases). The interpretation of droplet size and shape at a single time point is thus easily misleading. The seen effect can be explained by probing a different region in the phase diagram due to change in overall constituent composition, concentration, probably kinetics and experimental design.
Concluding from such a dataset that "MLF2 facilitates phase separating processes, revealing a hitherto unknown FG-Nup directed activity" is not based on the available data in my eyes.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

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TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding

technical terms, abbreviations, and active verbs..

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ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see https://www.nature.com/licenceforms/nrg/competing-interests.pdf.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

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must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

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• Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here http://www.nature.com/authors/policies/availability.html#data.

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We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

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FIGURES – Colour figure publication costs $600 for the first, and $300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

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- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the "unprocessed scans" Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – We are trying to improve the quality of methods and statistics reporting in our papers. To that end, we are now asking authors to complete a reporting summary that collects information on experimental design and reagents. The Reporting Summary can be found here https://www.nature.com/documents/nr-reporting-summary.pdf)If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

--------- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements ---------

Author Rebuttal to Initial comments

Point-by-Point Response, Prophet et al. (NCB-A46885A)

We appreciate the helpful and timely evaluation of our manuscript. The constructive criticism has led to a considerably improved manuscript. As detailed in our point-by-point responses below, we have constructively addressed all points of concern in this revision. The new results

are not only in excellent agreement with our original conclusions, but allow us to make a significantly more convincing case for a specific FG-Nup directed activity of the MLF2-HSP70-DNAJB6 complex. We hope that the reviewers agree that the revised manuscript is now acceptable for publication. Please note our responses to each reviewer comment (bold) are found in italicized text.

Reviewer #1:

Major concern:

Starting in line 203, the authors describe experiments to identify properties of MLF2 that make it localize to blebs. In one experiment, all (?) methionines of MLF2 are replaced with alanine. As a result, the protein does not localize to blebs anymore. Using the Alphafold model of MLF2, it is obvious that mutating all methionines to alanine almost certainly will result in misfolded protein, as several methionines are, expectedly, in the hydrophobic core. Thus, the experiment is not very well designed. In order to sensibly show which properties make MLF2 immerse into the bleb phase one has to use surface mutations that do not affect the protein structure. Given the preliminary nature of the results shown in Figs 7a,b, and the considerable effort it would take to make this more meaningful, I recommend leaving these experiments out. They are not necessary to convey the main message of this paper. Lines 306-312 in the discussion should be removed as well.

We thank the reviewer for this helpful comment and agree that the data shown in Figures 7a and 7b are somewhat preliminary. Therefore, we have elected to remove these experiments from the manuscript and modify the discussion accordingly.

Minor revisions:

1. Line 63 – missing word 'of' after word 'property.'

This was fixed in the revised version.

2. Figure 5e – change labeling to be consistent with the other figures ('Minus MLF2-HA' 'Plus MLF2-HA' not consistent with general style)

The figure labeling has been updated to be consistent with other labeling.

3. In lines 174-175 the authors write:

'Together, we conclude that the FG-Nup Nup98 is required for the formation of an unusual NE compartment consisting of K48-Ub, MLF2, specific FG-Nups, and

chaperones'. Is there are reason to say 'unusual NE compartments' rather than 'NE blebs'. If not, 'NE blebs' should be used to avoid confusion.

We agree and have updated the text accordingly.

4. Line 295: add 'the' after 'prevent'.

This text has been corrected.

5. Line 745: please explain 'brSUMO'. What does br stand for?

We thank the reviewer for pointing out this error. The text now reads "bdSUMO" as the SUMO is derived from Brachypodium distachyon.

Reviewer #2:

Major comments:

1. The observations that only class B JDPs are enriched in the "blebs" together with different members of Hsp70s is interesting and should be discussed. Does the prolonged G/F rich region of class B JDPs contribute to the interaction with Nups or MLF2? What exactly is the role of Hsp70? And how are the chaperones targeted to the "blebs"?

We have added the following text to the discussion: "Our proteomic experiments reveal a specific enrichment of class B HSP40s inside blebs. While future work will be required to understand whether an active exclusion of other HSP40 classes exists, we propose that DNAJB2 and DNAJB6 are recruited by unique and specific properties of these HSP40s. It will be interesting to test whether MLF2 interacts with other HSP40 classes and if other HSP40s can immerse into FG-rich phases like DNAJB6 (Fig. 6b)."

