Emerin self-assembly and nucleoskeletal coupling regulate nuclear envelope mechanics against stress

Anthony Fernandez, Markville Bautista, Liying Wu and Fabien Pinaud
DOI: 10.1242/jcs.258969

Editor: Jennifer Lippincott-Schwartz

Review timeline

Original submission:	27 May 2021
Editorial decision:	12 July 2021
First revision received:	7 December 2021
Editorial decision:	24 January 2022
Second revision received:	3 February 2022
Accepted:	14 February 2022

Original submission

First decision letter

MS ID#: JOCES/2021/258969

MS TITLE: Emerin self-assembly and nucleoskeletal coupling regulate nuclear envelope mechanics against stress

AUTHORS: Anthony Fernandez, Markville Bautista, and Fabien Pinaud
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Emerin is a protein that resides in the nuclear envelope and plays a key role in the maintenance of nuclear shape. This manuscript reports on the structural organization of emerin in cells, derived from various imaging technologies that achieve single-protein spatial resolution. The authors reveal emerin oligomerization in nano-clusters, measure the dynamics of emerin in live cells, and report interactions with other proteins including nuclear actin, lamin, BAF and SUN1. Upon mechanical stress, the authors find that emerin responds by a change in its oligomerization and dynamics, and that some protein interactions are perturbed. Finally, the authors investigate pathologically relevant emerin mutants, adding important information on the mechanism of dysregulation. The findings are summarized in a mechanistic model on nuclear shape regulation by emerin structural organization. Overall, the manuscript tackles an important question on how nuclear shape is maintained under conditions of mechanical stress; the date therein significantly improves our understanding on how emerin acts as a regulator. The authors applied a well-selected combination of biophysical methods, experiments are rigorous, and results are presented very clearly.

Comments for the author

At this point, I would like to disclose that my expertise lies in imaging technologies, which is why my comments and suggestions will largely focus on these aspects of the manuscript.
(1) The authors extract four diffusion states from the single particle tracking date; I have two comments that I ask the authors to address: (i) I am missing the minimal diffusion coefficient that, with respect to the localization error, can be determined (see e.g. Rossier 2012, with a cutoff of 8 x 10-3 um2/s); please add this information. Related to this, could the authors imagine that D3 and D4 are not distinguishable by their experimental approach, or that these two states are identical? (ii) D1 is populated to 1-4%; can the authors please comment how robustly this diffusion state may be determined in their analysis?
(2) The diffusion of emerin is responsive to lamin and actin downregulation (lines 109ff), and an increase in the diffusion coefficient of D3 and D4 is found in both cases. Different however is that D2 is only affected in case of depleting nuclear acting (through IPO3). Could the authors think of a possible explanation?
(3) The data on emerin clustering is exciting (figure 3). I wonder whether any assumption on molecular numbers within the oligomeric clusters can be made? This might be possible by either analyzing the number of blinking events, or the kinetics of the blinking events; since the data is available, this information might be accessible with reasonable effort (see e.g. PMID 32541966)?
(4) The authors report several interactions of emerin with other proteins. In order to assess the level and abundance of these interactions, it would be helpful to show two-color microscopy data. This does not have to be super-resolution data; the degree of perturbation of some interactions, e.g. in the presence of stress, might be already visible in confocal images.

Minor comments
- line 174, please add the original reference for dSTORM
- line 701, the authors reference Thompson et al. (#66) to calculate the localization precision; the formula therein has been corrected in Mortensen et al., Nat Meth 2010
- wherever the authors think it is possible, I ask the authors to consider avoiding bar plots and instead show the data points in a different representation; please also check whether a t-test is applicable (requires symmetric distributions); suggestions on graphical presentation with further helpful hints are e.g.
https://www.nature.com/articles/s41592-019-0470-3
https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1002128

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Fernandez et al. use single molecule tracking and super-resolution microscopy to investigate the nanoscale spatial organization of emerin at the inner and outer nuclear membranes in cells subjected to mechanical challenges. They demonstrate that emerin assembles into localized oligomeric nanoclusters that appear to be stabilized by A-type lamins and SUN1. The authors further show that the nanoscale spatial organization and mobility of emerin is also influenced by BAF and nuclear actin.
Interestingly, emerin's nanoscale spatial organization was found to change in response to culturing cells on different micropatterns to induce mechanical strain on the nucleus. These changes in emerin oligomerization and mobility were shown to be important determinants of nuclear shape adaption against force. Finally Fernandez et al. reveal that Emery-Dreifuss muscular dystrophy-associated emerin mutations, the expression of which results in abnormal nuclear envelope deformations, exhibit defects in their ability to form A-type lamin and SUN1-stabilized oligomers.
Overall, I found this manuscript to be well written and its results to be quite exciting. That beings said, I would like the authors to address several issues (see below) that I had with their work before I feel comfortable recommending this work for publication at the Journal of Cell Science.

Comments for the author

1) The authors need to provide a Western blot to verify that they are able to successfully knockdown IP09.
2) Is there anyway for the authors to provide similar data from the nuclear envelope that is furthest away from the coverslip? It would be very interesting to see if the nanoscale spatial organization of emerin at the inner nuclear membrane differs between the ventral and dorsal nuclear envelopes of a single cell.
3) I think that it is important for the authors to demonstrate that they can rescue the effect of knocking down lamin A/C, IPO9, BAD, or SUN1 by re-expressing an RNAi-resistant cDNA construct that encodes the wild type form of the depleted protein. This will allow the authors to rule-out any potential off-target effects caused by their siRNAs.
4) What happens if the authors over-express lamin A/C or SUN1 or increase the levels of nuclear actin on the nanoscale spatial organization of emerin at the inner nuclear membrane? Do they see the opposite effect of depleting these proteins?
5) The authors state that the impact of the depletion of SUN1 on emerin is evidence of the involvement of the LINC complex in the nanoscale spatial organization of emerin at the inner nuclear membrane. While this result may support their conclusion, it is far from definitive. To really demonstrate the involvement of the LINC complex in this process (and not SUN1 operating on its own), the authors need to test the effect of the over-
express a dominant negative LINC complex inhibitor construct (e.g. the KASH domain of nesprin-1/2 or the luminal domain of SUN1) on emerin organization. It would also be powerful if the authors were to rescue their SUN1-depleted cells with a SUN1 construct that cannot interact with the KASH peptides of nesprins (e.g. a construct lacking the SUN domain or one that cannot homo-oligomerize).
6) I am a bit concerned about the fractionation-based method used by the authors in Figure S2 to quantify the levels of nuclear actin and how they change in response to various RNAi treatments. My concern stems from the fact that actin associated with the outer nuclear membrane cannot be distinguished from actin present within the nucleus using this method. I would strongly suggest that they authors use the previously described anti-nuclear actin chromobody (see Plessner et al. 2015 J Biol Chem) to more carefully assess the levels (and organization) of nuclear actin in their cells.
7) How do the authors know that what they are referring to as "monomers" are actually monomers and not an oligomer with only one active fluorescent protein?

Reviewer 3

Advance summary and potential significance to field

A set of inner nuclear envelope proteins, including LEM-domain proteins, are described as exhibiting large unstructured regions (IDR). In the case of the LEM-domain protein emerin, one of

the most studied inner nuclear envelope proteins, IDRs are responsible for emerin oligomerisation. However, the molecular details of emerin oligomers, as well as the function of these oligomers, are not clear, and difficult to study. This manuscript reports a high resolution study of these oligomers and their function in response to a mechanical stress in cells. It is an important contribution to the field.

Comments for the author

At the inner nuclear membrane, LEM-domain proteins exhibit large intrinsically disordered nucleoplasmic regions, whose functions are still unclear. Emerin, one of the most studied LEM-domain proteins, is phosphorylated in its disordered region after a mechanical stress, and this contributes to nuclear adaptation to the stress. Emerin also oligomerizes in vitro and in cells. However, how do its disordered regions promote oligomerisation and what is the role of emerin oligomerization in the nuclear response to a mechanical stress
are still unanswered questions. This work addresses these questions using single molecule tracking and super resolution microscopy. An extensive description of emerin mobility and oligomeric states is provided the role of emerin interaction with its best known partners, as well as the impact of a mechanical stress, on these emerin properties is explored, and finally, emerin variants detected in patients with muscular dystrophy are characterized, to identify defects in emerin mobility, oligomeric states, as well as behavior after a mechanical stress. The experimental results are robust and novel. Only the discussion is sometimes difficult to follow, because it is based on a large number of (sometimes contradictory) experimental data previously published on emerin. Summarizing these previous data at the beginning of the discussion in a clear and condensed manner would have helped to follow the interpretation of the new data provided by this study.

Comments:
1) In the abstract, the authors write: "the abnormal nuclear envelope deformations induced by EDMD emerin mutants stem from a defective formation of lamin A/C and LINC complex-stabilized emerin oligomers". Is it really true for all the variants (and especially Q133H; see Fig. 6c) ? Also, no experimental results are obtained on the variants after depletion of lamin A/C and SUN1, so that this sentence seems to be not completely accurate.
2) The introduction is clear and well-focused.
3) Fig.2: how do the authors explain that, after depletion of nuclear actin, the mobility of emerin anchored at the outer nuclear membrane is significantly increased ? This emerin fraction is assigned to the outer nuclear membrane because depletion of lamin A/C does not affect its mobility > couldn't it be an emerin fraction at the INM that does not bind to lamin A/C (so is more mobile) ? Could you check using an independent experiment that in your cells, 10% of emerin is really located at the ONM ?
4) Fig.3: if lamin A/C and BAF were simultaneously depleted, would the impact be additive ? From the current data, as the impact of lamin A/C depletion is more important than that of BAF depletion, we can conclude that lamin A/C anchors emerin partly independently from BAF, however the opposite is unclear.
5) Fig.3: the data clearly show that depletion of either lamin A/C or SUN1 strongly decreases emerin oligomerization. But does SUN1 favor lamin binding to emerin, or lamin favor SUN1 binding to emerin ? Or are these binding events totally independent one from the other ?
6) Fig.4: Depletion of lamin A/C or nuclear actin causes the same nuclear shape defect after a mechanical stress (lack of adaptation to the mechanical stress). Why is the impact of depleting SUN1 and BAF, as performed in other experiments, not shown here ? Similarly, depletion of nuclear actin does not modify emerin mobility after a mechanical stress. What is the impact of depletion of lamin A/C in this same experimental set up ?
The interpretation of the lack of impact of nuclear actin depletion on emerin mobility after a mechanical stress is that the stress disengages nuclear actin from the nucleoskeleton/emerin complexes. Could this be related to phosphorylation of Tyr74/Tyr95 ?
7) Figs.6 & 7: interpretation of these data is complicate, especially because it is based not only on the new data reported by the authors, but also on the contradictory results found in the literature and obtained through very different experimental set up. Many arguments were unclear for this reviewer.
Interpretation of Q133H: the distribution of oligomers displayed in Fig 6c seems close to the WT distribution however the mobility of this variant at the INM is increased, how can these two observations can be consistently interpreted ? Also, the authors write: "Q133H mutation disrupts

emerin binding to actin but does not impede interactions with lamin A/C, SUN1 or BAF". I would be more cautious because these results have rarely been obtained on purified proteins. These are often more indications than clear demonstrations of (a lack of) binding. Other studies also report opposite results, as Herrada et al. (2015) concerning emerin binding to lamin A/C.
Also, the authors write: "The increased lateral diffusion of Q133H, only at the INM, therefore indicates that it does not bind nuclear actin" > it could be that it does not bind lamin A/C as well. Also, depletion of nuclear actin increases the mobility of the outer membrane fraction of emerin (Fig. 2a) whereas depletion of lamin A/C does not (Fig. 2a also). So why strongly correlating an increased lateral diffusion of Q133H to a lack of binding to nuclear actin ?
8) Figs. 6& 7: in the case of P183H, it is not clear for this reviewer how different the behavior of this variant is when compared to del95-99. And what are the green bars on Fig. 6g compared to the red bars ? Also, why is the study of the behavior of del95-99 under stress (Fig. 7) not shown also for P183H ?

First revision

Author response to reviewers' comments

Responses to Reviewers' comments

Reviewer 1

Advance Summary and Potential Significance to Field:
Emerin is a protein that resides in the nuclear envelope and plays a key role in the maintenance of nuclear shape. This manuscript reports on the structural organization of emerin in cells, derived from various imaging technologies that achieve single-protein spatial resolution. The authors reveal emerin oligomerization in nano-clusters, measure the dynamics of emerin in live cells, and report interactions with other proteins including nuclear actin, lamin, BAF and SUN1. Upon mechanical stress, the authors find that emerin responds by a change in its oligomerization and dynamics, and that some protein interactions are perturbed. Finally, the authors investigate pathologically relevant emerin mutants, adding important information on the mechanism of dysregulation. The findings are summarized in a mechanistic model on nuclear shape regulation by emerin structural organization. Overall, the manuscript tackles an important question on how nuclear shape is maintained under conditions of mechanical stress; the date therein significantly improves our understanding on how emerin acts as a regulator. The authors applied a well-selected combination of biophysical methods, experiments are rigorous, and results are presented very clearly.

Comments for the Author:
At this point, I would like to disclose that my expertise lies in imaging technologies, which is why mycomments and suggestions will largely focus on these aspects of the manuscript.

(1) The authors extract four diffusion states from the single particle tracking date; I have two comments that I ask the authors to address: (i) I am missing the minimal diffusion coefficient that, with respect to the localization error, can be determined (see e.g. Rossier 2012, with a cutoff of 8 x 10-3 um2/s); please add this information. Related to this, could the authors imagine that D3 and D4 are not distinguishable by their experimental approach, or that these two states are identical? (ii) D1 is populated to 1-4%; can the authors please comment how robustly this diffusion state may be determined in their analysis?

(i) To provide an estimate of the minimal diffusion coefficient that can be determined in our experiments, we attempted to imaged PA-TagRFP-emerin in fixed cells. Unfortunately, following paraformaldehyde (PFA) fixation, PA-TagRFP could not be photoswitched, consistent with previous observations that chemical fixation can affect the photophysical properties of some photoswitchable FPs (e.g. see comments in **Finan *et al.*, Methods Mol. Biol. 2013**). PA-Tag-RFP-emerin could be photoswitched following cell fixation with ice-cold acetone/methanol (**Fig. R1**), yet the distribution of localization precisions appeared slightly degraded compared to live cells,

again possibly indicating an altered photophysics of the switchable FP. In addition, we detected a significant amount of mobile PA-Tag-RFP- emerin in acetone/methanol-fixed cells, suggesting that this fixation protocol does not fully immobilize emerin as previously reported for other membrane proteins (**Tanaka *et al.*, Nature Methods 2010**). In effect, tracking of PA-Tag-RFP-emerin in acetone/methanol-fixed cells resulted in apparent PA-Tag-RFP- emerin displacements larger than in live cells (**Fig. R1**).

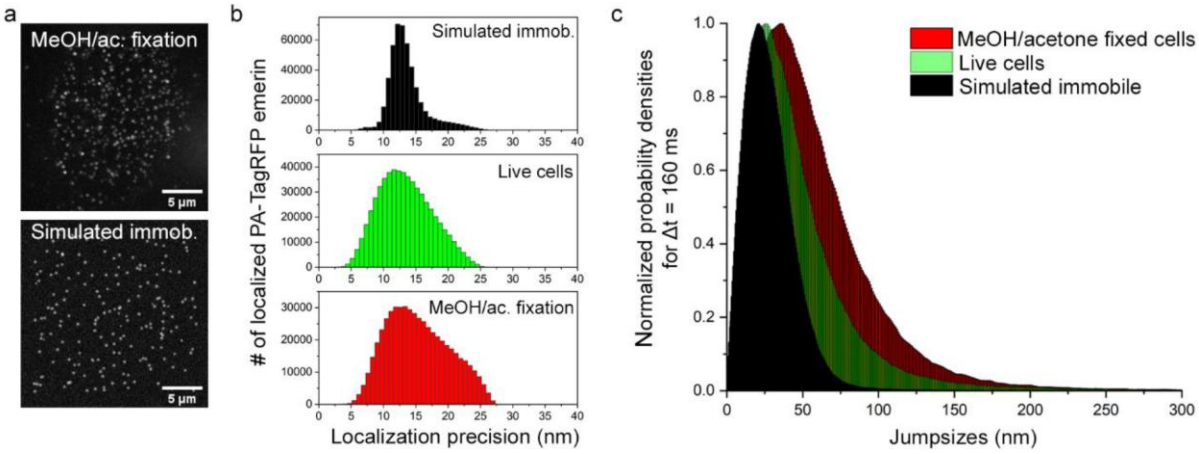

Fig. R1: a) Examples of PA-TagRFP-emerin single molecule signals acquired at the NE after cell fixation with methanol/acetone (top) or by simulating immobilized molecules with FluoSim (bottom). b) Comparison of the distribution of localization precisions for individual PA-TagRFP-emerin acquired in live cell, after methanol/acetone cell fixation or after simulation as immobile molecules. c) Comparison of the step size probability distributions at Δt = 160 ms, for simulated immobile PA-TagRFP emerin (black, n=345930 events), wild-type PA-TagRFP emerin in live cells (green, n=297037 events) and wild-type PA-TagRFP emerin in methanol/acetone-fixed cells (red, n=303602 events). The presence of large step sizes in the acetone/methanol- fixed cells is indicative of ineffective PA-TagRFP emerin immobilization by this fixation protocol.

As an alternative to studying PA-TagRFP in chemically fixed cells, we simulated immobile individual PA- TagRFP molecules using the software Fluosim (**Lagardère *et al.*, Scientific Reports 2020**), setting PSF properties, acquisition parameters, background and EMCCD readout noise, similar to our live cell imaging conditions (**Fig. R1**). We then localized and tracked ~10000 simulated molecules with SLIMfast, using the same parameters as for live cells. This resulted is a localization precision of 13 ± 3 nm, relatively similar to that of our experimental data (**Fig. R1**). Like in live cells, the apparent diffusion coefficient for these immobile molecules was determined using an analysis of the PDSD (in this case i = 1) and fitting the resulting $r^2(t)$ curve over the first four time lags (t1-t4) with equation (3) in the main text. Note that the number of events (N) used to build the PDSD curve at each time lag was fairly similar to experimental data (e.g. for Δt = 40 ms, $N_{immobile}$: 409189 events *vs.* $N_{wild\text{-}type\ emerin}$: 493378 events). Under these conditions the apparent diffusion coefficient of immobile PA-TagRFPs was $D_{immobile}$: $1.7\times10^{-4} \pm 2\times10^{-5}$ µm^{2}s^{-1}. This value is somewhat consistent with the lower limited expected for immobile particles, defined by Sergé *et al.* (Nature Methods, 2008) for the MTT tracking algorithm used in SLIMfast as:

$$D_{min} \sim \frac{\sigma^2}{4\tau} \quad (1)$$

where σ is the localization precision (13 nm), and τ the time interval to fit the $r^2(t)$ curve (4*0.04= 0.16 s), giving D_{min} ~ 2.6×10^{-4} µm^{2}s^{-1}.

Note that if fitting of the $r^2(t)$ curve for immobile PA-TagRFPs is done on slightly longer time lags (e.g. t1-t6), the difference between $D_{immobile}$ and D_{min} is reduced, with $D_{immobile}$: $1.4\times10^{-4} \pm 1\times10^{-5}$ µm^{2}s^{-1} and D_{min}~ 1.7×10^{-4} µm^{2}s^{-1}. This likely reflects the influence of our localization error on early time points. Refitting our sptPALM data between t1-t6 did not significantly impact our estimated diffusion coefficients, and we deem it unwarranted to recalculate all the diffusion coefficients in the manuscript with this slightly longer fit, as data interpretations would not change.

Based on these results, we have now provided a value of $D_{immobile}$: $1.7\times10^{-4} \pm 2\times10^{-5}$ µm^{2}s^{-1} in the manuscript and consider diffusion coefficients equal or inferior to this threshold as reflecting

emerin immobilization (**lines 865-869**). This is the case for the D4 values of mutated emerin Δ95-99 and P183H oligomers. The manuscript has therefore been modified accordingly (**lines 367 and 399**), and data interpretation are not affected by the classification of Δ95-99 and P183H oligomers as immobile. These results also indicate that the diffusion of emerin monomers (D3) and oligomers (D4) are distinguishable and correspond to two different diffusing states.

Regarding the Rossier *et al*. (**Nature Cell Biology 2012**) article mentioned by Reviewer 1, it is not clear to us why the authors use the spatial resolution as a parameter to define $D_{immobile}$. We also point to the fact that, in this 2012 study, diffusion coefficients are evaluated from the MSD of individual trajectories and the authors do not appear to include a localization error offset ($4\sigma^2$) when fitting MSDs, which could result in largely overestimated diffusion coefficients.
The theoretical basis to evaluate the minimal diffusion coefficient that can be measured given a specific localization error can be derived from the work of Michalet (**Physical Review E 2010**) and Michalet & Berglund (**Physical Review E 2012**), where the theoretical limit of diffusion coefficient uncertainty in individual MSD analyses was defined. As briefly discussed in Michalet 2010, this theoretical basis is applicable to ensemble MSD for multiple trajectories and to PDSD analyses (separation of ensemble MSDs from mixed populations across multiple trajectories).
For instance, to find the smallest D value (D_{min}) that can be safely estimated from experimental data (N trajectory points, NT trajectories) characterized by a localization uncertainty σ, a frame separation Δt and a frame exposure t_E, one can use equation (12) of Michalet & Berglund 2012, combined with results of Section VII.A of Michalet 2010, to obtain the Cramer-Rao lower bound for the error S(D) on D:

$$\frac{S(D)}{D} \geq \left[\frac{1+2\sqrt{1+2x}}{(N-1)N_T}\right]^{\frac{1}{2}} \quad (2)$$

where $x = \frac{\sigma^2}{D\Delta t} - 2R$ (Eq. 4, Michalet, 2012) and $R = \frac{t_E}{6\Delta t}$

We can then define a maximum tolerable relative error of S(D)/D = α < 1 in order to obtain D_{min}. A value α = 0.05 (5% error) would appear to be entirely satisfactory. The corresponding D_{min} can then be obtained by solving equation (2) for D, assuming all other parameters are known. We obtain:

$$Dmin = \frac{\sigma^2}{\Delta t\left[\frac{\left[(N-1)N_T\alpha^2-1\right]^2-4}{8}\right]+\frac{t_E}{3}} \quad (3)$$

For example, using N = 10, $N_T = 10^3$, α = 0.05, σ = 20 nm and $t_E = \Delta t = 40$ ms, we get $D_{min} = 1.7 \times 10^{-4}$ μm²/s. Using a larger number of trajectories, or longer trajectories, or a less stringent condition for the relative error for Dmin, this value would be diminished correspondingly. From equation (3), the number of trajectory points used to build an ensemble MSD curve (e.g. the length of trajectories (N) and the number of trajectories (N_T)) matters when trying to estimate a D value, including Dmin. In other word, provided that a sufficiently high number of trajectory points and trajectories are available, one can, in principle, measure any D no matter what the localization uncertainty is. We stress, however, that estimating a Dmin value for a S(D)/D = α value close to the Cramer-Rao lower bound, requires fitting the MSD on an optimal number of points, as discussed in Michalet 2010. In our case, we did not attempt to optimize the number of points used to fit our $r^2(t)$ curves and we therefore use the simulated data of immobile PA-TagRFPs and fit over t1-t4 for Dmin evaluation, as described above.

(ii) D1 effectively represents a small percentage of $r^2(i)$ in PDSDs at each time lag (~1-4%). Yet, the number of steps used to build each PDSDs in very large. For instance, with a median length of trajectories of 0.24s (6 steps) and ~70000 trajectories for wild-type PA-TagRFP-emerin, PDSDs are built with a median number of steps of 420000. Taking 1% of this value, D1 is thus evaluated from approximatively 4,200 steps. This is equivalent to building an MSD curve from a single diffusing particle with 4200 steps. As discussed in (i) above, we can use equation (2) to estimate S(D)/D = α, given our acquisition parameters. In our case, using the worse localization precision of σ = 25 nm, at

the right tail of our histogram of distribution (**Fig.R1b**), $t_E = \Delta t$ = 40 ms, N = 4200, NT = 1, and D1 = 2.21×10^{-1} $\mu m^2 s^{-1}$ for wild-type PA-TagRFP-emerin, we obtain $\alpha < 5\%$. Again, this assume that an optimal number of points was used to fit the $r^2(t)$ of D1. As presented in Michalet 2010, for x values < 1 (which is the case for our D1 values), a fit of $r^2(t)$ over 4 points (t1-t4) would be close to optimal to minimize the error in finding D for a trajectory of 1000 points and above. This indicates that under our imaging and analytical conditions we should be able to obtain value of D1 with less than 5% error from its true value, despite D1 representing only a small fraction of detected step events.