In the revised manuscript, we have also added new data and concretely investigated whether the G/F-rich and the S/T-rich regions of DNAJB6b contributes to its recruitment to blebs and interaction with MLF2. To avoid introducing major structural perturbations, we elected to mutate the phenylalanine residues to alanine within these regions instead of deleting large domains of DNAJB6b. By mutating the phenylalanine residues within the G/F-rich region of DNAJB6b (a.a. 72-131) to alanine, we find this region is dispensable for its recruitment to blebs (Extended Data Fig. 3d). In contrast, mutating the phenylalanine residues to alanine within DNAJB6b's disordered S/T-rich region (a.a. 132-195) prevents its recruitment to blebs (Extended Data Fig. 3d). These data are supported by the result that the G/F-rich region mutant maintains its interaction with MLF2, but the S/T-rich region mutant interacts with significantly less MLF2 (Extended Data Fig. 3c). We concluded that unlike the G/F-rich region, the S/T-rich region of DNAJB6 contributes to its recruitment to blebs and interaction with MLF2.

Moreover, we have added new experiments in the context of HSP70 and K48-Ub recruitment. When TorsinKO cells are treated with the HSP70 ATPase inhibitor VER-155008, which mimics the ADP-bound state of HSP70 (doi: 10.2174/1568026616666160413140911), HSP70 expression is elevated and significantly more K48-Ub localizes to blebs (Extended Data Fig. 3a, b). As ADP-bound HSP70 has a higher affinity for clients relative to its ATP-bound state, HSP70 may be driven into blebs by virtue of client binding and, along with JDPs, may serve to promote client solubility and prevent terminal aggregation.

2. Do the JDPs and Hsp70 chaperones act independently on Nups or do they cooperate? In the reconstituted system, the authors could address this question by using a HPD-mutant or simply starting by testing the effect of DNAJB6 +/-Hsp70+/-ATP or using a DNAJB6 variant lacking the J-domain.

We thank the reviewer for this insightful question. We have purified the WT human DNAJB6b, the H31Q DNAJB6b mutant, and HSP70 (HSPA1A), and performed a series of in vitro experiments with separate and combinatorial additions of chaperones to address these points.

To ascertain whether the HSP70 system effects FG-rich Nup condensates, we formed ScNup116 or TtMacNup98A particles in the presence of varying protein combinations. We found that alone, neither HSP70 nor DNAJB6b significantly affects the initial morphology of FG-rich Nup condensates (Fig. 6e, Extended Data Fig. 7b, c). We note, however, that DNAJB6b (WT and H31Q) may cause somewhat smaller condensates to form. Consistent with our original observations, we found that the MLF2:HSP70 complex promotes the formation of larger, morphologically homogenous condensates (Fig. 6a-e, Extended Data Fig. 7b).

Incorporating an additional suggestion from R#3, we also monitored FG-Nups over time, both with identical and additional readouts (see also our response to R#3 Q7). We observed that the FG-rich condensates lose integrity over time and shrink considerably in size (Fig. 6e-g, Extended Data Fig. 7a-c). While HSP70 and DNAJB6b alone had no effect on the particles' dissolution over time, the MLF2:70 complex effectively protects condensate integrity (Fig. 6e, Extended Data Fig. 7b). HSP70 in solution with WT DNAJB6b also had a robust ability to prevent FG-rich particles from dissolving after three hours at 30ºC in the presence of ATP (Fig. 6f, Extended Data Fig. 7c). The extent of particle preservation was similar to that for the MLF2:HSP70 complex (Fig. 6e,f, Extended Data Fig. 7b,c). We note that after three hours, the particles looked more regularly spherical and less clustered with the HSP70:DNAJB6b complex compared to the MLF2:HSP70 complex. The effect of DNAJB6b on HSP70 and MLF2 depended on its interaction with HSP70 as the H31Q mutant failed to protect particle morphology (Fig. 6f, Extended Data Fig. 7c). In fact, the H31Q-DNAJB6b appeared to have a dominant-negative effect as its combination with MLF2:HSP70 yielded worse particle preservation than MLF2:HSP70 without DNAJB6b (Fig. 6e,f).

We thus conclude that the MLF2-HSP70-DNAJB6 complex not only fosters condensate formation, but additionally maintains condensate integrity over time.