(2) The diffusion of emerin is responsive to lamin and actin downregulation (lines 109ff), and an increase in the diffusion coefficient of D3 and D4 is found in both cases. Different however is that D2 is only affected in case of depleting nuclear acting (through IPO3). Could the authors think of a possible explanation?
Unfortunately, we do not yet have a clear understanding as to why the lateral mobility of emerin at the ONM increases when nuclear actin is downregulated (or when nuclei are subjected to force). We have observed similar increases in ONM and INM diffusion of emerin when pulling on the NE in live cells via micropipette suction, consistent with the idea that these enhanced mobilities are linked to rapid changes at/around the NE, following force application.
Because a similar increase in ONM emerin mobility is seen when endogenous BAF is knocked-down and replaced by BAF^{L58R}, which does not bind the LEM domain of emerin, it is possible that nuclear actin depletion induces downstream changes in the interaction of ONM emerin with cytoplasmic BAF. This could be related (i) to the phosphorylation/glycosylation state of emerin (e.g. on the LEM domain) which can modulate binding to BAF (**Tifft *et al.*, Journal of Cell Science 2009; Berk *et al.*, Journal of Biological Chemistry 2013**) and (ii) to its redistribution towards the ONM (increased diffusion). Impeding emerin- LEM/BAF interactions can indeed result in a larger fraction of ONM emerin, as shown with BAF^{L58R} (Fig. 2A, 21% *vs.* 8% for population D2). During nuclear deformation after micropatterning, which likewise induces an increase in ONM emerin mobility, a significant amount of emerin also redistributes towards the ER, as we observed previously (**Bautista *et al.*, Micromachines 2018**). It would be of interest to evaluate if the pool of ONM emerin and its phosphorylation/glycosylation state change significantly after actin depletion, BAF^{L58R} or force application on micropatterns.
As for the emerin mutants, possible additional defects in their post-translational modifications, including phosphorylation/glycosylation (**Tifft *et al.*, Journal of Cell Science, 2009; Berk *et al.*, JBC 2013**), might preclude the detection of this faster ONM emerin pool.

To address the comment of Reviewer 1, we have modified the manuscript to underline that the faster ONM diffusion of emerin after IPO9 KD remains to be defined (**lines 127-128**).

(3) The data on emerin clustering is exciting (figure 3). I wonder whether any assumption on molecular numbers within the oligomeric clusters can be made? This might be possible by either analyzing the number of blinking events, or the kinetics of the blinking events; since the data is available, this information might be accessible with reasonable effort (see e.g. PMID 32541966)?

The ability to estimate the number of proteins in a cluster based on individual protein counts and blinking statistics of a fluorophore is indeed a very exciting development for the field. We feel, however, that in our case this information might not be particularly relevant considering our experimental conditions. Indeed, we do not image emerin expressed at endogenous levels, but SNAP-emerin fusions for which we do not tightly control the expression levels. This is the primary reason why we provide relative clustering densities of emerin as compared to the average density detected across the NE. This standardization allows us to compare emerin densities between different cells, without interferences from potential variations in emerin expression levels and even in cases where complete BG-A647 labeling might not have been achieved. We will, however, apply statistical analyses similar to those described in PMID 32541966 when we implement CRISPR insertions of our SNAP-emerin fusions in future studies, to achieve endogenous cellular expression levels.

(4) The authors report several interactions of emerin with other proteins. In order to assess the level and abundance of these interactions, it would be helpful to show two-color microscopy data. This does not have to be super-resolution data; the degree of perturbation of

some interactions, e.g. in the presence of stress, might be already visible in confocal images.
The interactions of emerin with other proteins we have discussed in the manuscript have been studied by different research groups over the years, using in vitro co-immunoprecipitation, binding affinity assays or, more recently, antibody proximity ligation assays in cells.
Because the optical resolution of two-color confocal microscopy is diffraction-limited, it will not likely provide evidence of productive or defective interactions of emerin with other proteins, unless it is used in the context of carefully designed FRET imaging experiments. At best, perturbations that induce changes in the interaction of emerin with some of its nucleoplasmic partners might be visualized by a partial re- localization of the emerin pool towards the ER membrane in confocal images (See for example the effect of SUN1 over-expression on emerin localization discussed in question 3 of Review 2). For instance, we have previously shown that nuclear stress in micropatterned cells induces a redistribution of emerin toward the ER membrane (**Bautista et al., Micromachines 2018**). Lamin A/C depletion and some emerin mutants that do not bind lamin A/C or actin were also shown to result in emerin localizing less efficiently at the NE, although emerin still displays significant NE association and mutants have similar FRAP diffusion characteristics compared to wild-type emerin, as we have shown. The apparent limited impact of interfering with emerin binding to its partners or expressing emerin mutants on the cellular distribution of emerin indicates that emerin interactions with its partners are complex, multiprong and likely take place on scales smaller than the optical diffraction limit. Thus, confocal imaging is not expected to clearly reveal changes in these interactions.
As suggested by Reviewer 1, we have nonetheless assessed if nuclear stress by cell micropatterning additionally triggers changes in the organization of emerin's binding partners, and if such changes might be noticeable in dual-color confocal images. As can be seen from **Fig. R2**, partial redistribution of emerin towards the ER membrane is observed when cells are grown on 15 µm micropatterns, as we previously reported (**Bautista et al., Micromachines 2018**). However, these perturbations do not result in obvious changes in the cellular organization of emerin's binding partners, including lamin A/C, SUN1, BAF or nuclear actin, as predicted. This confirms that dual-color confocal imaging is not a suitable approach to evaluate the level and the abundance of emerin interactions with other proteins. It also justifies the use of super-resolution imaging techniques, siRNA and emerin mutations to define how such interactions impact emerin organizations.

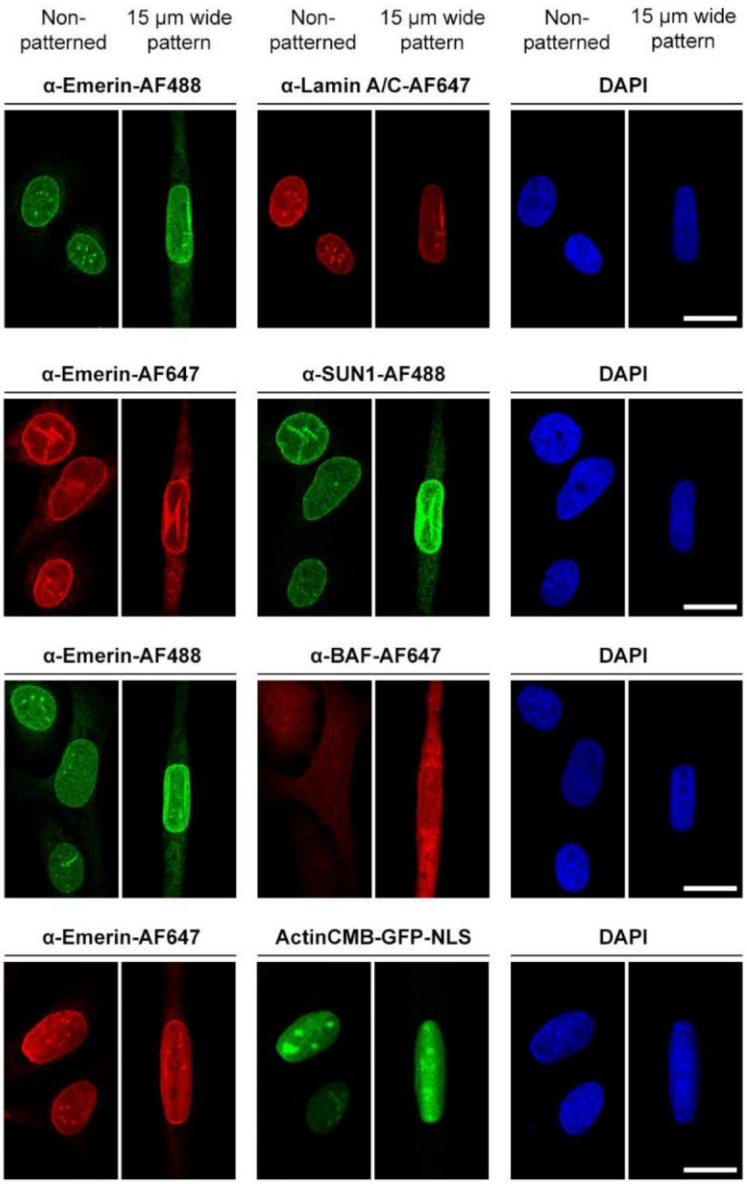

Fig. R2: Dual-color confocal imaging of emerin with lamin A/C, SUN1, BAF or nuclear actin in wild-type HDF grown randomly on fibronectin substrates or micropatterned on 15 µm-wide fibronectin strips to induce nuclear stress. Primary antibodies are described in the main text and secondary antibodies are labeled with Alexa fluor 488 (AF488) or Alexa fluor 647 (A647). Attempt to detect nuclear actin was done using a nuclear actin chromobody fused to tagGFP (ActinCMB-GFP-NLS). While emerin partially re-localizes toward the ER following micropatterning, there is not obviously changes in the distribution of lamin A/C, SUN1, BAF or nuclear actin compared to non-patterned cells at this imaging resolution. All scales: 20 µm.

Minor comments

- **line 174, please add the original reference for dSTORM**

The reference has been added. A reference to sptPALM with PA-TagRFP has also been added. (**lines 81-82**).

- **line 701, the authors reference Thompson et al. (#66) to calculate the localization precision; theformula therein has been corrected in Mortensen et al., Nat Meth 2010**

The reference has been added. (**line 839**)

- **wherever the authors think it is possible, I ask the authors to consider avoiding bar plots and insteadshow the data points in a different representation; please also check whether a t-test is applicable (requires symmetric distributions); suggestions on graphical presentation with further helpful hints are e.g. https://www.nature.com/articles/s41592-019-0470-3**

https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1002128
The bar plots presented through-out the manuscript report on the diffusion coefficient values or the clustering values determined for emerin under the multiple conditions described. Those individual values are obtained by least-square fitting of the $r^2(t)$ curve for each diffusing population or least-square fitting of the relative neighborhood density curves for super-resolution data. We believe that bar plots are the most comprehensive way to report these individual values as it allows a reader to easily identify different emerin subpopulations (ER, ONM, INM monomers and INM oligomers) or different clustering state (monomer and oligomers) and to rapidly compare different conditions. We try to avoid bar plots whenever possible, but in this case, we think those are essential.

We apologize for a typo indicating the F-tests were used to compare diffusion coefficients. All diffusion coefficients extracted by fitting $r^2(t)$ curves were compared using t-tests. The typo has been corrected in the manuscript. As mentioned by Reviewer 1, t-tests assume a symmetric distribution of the data underlying the process being studied. In our case, we extract a mean diffusion coefficient for each emerin sub-population by least-square fitting of a $r^2(t)$ curve over short time lags (t1-t4), after separation of each sub-population from our ensemble of trajectories. As such, the diffusion parameter extracted by least square fitting (diffusion coefficient value) and the use of t-tests to compare this parameter across samples, are valid as long as the probability distribution function of the mean square displacements for the selected time lags (t1-t4) is symmetric.
As previously reported by Michalet (**Physical Review E 2010**), the probability distribution function of the mean square displacements for an individual trajectory is quasi-Gaussian for small time lags, but become more asymmetric for larger time lags. The same observation was made by Saxton (**Biophysical Journal 1997**) when looking at the statistical distribution of single-trajectory diffusion coefficients obtained by fitting MSD curves over short or long time lags. Thus, provided that a $r^2(t)$ curve is fitted over a small number of points that are well averaged (e.g. t1-t4), as is the case for all our sptPALM data, the underlying distribution can be considered symmetric and the use of t-tests is appropriate.

The comment of Reviewer 1 also prompted us to verify that all the NSI values we report are distributed normally, as required for t-test statistics. This verification was done initially for most NSI measurements, but not systematically. Out of the 26 NSI measurements described in the manuscript we identified 6 conditions where the NSI values do not approximate a normal distribution. For the sake of rigor, we have now re-evaluated statistical comparisons across all samples using a non-parametric Wilcoxon rank-sum test that does not assume a normal distribution of the NSI. Using the Wilcoxon test, all the NSI data in Fig. 4C remain significantly different at $p<0.01$ and interpretation are unchanged. In Fig. 5b, significant differences also remained unchanged except for: (i) Δ95-99 in non-patterned cells ($p<0.05$, p value:0.0114) and (ii) Q133H in 15 um patterns ($p<0.01$, p value: 0.0008). Data interpretation is not affected by these changes, and Fig. 5b has been modified accordingly, together with the main text.

Reviewer 2

Advance Summary and Potential Significance to Field:
In their manuscript, Fernandez et al. use single molecule tracking and super-resolution microscopy to investigate the nanoscale spatial organization of emerin at the inner and outer nuclear membranes in cells subjected to mechanical challenges. They demonstrate that emerin assembles into localized oligomeric nanoclusters that appear to be stabilized by A-type lamins and SUN1. The authors further show that the nanoscale spatial organization and mobility of emerin is also influenced by BAF and nuclear actin. Interestingly, emerin's nanoscale spatial organization was found to change in response to culturing cells on different micropatterns to induce mechanical strain on the nucleus. These changes in emerin oligomerization and mobility were shown to be important determinants of nuclear shape adaption against force. Finally, Fernandez et al. reveal that Emery-Dreifuss muscular dystrophy-associated emerin mutations, the expression of which results in abnormal nuclear envelope deformations, exhibit defects in their ability to form A-type lamin and SUN1-stabilized oligomers. Overall, I found this manuscript to be well written and its results to be quite exciting. That beings said, I would like the authors to address several issues (see

below) that I had with their work before I feel comfortable recommending this work for publication at the Journal of Cell Science.

Comments for the Author:

1) The authors need to provide a Western blot to verify that they are able to successfully knockdown IP09.

We tried extensively to assess the knockdown of IPO9 by immunoblotting and immunofluorescence imaging, without real success. Indeed, none of the antibodies we tested (Abnova polyclonal #PAB0154, C-terminal epitope or Sigma #SAB4200155, N-terminal epitope) was capable of reliably detecting IPO9 by immunoblotting in our cells, and this despite extensive variations in conditions (concentrations, incubation times, immunoblot membranes, etc.). Those same antibodies also failed at reliably detecting IPO9 by immunostaining. As shown in **Fig. R3** below, after immunostaining with the Sigma #SAB4200155 anti-IPO9 antibody, fluorescent signals are barely above background levels. We did detect a small reduction in nuclear and cytoplasmic IPO9 staining intensities following treatment of cells with our IPO9 siRNA (**Fig. R3A**), which was confirmed, to some extent, by testing the effect of stably expressing a small hairpin RNA (shRNA) against IPO9 (**Fig. R3B**). However, in both case, fluorescent signals remained extremely low, suggesting either inefficient recognition of the Sigma anti-IPO9 antibody or low endogenous IPO9 expression levels in HDF. The Abnova anti-IPO9 antibody also gave immunostaining signals level barely above background. Again, we sampled many different conditions (concentrations, incubation times, fixation protocols, etc.) to optimize cell immunostaining with both anti-IPO9 antibodies, but we did not manage to improve signal levels above those seen in **Fig. R3**.

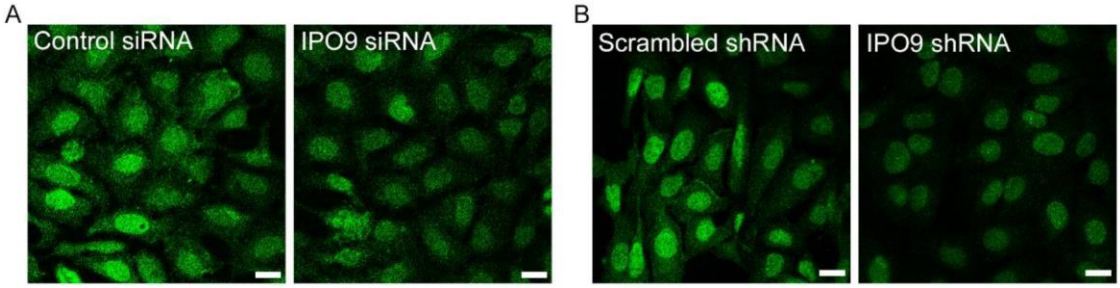

Fig. R3: **Immunostaining of HDF with Sigma SAB4200155 anti-IPO9 antibody**. (A) Confocal images of HDF after treatment with control or IPO9 siRNA. Scale: 20 µm. (B) Confocal images of HDF stably expressing a scramble shRNA or an IPO9 shRNA. Scale: 20 µm.

We note that the expression levels of IPO9 in primary skin cells and tissues appear to be much lower than in established cell lines (on the order of 50 to 100-fold lower expression) as per integrated proteomics data from the Genecards database (https://www.genecards.org/cgi-bin/carddisp.pl?gene=IPO9), which could explain our inability to reliably detect IPO9 in HDF.

The effects of IPO9 knockdown on nuclear actin content and its organization have been extensively characterized, and rather than spending time and money testing and identifying a functional anti-IPO9 antibody for HDF, we resorted to evaluating those effects more directly using nuclear actin probes, as suggested by Reviewer 2 in question 6. As shown and discussed in our answers to question 6, IPO9 siRNA effectively results in a reduced formation of short nuclear actin filaments, fully consistent with the reduced content in nuclear actin we had already established by immunoblotting against actin.

2) Is there anyway for the authors to provide similar data from the nuclear envelope that is furthest away from the coverslip? It would be very interesting to see if the nanoscale spatial organization of emerin at the inner nuclear membrane differs between the ventral and dorsal nuclear envelopes of a single cell.

The organization of emerin can be studied at the dorsal NE, but we generally prefer imaging at the ventral NE for a couple of technical reasons:

(i) The HILO sheet tends to broaden significantly at the large angles required to reach the dorsal NE, which reduces our signal-to-noise ratios during dSTORM and impacts localization accuracies.

(ii) We have found that the dorsal NE often display significant more folds than the ventral NE,

which limits the selection of areas with relatively homogenous z-ranges to study the two-dimensional cluster organization of emerin.

We have now provided an analysis of the nanoscale organization of emerin at the dorsal NE, in regions devoid of obvious membrane folds. As seen in **Fig. R4** below, emerin distributes and organizes as monomers and oligomers in a manner very similar to that observed at the ventral NE. These data have been added in Supporting Information and briefly mentioned in the main text. (**lines 195-196**)

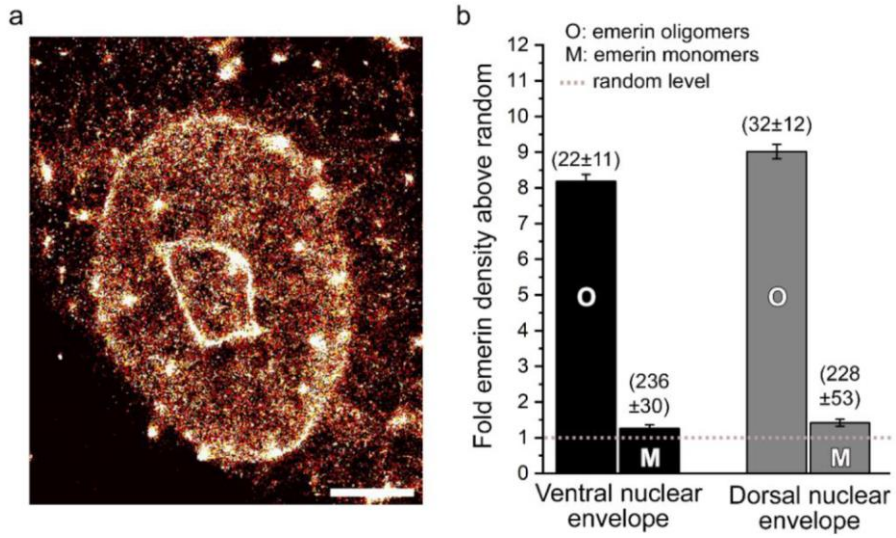

Fig. R4: Nanoscale organization of emerin at the dorsal nuclear envelope of human dermal fibroblasts. a) Two-dimensional rendering of wild-type SNAP-emerin imaged by 3D super-resolution at the dorsal nuclear envelope. Areas with very high densities of emerin (white spots and lines) correspond to local nuclear envelope folds often observed in the plane of the dorsal nuclear envelope and to the 2D-projection of different z-positions of emerin at the nuclear rim. Scale: 5 µm. b) Comparison of molecular densities above random (± s.e.m.) for wild-type emerin oligomers (O) and monomers (M) at the ventral (189331 localizations, 10 nuclei) or the dorsal (51387 localizations, 4 nuclei) nuclear envelope of human dermal fibroblasts. Values in parenthesis represent the length scale (± s.e.m.) of each domain in nanometers.

3) I think that it is important for the authors to demonstrate that they can rescue the effect of knocking down lamin A/C, IPO9, BAD, or SUN1 by re-expressing an RNAi-resistant cDNA construct that encodes the wild type form of the depleted protein. This will allow the authors to rule-out any potential off- target effects caused by their siRNAs.

Please note that we already assessed potential off-target or side effects of our siRNAs by immunoblotting and confocal imaging as described in Supplementary Fig. S2 and S3. Other than a reduction in nuclear actin after siRNA of lamin A/C, there is no significant off-target effect of our different siRNA treatments on the expression nor the localization of key emerin binding partners.
We have nonetheless additionally assessed the effects of rescuing lamin A/C expression (using a siRNA- resistant human lamin A/C) and rescuing SUN1 expression (using a siRNA-resistant human SUN1 fusion to EGFP) on the nanoscale distribution of emerin. For this we designed two new siRNA against lamin A/C and Sun1. As shown in **Fig. R5A-B** below, both siRNA induce the depletion of lamin A/C or SUN1, respectively.