3. The immunoprecipitation of HSPA1A in WT and TorsinKO cells (Fig. 5b) shows an enrichment of several proteins. How did the authors assign the corresponding protein bands? Of course it is an educated guess to predict that some of the bands represent the known interaction partners, MLF2 and DNAJB6 – yet an assignment based on the MW alone is not sufficient. In addition they noticed a new interaction with a so far unknown protein (black arrow in Fig 5b) that should be characterized. Ideally the IP should be repeated and combined with an MS-analysis. The identification of the additional interaction partner of HSPA1A in TorsinKO cells only could further strengthen this analysis. Notably this interaction appears to be gone upon depletion of MLF2. This could suggest that this protein is bound via MLF2 or that it is a PTM-form of MLF2 itself that migrates faster in the SDS-PAGE.

We thank the reviewer (also R#3, point #2) for this helpful comment as we agree that an educated guess of DNAJB6 based on MW is not sufficient. We have now performed a knockdown of DNAJB6 and found the band around 27 kDa no longer co-immunoprecipitates with HSPA1A (Fig. 4c). Furthermore, we dissociated the primary anti-HSPA1A IP, performed a Re-IP with an anti-DNAJB6 antibody, and confirmed that DNAJB6 is increasingly associated with HSPA1A in TorsinKO relative to WT cells (Figure 4c). We believe this confirms the identity of the band as DNAJB6.

As suggested by the reviewer, we additionally analyzed the HSPA1A IP experiment by mass spectrometry. This analysis revealed that within the 20-37 kDa region, only MLF2 and DNAJB6 are significantly enriched in the TorsinKO IP sample compared to the WT IP control (Extended Data Fig. 3e, Table S2). Beyond confirming the identity of the previously unconfirmed band as DNAJB6, it also demonstrates that the band migrating slightly faster than MLF2 represents a post-translationally modified version of MLF2: due its high methionine content (Extended Data Fig. 6a), MLF2 is prone to extensive methionine oxidation, and we detected an abundance of corresponding signature masses in samples derived from the 20-37 kDa region of the SDS-PAGE gel (Table S2). Since this oxidation might merely result from oxidizing conditions during our IP (we have not included reducing agents to preserve the integrity of disulfide-linked immunoglobulins), we have not commented on this aspect in our revised manuscript as it would distract from the main conclusions of this experiment. However, we could certainly include this discussion if the reviewer feels it would benefit the reader.

4. The observation that MLF2 expression can cause a cellular redistribution of DNAJB6 from protein aggregates to "blebs" is very interesting (Fig. 5e). Would such an MLF2 expression in turn lead to a higher polyQ aggregation and toxicity caused by polyQ (e.g. sequestration of endogenous proteins into polyQ aggregates, intercalation and

disruption of membranes etc)? And, would an overexpression of DNAJB6 be a fruitful strategy to ameliorate not just the polyQ aggregation but also the defects at the NE?

The main point we wanted to make with this "tug-of-war" experiment is that NE blebs have an enormous capacity to sequester chaperones. PolyQ inclusions have long been considered to be the most potent disease allele-specified inclusions in this regard and we used them as experimental tool/point of comparison. We were surprised to see that NE blebs have a far greater chaperone capacity resulting in a quantitative sequestration in blebs vs. PolyQ inclusions, underscoring the unexpected proteotoxic properties of NE blebs that were borne out by this study. While a functional dissection of MLF2/PolyQ interactions represent an interesting question for the future, we concur with the editor–and hope that this reviewer agrees–that an in-depth analysis in this direction is beyond the scope of the current study which is focused on disease etiology of Dystonia.

5. In figure 5d, the authors depict a trimeric complex of HSPA1A, MLF2 and DNAJB6. The way this cartoon is depicted suggests that MLF2 can interact with DNAJB6 and HSPA1A. What is the evidence for a direct interaction (i) and that they form a stable trimeric complex (ii) as opposed to two independent interactions? If they form a trimeric complex, than certainly all reconstituted assays should take this into account and should be performed with these 3 proteins + the substrate (Nup98 or Nup116) in Fig. 7.