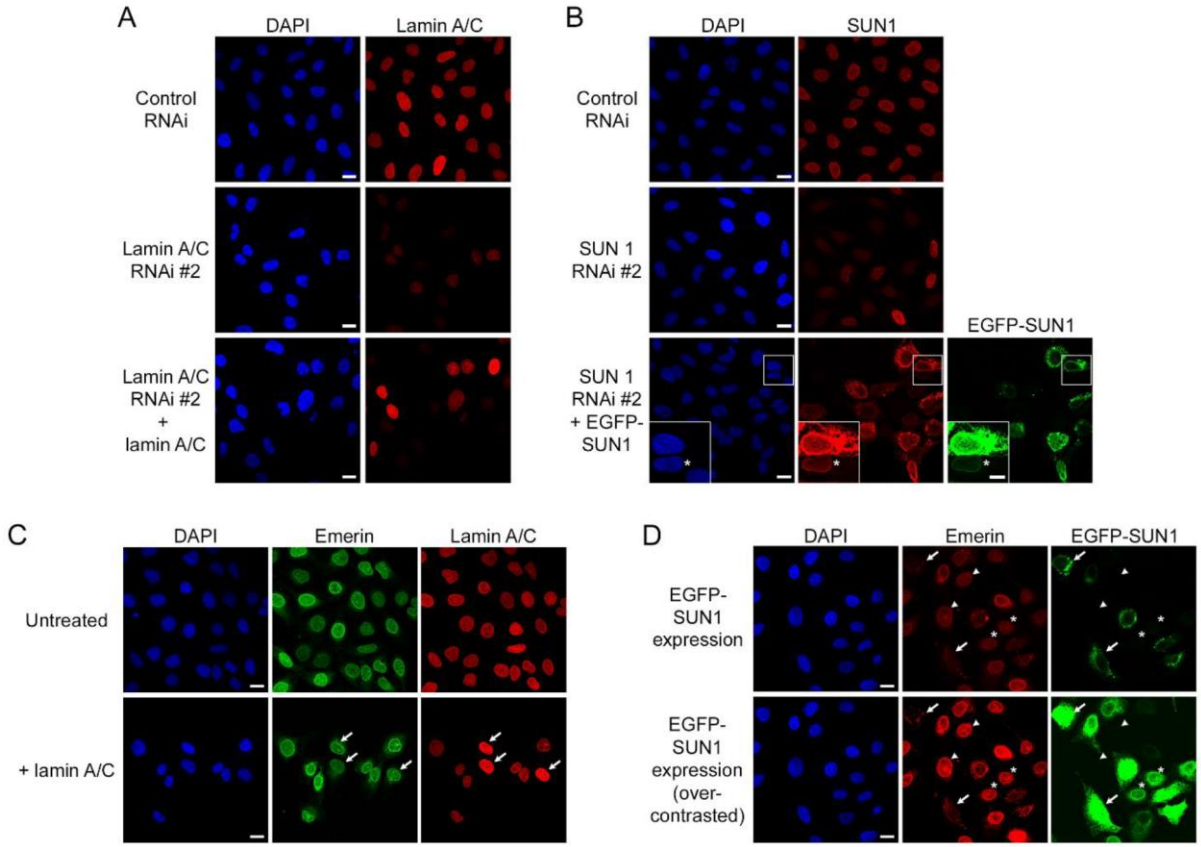

Fig R5: Rescue by expression of exogenous lamin A/C or EGFP-SUN1 after siRNA knockdown and effects of their over-expression on emerin localization. A) Confocal fluorescence imaging of lamin A/C and the nucleus (DAPI) after siRNA-induced depletion of lamin A/C and re-expression of exogenous and siRNA-resistant lamin A/C. Scales: 20 µm. B) Confocal fluorescence imaging of SUN1, EGFP-SUN1 and the nucleus (DAPI) after siRNA-induced depletion of endogenous SUN1 and re-expression of exogenous and siRNA-resistant EGFP-SUN1. The over-contrasted inset shows that overexpression of EGFP-SUN1 induces the mis-localization of SUN1 from the nuclear envelope, while cells with low EGFP-SUN1 expression display relatively normal nuclear envelope accumulation (star). Scales: 20 µm, inset: 10 µm. C) Confocal fluorescence imaging of lamin A/C, emerin and the nucleus (DAPI) after overexpression of exogenous lamin A/C. Cells overexpressing lamin A/C (arrows) display an apparently normal emerin localization at the nuclear envelope. Scales: 20 µm. D) Confocal fluorescence imaging of emerin, EGFP-SUN1 and the nucleus (DAPI) after overexpression of exogenous EGFP-SUN1. EGFP-SUN1 overexpression induces a mis-localization of emerin from the nuclear envelope (arrows), while low EGFP-SUN1 expression levels (stars) result in relatively normal emerin localization at the nuclear envelope compared to non-expressing cells (arrowheads). Scales: 20 µm.

Expression of siRNA-resistant exogenous lamin A/C or siRNA-resistant exogenous EGFP-SUN1 after knocking down each endogenous protein also induces a re-enrichment of lamin A/C and SUN1 at the nuclear envelope, respectively. Notice however that, despite knock-down of endogenous SUN1, excessive expression of exogenous EGFP-SUN1 leads to its mis-localization from the NE, while cells with low EGFP- SUN1 expression display a relatively normal NE accumulation of SUN1 (**Fig. R5B**). This indicates that INE sites where SUN1 normally assembles to form LINC complexes are saturable and that overexpression of EGFP-SUN1 induces a spillage of SUN1 toward other internal cell membranes, as previously reported (**Haque *et al*. Molecular and Cellular Biology 2006; Chen *et al*., Cell 2012**).

Notice also that, as a consequence of EGFP-SUN1 overexpression and SUN1 spillage, endogenous emerin significantly mis-localizes away from the NE (**Fig. R5D**). This is not the case when lamin A/C is overexpressed (**Fig. R5C**). During dSTORM experiments of SNAP-emerin, under conditions where exogenous EGFP-SUN1 is expressed after siRNA of endogenous SUN1, we therefore limited our imaging to cells expressing low levels of EGFP-SUN1, as determined by EGFP imaging. A similar approach was used during dSTORM of SNAP emerin where exogenous lamin A/C is expressed after siRNA of endogenous lamin A/C, as determined by lamin A/C immunostaining.

Having verified the effectiveness of knocking down endogenous lamin A/C and SUN1 with new siRNA designs and having characterized the effects of rescuing expressions with siRNA-resistant

constructs or of inducing overexpression of exogenous lamin A/C and EGFP-SUN1, we then studied the impact of both rescues and overexpression on the nanoscale organization of wild-type emerin.
Lamin A/C rescue and overexpression: As shown in **Fig. R6** below, the loss of emerin oligomers and the wide dispersion of emerin monomers we observed after knocking down endogenous lamin A/C are reversed to near normal levels upon rescue by exogenous expression of a siRNA-resistant human lamin A/C. This confirms the specificity of our siRNA treatment, as already implied by our confocal and immunoblotting data. Interestingly, lamin A/C overexpression (no siRNA) induces a loss of emerin oligomers (**Fig. R6**), indicating that a balanced expression of lamin A/C is important to promote the self- association of emerin at the INM. Lamin A/C overexpression was previously shown to trigger a nuclear accumulation of BAF and its enrichment at the nuclear rim (**Loi *et al.*, Oncotarget 2016**). It is possible that the observed decreased oligomerization of wild-type emerin stems from excessive interaction of emerin with accumulated nuclear BAF, consistent with the need to modulate emerin/BAF/lamin A/C interactions

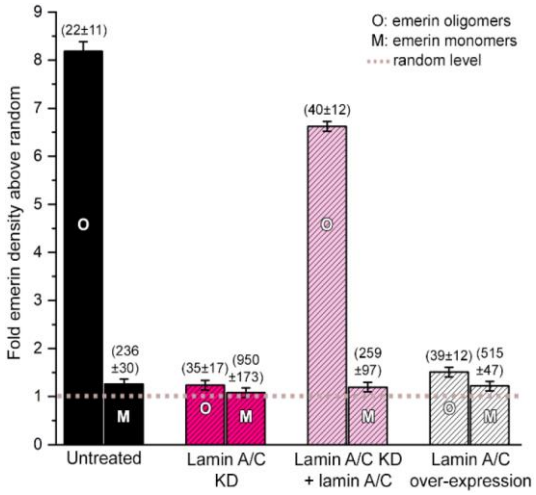

Fig R6: Effects of rescuing lamin A/C expression and of lamin A/C overexpression on the nanoscale organization of emerin. Molecular densities above random (± s.e.m.) for wild-type emerin in untreated cells, after lamin A/C knock down, lamin A/C knock down and exogenous expression of lamin A/C (118859 localizations, 6 nuclei) or overexpression of lamin A/C (204532 localizations, 5 nuclei). Values in parenthesis represent the size (± s.e.m.) of each domain in nanometers.

to promote the self-assembly of emerin into oligomers, as we have already discussed the context of the Δ95-99 and P183H emerin mutants.
These new data have now been added to Fig. 3 in the main text and as supplementary figures. They have also been briefly discussed in the manuscript (**lines 210-213; 508-511**). Materials and Methods have also been updated.

SUN1 rescue and overexpression: As shown in **Fig. R7** below, the loss of emerin oligomers we previously observed after SUN1 knockdown is partially reversed upon low level expression of an exogenous and siRNA resistant EGFP-SUN1. Indeed, the density of oligomers returns to 4.2 fold above random in 38±13 nm nanodomains, while the distribution of emerin monomers is not impacted. However, when exogenous EGFP-SUN1 is overexpressed (no siRNA), the formation of emerin oligomers is disrupted (1.4 fold above random in 61±20 nm nanodomains) and emerin monomers are distributed over much larger NE domains. This is fully consistent with our confocal microscopy observations that SUN1 overexpression induces a mis-localization of emerin from the NE and indicates that, like for lamin A/C, a balanced cellular expression of SUN1 is required: **(i)** to ensure a proper NE localization of emerin and **(ii)** to promote its self-assembly into oligomers at the INM. We note that, compared to untreated or mock cells, emerin densities did not return to ~8 fold above random, likely because of our inability to precisely control the knockdown levels of endogenous SUN1 and the re-expression level of EGFP-SUN1. However, these results confirm that our SUN1 siRNA effects are specific and that, as we initially proposed, interactions of emerin with SUN1 plays a critical role in stabilizing emerin oligomers at LINC complexes in the INM.

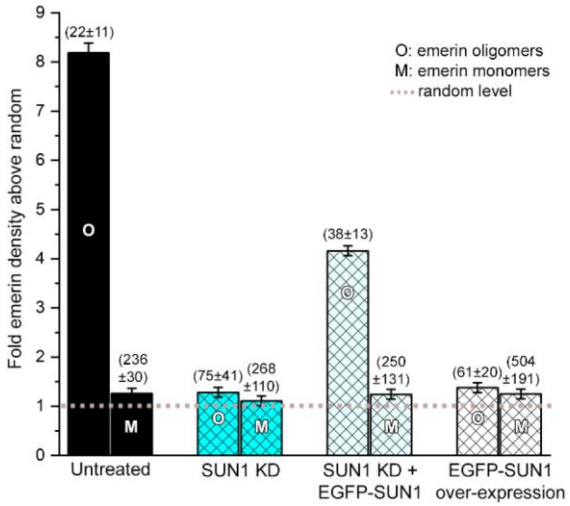

Fig R7: **Effects of SUN1 expression rescue and SUN1 overexpression on the nanoscale organization of emerin.** Molecular densities above random (± s.e.m.) for wild-type emerin in untreated cells, after SUN1 knock down (258300 localizations, 6 nuclei), SUN1 knock down and exogenous expression of EGFP-SUN1 (85210 localizations, 5 nuclei) or overexpression of EGFP- SUN1 (288522 localizations, 7 nuclei). Values in parenthesis represent the size (± s.e.m.) of each domain in nanometers.

These results have now been added to Fig. 3 of the manuscript, the Supplemental Information and they have been briefly discussed in the main text. (**lines 243-248**).

Please note that we did not test the effect of rescuing BAF, as our experimental design is already equivalent to a rescue of endogenous BAF depletion with BAF^{L58R}. As an alternative to rescuing IPO9 depletion, we have studied the effect of depleting the nuclear actin exporter exportin-6 (XPO6) and increasing nuclear actin levels. Those results are described in response to question 4 of Reviewer 2 below.

4) What happens if the authors over-express lamin A/C or SUN1 or increase the levels of nuclear actinon the nanoscale spatial organization of emerin at the inner nuclear membrane? Do they see the opposite effect of depleting these proteins?

For our answers on lamin A/C and SUN1 overexpression, please see question 3 of Reviewer 2 above. In addition to studying the overexpression of lamin A/C and SUN1, we also evaluated the impact of increasinglevels of nuclear actin by depleting the nuclear actin exporter XPO6 with siRNA.
Please note that we first verified that siRNA against XPO6 effectively induces an accumulation of actin tothe nucleus. This was done as part of a set experiments performed in response to question 6 from Reviewer 2 and aimed at better characterizing the levels of nuclear actin following siRNA against IPO9 and XPO6. As shown in **Fig. R11**, siRNA against XPO6 effectively induces an increase formation of short nuclear actin filament consistent with an increase in nuclear actin content compared to an siRNA control.
Cells treated with siRNA against XPO6 were then imaged by dSTORM to evaluate the impact of increasing nuclear actin on the nanoscale organizations of emerin. As seen in **Fig. R8** below, inducing nuclear accumulation of actin does not affect the organization of emerin monomers, but oligomer densities decrease to 1.5±0.1 fold above random in 49±16 nm nanodomains. This indicates that excess nuclear actinimpedes the formation of dense emerin oligomers, which is consistent with our earlier data and our conclusion that unbinding of emerin from nuclear actin facilitates the formation of oligomers. We note that some nuclei appeared locally crumpled after siRNA of XPO6.