We have elected to remove the schematic in Figure 5d as we agree more evidence is required to depict such a trimeric complex. Nonetheless, we have made comparisons between all three proteins (MLF2, HSP70, and DNAJB6) in in vitro *experiments in Figure 6.*

Minor comments:

1. The „bleb" enrichment analysis revealed a high abundance of molecular chaperones: e.g. Hsp70s (HSPA1A, Hsc70, HSPA2, HSPA4) and JDPs (DNAJB2, DNAJB6, DNAJB12). The authors test most of these hits by co-localization studies as well as knockdown/ectopic expression. Yet they jump quickly on DNAJB6: line 89: "First, we validated that delta133ORF stably interacts with DNAJB6 exclusively in TorsinKO cells….". This sentence is followed by a summarizing statement: line 91: "These data suggest that beyond K-48-Ub, MLF2, and FG-Nups, blebs harbor specific members of the Hsp70 and Hsp40 families". At this point, the authors have not yet validated the localization of any other chaperone apart from DNAJB6 in these "blebs". Only in the next paragraph with a separate header starting at line 94 they validate the location of the other chaperones. This section needs rephrasing / sorting.

We thank the reviewer for pointing out this confusing section and have sorted out the text to better fit the headers and logical flow.

2. How defined and characterized are „blebs". This term is frequently used throughout the manuscript and this reviewer wonders if "blebs" are unique structures with the same content as well as morphological and physical parameters in this work and the literature? This is relevant as the authors have analysed enriched proteins in these "blebs" by proximity labelling and "bleb"-specific probe, delta133ORF10. The authors should comment if similar data sets exist and how their proteomic data compare to potential other data sets.

We agree that "bleb" is a loose term and we have clarified this point to make the text more accessible to the broad readership of NCB. To this end, we have included a new schematic diagram of NE blebs in the context of Torsin perturbation (Extended Data Fig. 1a). We have also explained in the revised introduction that these blebs are omega-shaped herniations of the inner nuclear membrane that protrude into the perinuclear space and are constricted by an NPC-like structure at their base stemming from defective NPC assembly. We further added that NE blebs represent the hallmark phenotype common to a wide variety of animal and cell-based models with perturbed Torsin function (dominant-negative alleles, Torsin-null alleles, siRNA), and are also observed in yeast upon mutation of specific Nups or proteins involved in NPC assembly/nuclear envelope fusion.

To our knowledge, our study is the first to present a detailed compositional analysis of NE blebs. Here, we establish the first bleb-specific probes in the field, opening the door for future functional analysis by us and others across model systems. Please note that our prior proteomic characterization (Rampello et al. JBC) relied on a comparison of bulk NE isolates enriched for ubiquitylated species (but not specifically blebs), however we compare both this prior and our two new datasets relying on MLF2-APEX2 and Δ133 ORF10 in our study (Fig. 2e).

Having identified, validated, and functionally analyzed several of the highest-ranking bleb resident proteins in cells and in vitro*, we believe that our dataset provides a robust foundation for future studies to explore NPC assembly and its interplay with disease.*

Reviewer #3:

1. In figure 3 they report on DNAJB6 immunogold labelling inside the bleb lumen. The data lacks quantitation.

We have quantified the number of anti-DNAJB6- and HSPA1A-conjugated immunogold beads associated with nuclear envelopes from wild type and TorsinKO cells. These data have been included as new panel (Fig. 3c).

2. In figure 5 the correlation between the blots in panel b and c is weak, as the DNAJB6 bands in b are hardly recognizable. A more direct validation would be helpful

We fully agree and now confirm the identity of this band by several complimentary approaches. Please see our response to point #3 by Reviewer 2 who expressed the same concern.

3. In figure 6a to c, the authors follow up their APEX data which suggest also the presence of various Nups near MLF2. The data reveals large micrometer sized aggregates starining positive for Nups, MLF2, DNAJB6 and K48 in TorsinKO cells and knock downs of Nup98. Nup98 is a vital component of the NPC, and downregulating it can have severe effects on whole cell physiology and function and is certainly a stress signal for the cell.

We appreciate this concern given Nup98's critical role in the NPC. However, Nups are very stable proteins and repeated doses of siRNA over several days are often required to elicit significant phenotypic consequences. Please note that we already excluded off-target effects in the original manuscript via complementation analysis (now Extended Data Fig. 4d,e). In the revised manuscript, we have additionally tested for possible nuclear transport defects. We do not find that Nup98 knockdown for 48 hours significantly perturbs nucleo-cytoplasmic transport during this relatively short timeframe: first, we have quantified the nuclear/cytoplasmic ratio of a GFP-NES construct under siNT or siNup98 conditions in TorsinKO cells (Extended Data Fig. 4h). Second, we did not observe significant differences in Ran localization upon Nup98 depletion (Extended Data Fig. 4g). While it is difficult to entirely rule out non-specific effects in a cellular setting–a fact that we now clearly state in the revised text–we hope the reviewer agrees that these newly added controls are adequate and argue against non-specific consequences of stress or gross NPC malfunction.