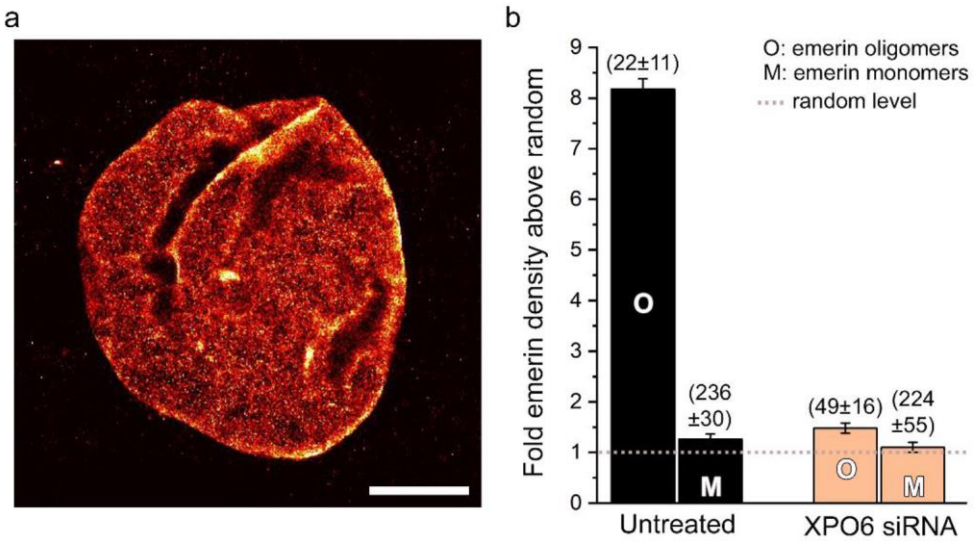

Fig. R8: **Nanoscale organization of wild-type emerin following increase in nuclear actin levels by siRNA of exportin-6**. a) Two-dimensional rendering of wild-type SNAP-emerin imaged by 3D super-resolution in HDF cells treated with siRNA against exportin-6 (XPO6). Some nuclei appeared locally crumpled after siRNA of XPO6. Scale: 5 µm. b) Molecular densities above random (± s.e.m.) for wild-type emerin oligomers (O) and monomers (M) in untreated cells or after XPO6 knock down to increase nuclear actin levels (315527 localizations, 7 nuclei). Values in parenthesis represent the length scale (± s.e.m.) of each domain in nanometers.

These data have now been added in Supplementary information and described briefly in the main text. (**lines 223-226; 361-362**).

5) The authors state that the impact of the depletion of SUN1 on emerin is evidence of the involvement of the LINC complex in the nanoscale spatial organization of emerin at the inner nuclear membrane. While this result may support their conclusion, it is far from definitive. To really demonstrate the involvement of the LINC complex in this process (and not SUN1 operating on its own), the authors need to test the effect of the over-express a dominant negative LINC complex inhibitor construct (e.g. the KASH domain of nesprin-1/2 or the luminal domain of SUN1) on emerin organization. It would also be powerful if the authors were to rescue their SUN1-depleted cells with a SUN1 construct that cannot interact with the KASH peptides of nesprins (e.g. a construct lacking the SUN domain or one that cannot homo-oligomerize).

We have now tested the influence of disrupting the LINC complex on the nanoscale organization of emerin. This was done by expressing a LINC complex inhibitor, in the form of a mCherry fusion to a dominant negative nesprin1α KASH domain (mCherry-DN-KASH, **Lombardy *et al.*, JBC 2011**). mCherry- DN-KASH disrupts LINC complexes by binding to SUN proteins, effectively impeding the interaction of SUN proteins with endogenous KASH-domain proteins, including nesprins at the NE. As shown in **Fig. R9** below, the oligomerization of emerin is disrupted at a level equivalent to SUN1 siRNA when emerin is imaged by super-resolution microscopy in cells expressing mCherry-DN-KASH. These data confirm that the oligomerization of emerin at the NE not only requires the expression of SUN1 but also necessitates functional LINC complexes capable of connecting the nucleoskeleton and the cytoskeleton via SUN protein interactions with KASH domain proteins, including nesprins.
Data from panel (C) in **Fig. R9** have been added to the manuscript and discussed briefly in the main text (**lines 254-261**). **Fig. R9** it-self has been added in Supplementary Information.

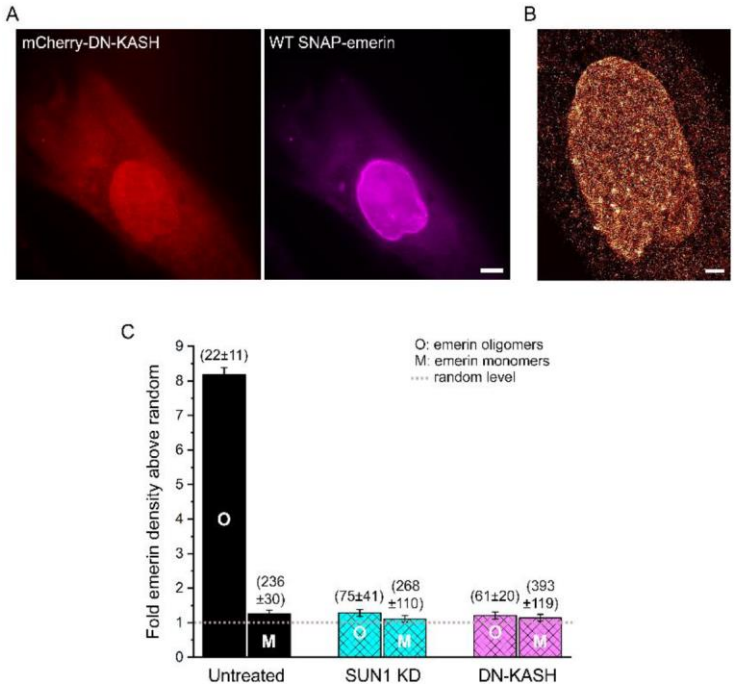

Fig. R9: Disruption of LINC complex formation by expression of domain negative KASH domain reduces emerin oligomerization at the nuclear envelope. a) Wide field fluorescence images of an emerin-null HDF co-expressing mCherry-DN- KASH (red) and wild-type SNAP-emerin labeled with BG-A647 (magenta). Scale: 5 µm. b) Two-dimensional rendering of wild- type SNAP-emerin imaged by 3D super-resolution in the cell in (a). Scale: 2 µm. C) Molecular densities above random (± s.e.m.) for wild-type emerin oligomers (O) and monomers (M) in untreated cells (189331 localizations, 10 nuclei), SUN1 knock down (258300 localizations, 6 nuclei) or after expression of mCherry-DN-KASH (492365 localizations, 5 nuclei). Values in parenthesis represent the length scale (± s.e.m.) of each domain in nanometers.

We have also tested the influence of a siRNA-resistant EGFP-SUN1^{W676E} mutant on the nanoscale organization of emerin. Amino acid W676, in the "KASH-lid" domain of SUN1, mediates hydrophobic interactions with the KASH domain of KASH proteins, including nesprins (**Gurusaran and Davis, Elife 2021**). The W676E point mutation was recently shown to disrupt the formation of SUN1-KASH1, SUN1-KASH4 and SUN1-KASH5 complexes (**Gurusaran and Davis, Elife 2021**). Like for the re-expression of the siRNA resistant EGFP-SUN1 described above, only cells that expressed low levels of the siRNA resistant EGFP- SUN1^{W676E} mutant after knockdown of endogenous SUN1 were imaged during dSTORM of SNAP-emerin. As shown in **Fig. R10** below, the formation of emerin oligomers is abolished at levels equivalent to that of mCherry-DN-KASH, confirming that SUN1 interactions with KASH domain proteins from the LINC complex are essential for the self-association of emerin at the INM. Again, these data indicate that emerin oligomerizes at SUN1 LINC complexes, in a process that requires SUN1-KASH domain interactions and a functional LINC complex capable of sensing nuclear envelope tensions.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

6) I am a bit concerned about the fractionation-based method used by the authors in Figure S2 to quantify the levels of nuclear actin and how they change in response to various RNAi treatments. My concern stems from the fact that actin associated with the outer nuclear membrane cannot be distinguished from actin present within the nucleus using this method. I would strongly suggest that they authors use the previously described anti-nuclear actin chromobody (see Plessner et al. 2015 J Biol Chem) to more carefully assess the levels (and organization) of nuclear actin in their cells.

As briefly discussed by Hurst *et al.* (**Trends in Cell Biology 2019**) nuclear fractionation does carry a risk of some cytoplasmic actin filaments collapsing on nuclei during cell wash and being carried-

over into the nuclear fraction. However, this issue can be minimized by placing cells on ice to induce F-actin disassembly and by using low salt and hypotonic buffers to prevent spontaneous actin polymerization. The cell and nuclear fractionation protocol we have employed is the same as that described by Berk *et al.* (**Journal of Biological Chemistry 2013**) to characterize emerin association with various nucleoskeletal "niches", including nuclear actin. It includes the conditions described above to limit contamination of the nuclear fraction by cytoplasmic actin. Note that this protocol also includes extra washing steps of the nuclear pellet to remove residual cytoplasmic components before nuclear lysis. As such, adequate precautions were taken to limit as much as possible contamination of our nuclear fraction with cytoplasmic actin. Please also note that the decrease in nuclear actin levels we have observed is similar to that reported when IPO9 siRNA treatments are performed in other mammalian cell types (**Dopie *et al*. PNAS 2012**).

Nevertheless, as suggested by Reviewer 2, we have tried to better characterize the level of nuclear actin in our HDF using a nuclear actin-chromobody fused to tagGFP and after fibronectin stimulation of cells as previously described (**Plessner *et al*., Journal of Biological Chemistry 2015**). Despite extensive efforts, that included the production of a stable cell lines and numerous assays, we concluded that, unfortunately, this nuclear actin-chromobody does not work in our cells as it is primarily enriched in nucleoli (as would a NLS-tagGFP fusion) without clear or reproducible evidence of filamentous nuclear actin detection, even under the various assays described in **Plessner *et al*.**.

We therefore resorted to employing a different nuclear actin probe called Utr230-EN and described by Belin et al. (**Belin et al., Molecular Biology of the Cell 2013**), that reports on short nuclear actin filament contents by producing nuclear puncta upon cellular expression. For our assays, we generated HDF(EMD$^{+/y}$) stably expressing Utr230-EN and treated them with control siRNA, IPO9 siRNA or XPO6 siRNA, under the same condition as our dSTORM and sptPALM measurements. Chemically fixed cells were then imaged by confocal microscopy and, as shown in **Fig. R11A** below, the nuclear localization pattern of Utr230-EN was classified into 3 groups: (i) small puncta and diffuse, (ii) diffuse or (iii) large foci, based on a similar classification by others (**Belin et al., Molecular Biology of the Cell 2013; Belin et al., Elife 2015; Xing et al., Journal of Cell Biology 2019**) to reflect different nuclear actin filament contents across cells. Data were pooled from three independent assays for each condition. As shown in **Fig. R11B**, the fraction of cells displaying a diffuse nuclear localization pattern of Utr230-EN increases upon IPO9 siRNA treatment compared to control siRNA, which is consistent with a reduction in nuclear actin content, as previously reported for similar IPO9 knockdown by other groups (**Belin et al., Molecular Biology of the Cell 2013; Belin et al., Elife 2015; Xing et al., Journal of Cell Biology 2019**). Inversely, the fraction of cells displaying enlarged Utr230-EN foci increases at the expense of other fractions upon XPO6 siRNA treatment compared to control siRNA, again consistent with an increase in nuclear actin content as previously reported for similar XPO6 knockdowns by others (**Belin et al., Molecular Biology of the Cell 2013; Belin et al., Elife 2015**).

These results confirm that our siRNA against IPO9 and XPO6 effectively modulate nuclear actin contents in HDF, as already established by our immunoblotting assay in the case of IPO9 siRNA.

These data have now been added to the Supplementary Information and methods have been mentioned in Materials and Methods.

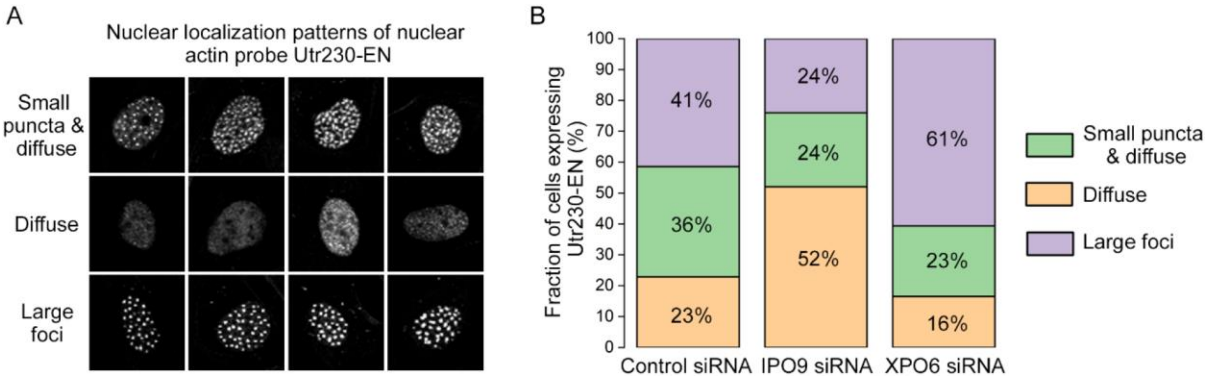

Fig. R11: Effects of IPO9 siRNA and XPO6 siRNA on nuclear actin organization. (A) Examples of nuclear localization patterns for the short nuclear actin filament probe Utr230-EN in HDF cells. Patterns are classified as: (i) small puncta and diffuse, (ii) diffuse or (iii) large foci, reflecting variations in nuclear actin filament content across cells. (B) Distribution of nuclear actin filament classes after control siRNA

treatment (n = 635 nuclei), IPO9 siRNA (n = 663 nuclei) or XPO6 siRNA (n = 625 nuclei). Knockdown of IPO9 results in the majority of cells displaying a diffused Utr230-EN pattern, indicative of lower nuclear actin filament contents. Inversely, knockdown of XPO6 results in most cells displaying larger and brighter foci compared to control siRNA, indicative of increased nuclear actin filament contents.

7) How do the authors know that what they are referring to as "monomers" are actually monomers and not an oligomer with only one active fluorescent protein?

This was indeed a concern when we first analyzed the diffusion properties of wild-type PA-TagRFP-emerin and it is the primary reason why we additionally used CALM imaging and co-expression of emerin fused to complementary split-GFP fragments to identify possible dimeric and oligomeric forms of emerin at the NE. As depicted in Fig. 2b, CALM imaging can identify both dimers and higher-order emerin oligomers, because split-GFP complementation between two emerin monomers and activation of GFP fluorescence signals take place if those emerin monomers are in very close proximity (e.g. forming dimers or higher- orders oligomers). As shown in Fig. 2d, tracking by CALM reveals that the overwhelming majority of complemented emerin species (90%) pertain to diffusing population D4 (oligomers), while a minority (10%) pertain to diffusing population D2 (likely split-GFP induced emerin dimers that cannot access the INM). Because we did not detect complemented emerin species pertaining to the population D3 we previously observed by sptPALM, we concluded that this population is unlikely to represent dimers or oligomers and thus can be classified as monomers.
As such, despite the fact that sptPALM with PA-tagRFP-emerin cannot differentiate between actual monomers or dimers/oligomers with only one activated PA-tagRFP, our CALM measurements with split- GFP fragments unambiguously indicate that population D3 pertains to emerin monomers, while population D4 represents at least emerin dimers or, more likely, emerin oligomers, as suggested by our initial observations that complemented emerin-GFP-emerin trajectories from population D4 often spatially overlap and as confirmed by our super-resolution imaging data.
We note that the capacity of split-GFP fragments and CALM imaging to detect relevant dimerization/oligomerization states of emerin was confirmed by our study of the P183H mutant. This emerin mutant was shown to have a strong capacity to self-assemble in vitro and was predicted to form dimers in cells (**Herrada *et al.*, ACS Chemical Biology 2015**). P183H emerin was also shown to alter emerin's nuclear localization to a more random distribution towards other subcellular membranes (lysosomes, plasma membrane, mitochondria) in muscle cells from EDMD patients (**Ellis *et al.*, Human Genetics 1999**). Consistent with these results, our CALM imaging of P183H fused to split-GFP fragments showed that, indeed, it has a strong tendency to form dimers/oligomers at the ER, the ONM and the INM compared to wild-type emerin (Fig. 6g and Fig. 2d). These P183H assemblies, when they are further stabilized by the irreversible complementation of the split-GFP fragments, prevent ~40% of the detected emerin mutant pool from accessing the INM (41% of complemented P183H are found at the ER membrane or the ONM, Fig. 6g). While we do not know if a similar excessive formation of P183H dimer/oligomers takes place in muscle cells from EDMD patients, their inability to efficiently reach the INM compared to wild-type emerin, as we have shown, would likely lead to their observed redistribution towards other subcellular membranes in EDMD patients (**Ellis *et al.*, Human Genetics 1999**).

Reviewer 3

Advance Summary and Potential Significance to Field:
A set of inner nuclear envelope proteins, including LEM-domain proteins, are described as exhibiting large unstructured regions (IDR). In the case of the LEM-domain protein emerin, one of the most studied inner nuclear envelope proteins, IDRs are responsible for emerin oligomerisation. However, the molecular details of emerin oligomers, as well as the function of these oligomers, are not clear, and difficult to study. This manuscript reports a high resolution study of these oligomers and their function in response to a mechanical stress in cells. It is an important contribution to the field.

Comments for the Author:
At the inner nuclear membrane, LEM-domain proteins exhibit large intrinsically disordered nucleoplasmic regions, whose functions are still unclear. Emerin, one of the most studied LEM-

domain proteins, is phosphorylated in its disordered region after a mechanical stress, and this contributes to nuclear adaptation to the stress. Emerin also oligomerizes in vitro and in cells. However, how do its disordered regions promote oligomerisation and what is the role of emerin oligomerization in the nuclear response to a mechanical stress are still unanswered questions. This work addresses these questions using single molecule tracking and super resolution microscopy. An extensive description of emerin mobility and oligomeric states is provided, the role of emerin interaction with its best known partners, as well as the impact of a mechanical stress, on these emerin properties is explored, and finally, emerin variants detected in patients with muscular dystrophy are characterized, to identify defects in emerin mobility, oligomeric states, as well as behavior after a mechanical stress. The experimental results are robust and novel. Only the discussion is sometimes difficult to follow, because it is based on a large number of (sometimes contradictory) experimental data previously published on emerin. Summarizing these previous data at the beginning of the discussion in a clear and condensed manner would have helped to follow the interpretation of the new data provided by this study.

1) In the abstract, the authors write: "the abnormal nuclear envelope deformations induced by EDMD emerin mutants stem from a defective formation of lamin A/C and LINC complex-stabilized emerin oligomers". Is it really true for all the variants (and especially Q133H; see Fig. 6c) ? Also, no experimental results are obtained on the variants after depletion of lamin A/C and SUN1, so that this sentence seems to be not completely accurate.

By using the word "defective" we meant to describe both over-oligomerization (Q133H) and reduced of oligomerization (P183H, Δ95-99) of emerin mutants. For each mutant (including Q133H), abnormal nuclear shapes (Fig. 5b non-patterned) are linked to an improper formation of emerin oligomers (Fig. 6), whose stabilization requires lamin A/C, SUN1 and functional SUN1 LINC complexes, as shown in cells expressing wild-type emerin (Fig. 3). We have also shown that maintenance of these same lamin A/C and SUN1-stabilized oligomers is important for nuclear deformation against force (wild-type emerin in micropatterns, Fig. 4), and that their improper formation leads to a lack of nuclear shape adaptation in cells expressing Δ95-99 emerin compared to cell expressing wild-type emerin under the same conditions (Fig. 7). We thus believe that the sentence, as written, is accurate and reflective of our data. Yet, for clarification, we have now changed "*defective formation*" to "*improper formation*" in the abstract. (**line 28**).
We agree that investigating the cumulative effects of lamin A/C and SUN1 depletion on the behavior of emerin mutants might be interesting, and we have indeed done so to study the importance of lamin A/C on the nanoscale organization of Q133H (see below, **Fig. R13**). Yet, as we have described above, we believe that our current results are sufficient to state that improperly formed lamin A/C and LINC complex- stabilized emerin oligomers (over- or under-oligomerization) in emerin mutants induce abnormal nuclear shapes.

2) The introduction is clear and well-focused.

3) Fig.2: how do the authors explain that, after depletion of nuclear actin, the mobility of emerin anchored at the outer nuclear membrane is significantly increased ? This emerin fraction is assigned to the outer nuclear membrane because depletion of lamin A/C does not affect its mobility > couldn't it be an emerin fraction at the INM that does not bind to lamin A/C (so is more mobile) ? Could you check using an independent experiment that in your cells, 10% of emerin is really located at the ONM ?

Please refer to our answer to a similar question by Reviewer 1 (question #2). Unfortunately, at this time, we do not have a complete understanding as to why the lateral mobility of emerin at the ONM increases when nuclear actin is downregulated.
It is unlikely that population D_2, which we identified as ONM emerin, could be an INM emerin fraction that does not bind lamin A/C. If that was the case, lamin A/C depletion by siRNA would have significantly increased the fraction of population D_2, for instance by inducing a disappearance of INM populations D_3 and/or D_4 as they merge with population D_2. This is not what we observed. Similarly, we did not observe a merging of population D_3 and D_4 with D_2 when we studied the emerin mutant Δ95-99, which has been well characterized has having lost the ability to bind lamin A/C. Inversely, emerin mutant P183H, which appears to display slightly enhanced binding to lamin A/C, based on *in vitro* blot overlay assays published by Lee *et al.* (**Journal of Cell Science 2001**),

did not result in a modified diffusion coefficient nor in a change in the fraction of population D2. As such, population D2 appears to behave independently of whether emerin binds or does not bind lamin A/C.

As suggested by Reviewer 3, we have now independently confirmed that ~10% of emerin is expected at the ONM in human dermal fibroblasts. To estimate the fraction of emerin at the ONM, we re-analyzed 3D confocal images of non-micropatterned wild-type human dermal fibroblasts (*EMD*$^{+/y}$) stained with an anti- emerin antibody (Abcam Inc, USA) as presented in Bautista *et al.* (**Micromachines 2018**). In this set of experiments, cells were permeabilized with saponin to image emerin associated only with the ONM and the ER (ONM+ER) or with Triton X-100 to image the entire emerin pool (INM+ONM+ER). Lamin A/C was also immunostained to identify cells where saponin or fixation might have inadvertently led to nuclear permeabilization and to verify effective nuclear permeabilization following Triton X-100 treatments (**Fig. R12**). Immunostaining and whole cell volume imaging by 3D confocal microscopy were performed under the exact same conditions for saponin or Triton X-100 permeabilizations.

To quantify the total amount of ONM and ER emerin per cell after saponin treatment, the entire cell volume (all acquired confocal z-planes) was combined into a single image by sum intensity z-slices projection and an ROI delineating the whole cell (ROI 1, **Fig R12**) was drawn to extract emerin fluorescence intensities after background correction. Similarly, the specific amount of ONM emerin can, in principle, be estimated from the same sum intensity z-slices projection using an ROI delineating only the nucleus (ROI 2, **Fig R12**). However, ROI 2 also includes contribution from ER emerin above and below the nucleus, that are also projected onto the nucleus by sum intensity z-slices projection. To correct for ER emerin contribution to the nuclear ROI 2, we first calculated the mean ER-only emerin intensity per area from ROI 1 - ROI 2. We then multiplied this mean intensity by the area of ROI 2, and subtracted this ER-only integrated intensity from the integrated intensity of ROI 2. This provides nuclear ROI 2 integrated intensities representing the amount of emerin only at the ONM.

The same process was used for image analyzes after Triton X-100 treatment to obtain nuclear ROI 2 integrated intensities representing the amount of emerin at both the INM and ONM. Finally, using a representative set of 20 cells for both saponin and Triton X-100 treatments, we calculated the fraction of ONM emerin per cell using:

$$\text{ONM fraction} = ER_{corrected}\ ROI\ 2_{saponin\ (ONM)} \ /\ ER_{corrected}\ ROI\ 2_{Triton\ x\text{-}100\ (INM+ONM)}$$

In this case, we make the reasonable assumption that cell nuclear volumes and emerin epitope recognition remain relatively similar between saponin and Triton X-100 treatments.

As shown from the distribution of calculated ONM fractions for those cells in **Fig. R12**, the mean ONM fraction at the nuclear envelope is **13±6 %**, in good agreement with the ONM emerin fraction detected in our sptPALM experiments, and consistent with previous observations that a non-negligeable fraction of emerin localizes at the ONM in human dermal fibroblasts (**Salpingidou *et al.*, Journal of Cell Biology 2007**).