4. One of the key claims of the paper is, that Nup98 forms a phase separated compartment inside the blebs. The only experimental evidence in that direction is coming from hexanediol experiments. Hexandiol experiments are widely used to interfere with hydrophobic interactions, but they are always just supportive, since adding 5% of an bi-alcohol can have all types of effects, including the most known effect to permeabilize the entire nuclear envelope. Seeing suddenly a fuzzy looking cell instead of dotty pattern is interesting but is in my eyes not sufficient evidence to built an entire biological mechanism on this, i.e. Nup98 is condensated inside the blebs and is suddenly released.

It seems that our original phrasing was potentially misleading and we have therefore improved this section in the revised version. It was not our intention to convey that the luminal space consists of a homogenous Nup98 phase. Rather, we identified several proteins including FG-Nups in the bleb, with Nup98 being most prominent compared to other FG-Nups (Nup62, Nup358, Nup153, and Nup214), as judged by peptide count. However, to reconstitute the entire complexity of this dynamic structure in authentic molar ratios in vitro *is not presently feasible*

(please note that about one third of Nups are FG-rich). Thus, our in vitro *analysis and several corresponding experiments in a cellular context are focused on Nup98.*

Interestingly, we found that Nup98 is required to target other FG-Nups and components of blebs to NE herniations. The knockdown of Nup98 does not prevent the TorsinKO-specific FG-rich foci from forming, rather, it causes these foci to ectopically accumulate in the cytosol instead of the bleb lumen (Fig. 5b,c). These cytosolic foci that form upon Nup98 depletion, like blebs, are stained by the Mab414 antibody and harbor MLF2, DNAJB6, and K48-Ub (Fig. 5b,c).

While we agree that the initial hexanediol experiments were insufficiently controlled, we respectfully disagree with the statement that 5% hexanediol permeabilizes the NE within five minutes. That 5% 1,6-hexanediol preserves the NE membrane and selectively perturbs the FG-Nup mediated permeability barrier has been reported previously and was in fact a reason to propose a selective phase model (doi: 10.1093/emboj/21.11.2664 and 10.7554/eLife.04251.001). To confirm this in our system, we now included side-by-side images of the intact NE under 5% 1,6-hexanediol by lamin A and emerin (Fig. 5h), as well as LBR, nucleoporins (Mab414), and Sun2 (Extended Data Fig. 5e).

We note that although the NE remains intact, the barrier established by the NPC is partially broken down under 5% 1,6-hexanediol treatment as Ran begins to "leak" out of the nucleus under this condition (Extended Data Fig. 5d). As 1,6-hexanediol is known to disrupt the hydrophobic contacts required for the cohesion of FG-Nups within the central channel of the NPC (doi: 10.7554/eLife.04251), this observation is in excellent agreement with previous findings showing that 1,6-hexanediol disrupts FG-driven compartments.

We have included new data showing that while 1,6-hexanediol selectively dissolves K48-Ub and MLF2-HA within blebs, the alcohol does not dissolve polyQ-97-GFP aggregates or the aggregated K48-Ub within them (Extended Data Fig. 5a,b). Thus, it is highly unlikely that blebs harbor terminally aggregated protein. Given their FG-rich nature and sensitivity to hexanediol, we propose blebs harbor FG-rich phase separated condensates. In addition, the bleb content is not dissolved by the related alcohol 2,5-hexanediol (new Extended Data Fig. 5c), which is not associated with dissolving phase separated compartments, further arguing against non-specific solubilization effects.

5. Figure 7 aims to provide additional in vitro data to support the claims made in Figure 6. Here I feel that I spotted a few misinterpretations in experimental design to study permeability function in vitro.

i) Since MLF2 forms a bight ring to the Nup98 droplets, it can bind Nup98 very tightly. The discussion on relevance of glycosylation is potentially misleading. Droplets can be liquid, gel or amyloid rich. They do not classify their droplets. Metazoan Nups are glycosylated,. Depending on experimental conditions different outcomes with different

permeability barrier properties can be achieved. Nup116 was not one of their APEX hits, and is certainly not identical to Nup98. As they try to identify a specific role of Nup98, swapping it against a different Nup without knowing what element might be key makes the sudden SWAP to get around an experimental difficulty hard to interpretate.

We agree that the term "droplet" can refer to a number of distinct biophysical properties (liquid, gel, etc.). The FG-rich phases we are presently using as in vitro *models of the bleb lumen have been extensively characterized by Görlich and colleagues (10.7554/eLife.04251.001) and we recapitulate their selective permeability properties using GFP derivates kindly shared by the Görlich lab (Fig. 6a). We also find the FG-rich condensates to be readily reversible with 2 M urea (Extended Data Fig. 7g).*

As discussed below (see response to point 7), we have followed the reviewer's suggestion of monitoring the condensates over time. We have included new data demonstrating that while neither Homo sapiens *Nup98 nor* Tetrahymena thermophila *MacNup98A form amyloid structures over 24 hours,* Saccharomyces cerevisiae *Nup116 does (Extended Data Fig. 7g). This difference in amyloid potential has been reported by the Görlich group (10.7554/eLife.04251.001). Because FG-rich phases exhibit differences in biophysical properties, we agree with the reviewer that our usage of the term "droplet" may not be the most accurate nomenclature. We have therefore updated our manuscript to use the more neutral term "FG-rich condensate."*

Rather than assigning a specific role to HsNup98, our goal with the in vitro *experiments was to establish a reductionistic, tractable system to determine whether MLF2, DNAJB6 or HSP70 interacts with FG-rich phases and possesses any FG-directed activity. We agree, of course, that the ScNup116 is not identical to the HsNup98.*

We also agree that the discussion surrounding Nup glycosylation could be misleading and have removed lines 227-235 of the results. Having discussed the issue of glycosylation with several leading laboratories in the field during our revision, we learned that while it is potentially possible to glycosylate Nups in vitro*, this modification introduces artifacts as glycosylation reactions can't be properly controlled. This results in significant hyper-glycosylation. The resulting condensates are hypoadhesive and consequently, do not accurately recapitulate the levels of glycosylation and properties in cells (personal communications with Dirk Görlich).*

Therefore, to better generalize our findings of an FG-directed activity for MLF2, we have purified a third FG domain from the ciliate Tetrahymena thermophila *(TtMacNup98A) (Fig. 6) since Nup glycosylation is less critical in lower eukaryotes. Using this Nup as a model along with yeast ScNup116, we have corroborated our original conclusions and found that MLF2 strongly interacts with FG domains and maintains FG phase integrity over time (Fig. 6, Extended Data Fig. 7).*

We hope that the reviewer agrees that our strategy with a significantly expanded in vitro *analysis testing MLF2, DNAJB6 and/or Hsp70 in the context of FG-Nups derived from three phylogenetically distinct species is an advantage as it allows us to generalize our key conclusion that these chaperones have a previously unknown FG-Nup directed activity.*

6. Panel e: There is an alternative interpretation to Figure e that needs to be considered. Most likely, their droplets are solid, that is either gel or amyloid like. Thus, most proteins will behave as clients in the gel/solid, and not change the phase separated state itself. MLF2 can bind to the gel/solid and will also occupy space in the gel/solid. Same for JB6. Thus, due to an excluded volume effect or simply limited binding sites less GFP can enrich. This explains their observed drops in fluorescence signal. Consequently their interpretation that permeability properties are changed are most likely wrong, as what they see is simply a competitio and or excluded volume effect.

We agree with most of those points and have in fact stated in the original text that a competition mechanism is a likely explanation. Still, even if a competition mechanism or excluded volume effects are at work, the net result is ultimately that the permeability for karyopherin-mediated cargo will be materially reduced. It is tempting to speculate that chaperone binding prevents premature engagement of karyopherins with FG-Nups during interphase NPC assembly, which we mention in the discussion as a possible mechanism warranting future investigation. Regardless, we have carefully scrutinized the text in revision and are now more explicit in mentioning these alternate mechanistic interpretations including excluded volume effects.

7. Panel g) I feel there is a misconception here. If only thermodynamics area at play, which is what would be the case if all effects can be accounted for only towards phase separation, then we would only see one big droplets after long time, as phase separation always leads to one single droplet (i.e. two phases). The interpretation of droplet size and shape at a single time point is thus easily misleading. The seen effect can be explained by probing a different region in the phase diagram due to change in overall constituent composition, concentration, probably kinetics and experimental design.

We thank the reviewer for the helpful suggestion to examine the FG-rich particles over time. We have included new data of the FG-rich condensates after three hours of incubation with buffer alone, MLF2, HSP70, or DNAJB6b (Fig. 6e-h, Extended Data Fig. 7a-c). We have described these findings in detail in our response to Reviewer 2's major comment #2. Briefly, we find that FG-rich condensate morphology loses integrity over time. However, in the presence of the MLF2-HSP70 or the HSP70-DNAJB6b complex, the condensates are significantly stabilized (Fig. 6e-h, Extended Data Fig. 7a-c). This protective effect is strongest when the MLF2-HSP70-DNAJB6b complex is present, which nearly completely prevents the disassembly of ScNup116 and TtMacNup98A condensates (Fig. 6f,h).

In addition to examining FG-rich condensates after three hours, we have taken advantage of the known propensity of ScNup116 to form cross-β amyloid structures. As reported previously (doi: 10.7554/eLife.04251.001 and 10.1038/s41467-021-24292-5), neither HsNup98 nor TtMacNup98A form amyloid structures (Extended Data Fig. 7d) due to differences in amino acid residue content. We determined whether MLF2, HSP70, or DNAJB6b affect the amyloid formation of ScNup116 over the course of 24 hours. To detect amyloids, we used the dye Thioflavin-T (ThT) that fluoresces in the presence of cross-β structures. By monitoring ThT fluorescence over time, we observe ScNup116 transition into amyloid structures (Extended Data Fig. 7d-f).

After 24 hours, neither HSPA1A nor DNAJB6b alone had any ability to prevent the formation of ScNup116 amyloids (Extended Data Fig. 7e). However, in the presence of ATP, the MLF2:HSP70 complex reduced the ThT-reactive cross-β structures even at a sub-stoichiometric concentration (Extended Data Fig. 7e). When DNAJB6b was included with the MLF2:HSP70 complex, the ability to prevent ScNup116 amyloid formation no longer depended on ATP and could prevent amyloid formation by about half even without ATP (Extended Data Fig. 7f). In contrast, HSP70 in complex with DNAJB6b strictly required ATP to prevent amyloid formation (Extended Data Fig. 7f). We interpret these data to suggest that MLF2 and DNAJB6b together have an ATP-independent Nup-directed activity that allows this complex to suppress amyloid formation. With HSP70, MLF2 has an ATP-dependent Nup-directed activity that suppresses ScNup116 amyloid formation most efficiently (Extended Data Fig. 7f).

Concluding from such a dataset that "MLF2 facilitates phase separating processes, revealing a hitherto unknown FG-Nup directed activity" is not based on the available data in my eyes.

We provide two new independent readouts of the in vitro *activity of the MLF2:HSP70 complex on FG-rich Nups. First, we demonstrate that the MLF2:HSP70 complex preserves FG particle morphology/integrity over time (Fig. 6e-h, Extended Data Fig. 7a-c). This effect is enhanced by the addition of WT DNAJB6b. Second, the MLF2:HSP70 complex significantly delays ScNup116 amyloid formation in an ATP-dependent manner (Extended Data Fig. 7d-g). HSP70 alone, in contrast, has no such effect. The ability for MLF2:HSP70 to decrease amyloid formation becomes ATP-independent when DNAJB6b is present. Despite also immersing within FG-rich particles, DNAJB6 alone had no effect on preventing amyloid formation.*

In addition to these in vitro *data, we already showed in the original version as a third line of evidence that overexpression of MLF2 in cells prevents ectopic FG-Nup accumulation (Fig. 5d,c). Evidence for a functional complex composed of MLF2, HSP70, and DNAJB6 is further strengthened by the observation that knocking down DNAJB6 or MLF2 influences how much of the other protein associates with HSPA1A, e.g., knocking down DNAJB6 greatly reduces the association of MLF2 with HSPA1A (Fig. 4b,c).*

Considering the plethora of new data obtained by time-resolved assays using a variety of independent readouts and multiple FG-Nups from distinct species, we hope the reviewer agrees that the MLF2-HSP70 complex has an FG-Nup directed activity that is enhanced in the presence of DNAJB6b.

Decision Letter, first revision:

9th August 2022

Dear Dr. Schlieker,

Thank you for submitting your revised manuscript "Atypical nuclear envelope condensates linked to neurological disorders reveal nucleoporin-directed chaperone activities" (NCB-A46885A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology. Please do not hesitate to contact me if you have any questions. Thank you again for your efforts revising the study,

Sincerely,

Melina

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns. I support publication of the revised manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my questions in a very satisfactory manner. Congratulations to the authors on this excellent publication.

Reviewer #3 (Remarks to the Author):

This is an excellent revision. All my comments have been adressed with high rigor and I highly recommend publication of this and the accompanying manuscript in NCB. The community will appreciate to learn about this new mechanism to chaperone FG Nups and this even in a desease relevant scenario!

This is up to the authors, as it does not affect the overall conclusions. However, I still highly caution how solid one can draw conclusion from the shape changes of condensates as done between line 258 and 272. As I mentioned before, the fact that we see more then one giant droplet, shows that the system has not reached thermodynamic equilbrium. So whatever changed shape, is some other effect (and I can think of many) and this is not necessarily the same as suggesting "that the MLF2:HSP70 complex possesses an FG-directed activity that facilitates the phase separation process".

17th August 2022

Dear Dr. Schlieker,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Atypical nuclear envelope condensates linked to neurological disorders reveal nucleoporin-directed chaperone activities" (NCB-A46885A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within one week). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Atypical nuclear envelope condensates linked to neurological disorders reveal nucleoporin-directed chaperone activities". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Cell Biology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

Best regards,

Nyx Hills
Staff
Nature Cell Biology

On behalf of

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: https://orcid.org/0000-0003-2389-2243

Reviewer #1:
Remarks to the Author:
The authors have addressed my concerns. I support publication of the revised manuscript.

Reviewer #2:

Remarks to the Author:
The authors have addressed all my questions in a very satisfactory manner. Congratulations to the authors on this excellent publication.

Reviewer #3:
Remarks to the Author:
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This is up to the authors, as it does not affect the overall conclusions. However, I still highly caution how solid one can draw conclusion from the shape changes of condensates as done between line 258 and 272. As I mentioned before, the fact that we see more then one giant droplet, shows that the system has not reached thermodynamic equilbrium. So whatever changed shape, is some other effect (and I can think of many) and this is not necessarily the same as suggesting "that the MLF2:HSP70 complex possesses an FG-directed activity that facilitates the phase separation process".

Author Rebuttal, first revision:

Rebuttal addressing the suggestion of Reviewer #3 (bolded):

This is up to the authors, as it does not affect the overall conclusions. However, I still highly caution how solid one can draw conclusion from the shape changes of condensates as done between line 258 and 272. As I mentioned before, the fact that we see more then one giant droplet, shows that the system has not reached thermodynamic equilbrium. So whatever changed shape, is some other effect (and I can think of many) and this is not necessarily the same as suggesting "that the MLF2:HSP70 complex possesses an FG-directed activity that facilitates the phase separation process".

We thank the reviewer for this important comment and have revised this section as follows to avoid making specific statements regarding MLF2's direct effect on phase separation:

Original text, line 258:

"The MLF2:HSP70 complex promotes the formation of large FG-rich condensates in vitro."

Revised text, line 216:

“Condensates have distinct properties when formed with MLF2.”

Original text, line 272:

“This suggests that the MLF2:HSP70 complex possesses an FG-directed activity that facilitates the phase separation process.”

Revised text, line 227-228:

“This suggests that MLF2:HSP70 possesses an FG-Nup directed activity.”

These statements are supported by a several independent experiments within our manuscript and avoids controversies regarding phase separation terminology.

Final Decision Letter:

Dear Dr. Schlieker,

I am writing on behalf of my colleague, Dr. Melina Casadio, who is out of the office.

I am pleased to inform you that your manuscript, "Atypical nuclear envelope condensates linked to neurological disorders reveal nucleoporin-directed chaperone activities", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Cell Biology style. Once your paper is typeset, you will receive an email with a link to choose the

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Please feel free to contact us if you have any questions.

With best regards,

Christina

--
Christina Kary, PhD
Chief Editor
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