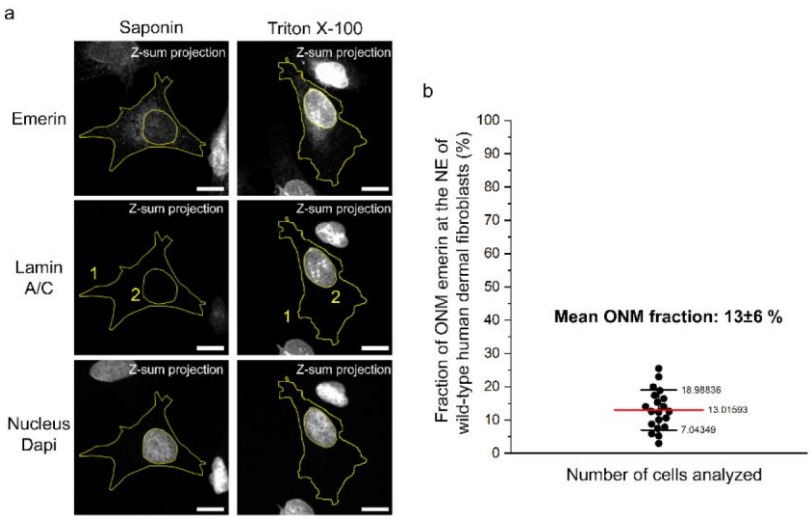

Fig. R12: **Quantification of ONM emerin fraction in human dermal fibroblasts from confocal Z-stacks.** a) Example of z-stack sum intensity projections for fibroblasts permeabilized with saponin or triton X-100 and immunostained for emerin, lamin A/C and Dapi. Region of interests (ROIs) used to measure fluorescence intensities from emerin immunostaining are shown in yellow. Scale bars: 15 µm. b) Histogram of emerin ONM fraction determined from confocal z-stack projections and nuclear ROI quantifications of saponin treated cells (N=20) and Triton X-100 treated cells (N=20). Mean is indicated by the red bar. Standard deviation of the mean is indicated by the black bars.

Please note that per the Z-projections presented in **Fig. R12**, there is significantly more ER-associated emerin in an entire cell than what we detect by HILO imaging in our single molecule experiments. Indeed, because our HILO light sheet illumination is directed to the bottom nuclear envelope, we sample only a very small fraction of the total pool of ER-associated emerin.
These data confirm that the ~10% population D2 we detect pertains to ONM emerin. Those immunostaining and quantitation results have now been added in Supplementary information and mentioned in the main text (**lines 128-130**). Methods are also described in Materials and Methods.

4) Fig.3: if lamin A/C and BAF were simultaneously depleted, would the impact be additive ? From the current data, as the impact of lamin A/C depletion is more important than that of BAF depletion, we can conclude that lamin A/C anchors emerin partly independently from BAF, however the opposite is unclear.

Please note that we did not image cells under strict BAF depletion, and BAF siRNA-treated cells expressed BAF^{L58R}, which does not bind the LEM domain of emerin. The L58R mutation does not interfere with BAF dimerization and DNA binding (**Samwer *et al.*, Cell 2017**) and does not appear to interfere with BAF binding to lamin A/C (**Halfmann *et al.*, Journal of Cell Biology 2019**). A mention of BAF^{L58R} properties hasbeen added to the manuscript. (**line 154**).
Although we have not tested a simultaneous depletion of lamin A/C together with a depletion of BAF + BAF^{L58R} expression, potentials for additive impacts can be extrapolated from our data and the work of Samson *et al.* (**Nucleic Acids Research 2018**), who showed that lamin A/C binds directly to emerin oligomers but interacts only indirectly with emerin monomers via BAF dimers. Indeed, our observations that lamin A/C depletion has a lesser impact on emerin diffusion than BAF siRNA+BAF^{L58R} are consistent with the results of Samson *et al.*.
In the case of emerin monomers, their INM mobility increases by ~2-fold after lamin A/C depletion but by ~5.5-fold after BAF siRNA+BAF^{L58R}. The 2-fold increase in emerin monomer diffusion indicates that the contribution of lamin A/C to potential emerin/BAF/lamin A/C tripartite interactions are reduced, but thatretained emerin/BAF interactions continue to partially slow down emerin monomers at the INE, likely via BAF binding to chromatin, as we discussed in the manuscript. The 5.5-fold mobility increase of emerin monomers in cells expressing BAF^{L58R} further indicate that those emerin/BAF interactions have a stronger influence than lamin A/C on the diffusion of monomers, which appears consistent with the observations of Samson *et al.* that lamin A/C only binds emerin monomers indirectly via BAF. As such, lamin A/C and BAF effects could be additive in the case of emerin monomers with BAF anchoring emerin monomers at the INM: (i) in part via the indirect effect of lamin A/C and (ii) primarily via ternary interactions with othernuclear components, most likely chromatin.
In the case of emerin oligomers, the INE mobility increases again by ~2-fold after lamin A/C depletion, but only by ~3.5-fold after BAF siRNA+BAF^{L58R}. The ~2-fold increase indicates that emerin/BAF interactions are retained at a level similar to that observed for monomers when the lamina is destabilized, which is consistent with the disrupted formation of oligomers we observed by super-resolution imaging. In other words, if lamin A/C cannot directly stabilize bridging interactions of the LEM domain with LEM binding sites on other emerin, individual emerin within unstabilized oligomeric nanodomains behave like monomers, with their LEM domain undergoing more frequent interactions with BAF. Note that they nonetheless diffuse more slowly than emerin monomers outside oligomeric domains, likely because theyinteract with additional partners such as LINC complex proteins, including SUN1. The ~3.5-fold increase after BAF siRNA+BAF^{L58R}, also suggests that lamin A/C has more influence on the mobility of emerin in oligomers than on emerin monomers when binding to BAF is prevented (5.5-fold vs. 3.5-fold increase), which is consistent with a direct binding of lamin A/C to oligomers but not to monomers, as reported by Samson *et al.* The decreased oligomerization of emerin when it cannot bind BAF (Fig. 3c) and its increasedmobility within oligomers (Fig. 2a), both indicate that, in addition to lamin A/C stabilization, interactions with BAF can modulate the organization of emerin oligomers, as we have discussed. As such, lamin

A/C and BAF effects could also be potentially additive in the case of emerin oligomers, which are stabilized by direct binding to lamin A/C and are further modulated by emerin binding to BAF.

5) Fig. 3: the data clearly show that depletion of either lamin A/C or SUN1 strongly decreases emerin oligomerization. But does SUN1 favor lamin binding to emerin, or lamin favor SUN1 binding to emerin ? Or are these binding events totally independent one from the other ?

Those are very good questions and there could indeed be a hierarchical influence of SUN1 and lamin A/C on the ability of oligomerized emerin to bind both proteins, notably since previous work by Haque *et al*. (**Journal of Biological Chemistry 2010**) has shown that SUN1 can bind both emerin and lamin A/C on different part of its N-terminal domain. While our siRNA rescue and overexpression experiments indicate that a balanced expression of both SUN1 and lamin A/C is required for the formation and the stabilization of emerin oligomers, they cannot explicitly define if SUN1 and lamin A/C act in a codependent or in an independent manner on the formation emerin oligomers.

As mentioned in our response to question #4 of Reviewer 3, Samson *et al*. (**Nucleic Acids Research 2018**) have shown that, *in vitro*, lamin A/C can only bind directly to emerin oligomers and not to monomers. In these *in vitro* assays SUN1 was not present, which suggests that, once emerin oligomers are formed, SUN1 is not needed for the direct binding of lamin A/C to oligomerized emerin. However, if SUN1 is also required for an efficient formation of emerin oligomers in cells, as our results indicate (Fig. 3), it might then indirectly favor lamin A/C binding to emerin oligomers and their stabilization. The argument that SUN1 could promote emerin oligomerization somewhat independently of emerin oligomers/lamin A/C direct interactions can be made from our study of the Δ95-99 emerin mutant. Indeed, this mutant does not bind lamin A/C nor actin, does not form oligomers, but does bind SUN1 *in vitro*. As we have shown, Δ95-99 emerin forms fewer and less dense emerin oligomers in non-patterned cells compared to wild-type emerin. Yet, in mechanically challenged cells having intact lamin A/C and SUN1, Δ95-99 emerin is capable of forming oligomers, albeit at a significantly reduced density compared to wild-type emerin (Fig. 7). It is unlikely that lamin A/C directly participates in the formation of these oligomers, because Δ95-99 emerin does not bind lamin A/C. Those oligomers could thus be induced by binding to SUN1, which, as part of the LINC complex, is expected to be directly involved in nuclear responses to forces. While we cannot completely rule out the involvement of other factors, this could indicate that SUN1 binding to emerin might induce some level of emerin oligomerization independently of a downstream stabilization by lamin A/C, in particular during nuclear responses to force. Such a scaffolding role of SUN1 is compatible with previous observations that it can self-assemble into nearly immobile oligomeric platforms, as mentioned in the manuscript. Also note, that, in confocal images of cells over-expressing EGFP-SUN1 (**Fig. R5D**, above), we have observed the formation of wild-type emerin puncta/aggregates that co-localize with EGFP-SUN1 puncta/aggregates in cytoplasmic membranes other than the NE, consistent with the possibility that SUN1 might induce emerin oligomerization independently of lamin A/C.
Together, these observations support the idea that SUN1 favors lamin A/C binding to emerin oligomers by serving as an initial scaffold for emerin self-assembly. High-resolution FRET experiments would however be needed to clearly define if SUN1 and lamin A/C binding to emerin oligomers are dependent or independent from one another. We believe that those are beyond the immediate scope of this manuscript.

6) Fig. 4: Depletion of lamin A/C or nuclear actin causes the same nuclear shape defect after a mechanical stress (lack of adaptation to the mechanical stress). Why is the impact of depleting SUN1 and BAF, as performed in other experiments, not shown here ? Similarly, depletion of nuclear actin does not modify emerin mobility after a mechanical stress. What is the impact of depletion of lamin A/C in this same experimental set up?

The purpose of studying the effects of lamin A/C and nuclear actin depletion on the NSI of nuclei was:

(i) To validate that these two major nucleoskeletal proteins participate in nuclear shape maintenance in our human dermal fibroblasts, as others have shown before in different cells. Indeed, we showed that the NSI is significantly modified following lamin A/C or IPO9 siRNA in non-

micropatterned cells (Fig. 4c).
(ii) To demonstrate that our micropatterning technique effectively induces increased nuclear stress and that nuclear deformations observed in micropatterned cells reflect a mechanical adaptation of nuclei against forces that implicate the nucleoskeleton. Indeed, we showed that nuclei become increasingly deformed has micropatterns get narrower and that wild-type-like nuclear deformations require an intact nucleoskeleton (Fig. 4c).

To our knowledge, neither SUN1 nor BAF are classified as nucleoskeletal proteins. The effects of BAF and SUN1 depletion on the NSI of our cells were therefore not studied, as we felt we could not directly link their potential effect to changes in nucleoskeletal adaptation against force.

Reviewer 3 mentions that we studied the organization of emerin after mechanical stress for cells depleted for nuclear actin. This is incorrect. We have studied the organization of emerin after mechanical stress for intact cells (15 and 10 µm micropatterns, Fig. 4d and 4e) and have separately studied the organization of emerin in non-micropatterned cells after lamin A/C depletion, nuclear actin depletion, SUN1 depletion or BAF siRNA+BAF^{L58R} (Fig. 2a and 3c). We noticed that both the mobility and the nanoscale organization of emerin in micropatterned cells are strikingly similar to that of emerin in non-micropatterned cells after nuclear actin depletion. We thus inferred that nuclear shape remodeling in micropatterns entails a disengagement of emerin/nuclear actin interactions.
It is not clear to us how emerin tracking and super-resolution imaging experiments in cells where micropatterning is combined with lamin A/C, nuclear actin, BAF or SUN1 depletion would capture easily interpretable organizations of emerin when those are already significantly impacted in non-patterned cells following such treatments.

The interpretation of the lack of impact of nuclear actin depletion on emerin mobility after a mechanical stress is that the stress disengages nuclear actin from the nucleoskeleton/emerin complexes. Could this be related to phosphorylation of Tyr74/Tyr95 ?

As already mentioned above, we did not study the impact of nuclear actin depletion after mechanical stress. We interpreted the increased mobility of emerin and changes in the formation of oligomers after mechanical challenges (without nuclear actin depletion) as stemming from a disengagement of nuclear actin from nucleoskeleton/emerin complexes, based on the striking similarities in emerin diffusion and nanoscale organizations between intact micropatterned cells and non-micropatterned cells depleted for nuclear actin.
As we discussed in the manuscript, there could be a link between emerin phosphorylation, its oligomerization and its stabilization by lamin A/C at SUN1 LINC complexes. How nuclear actin fits in these processes remains to be fully determined. It is possible that emerin phosphorylation happens first and then triggers a disengagement of emerin/nuclear actin interactions, ultimately resulting in faster emerin membrane diffusion and enhanced oligomerization. Alternatively, unbinding of emerin from nuclear actin might happen first, which could then favor the phosphorylation of emerin along its IDR by unmasking potential phosphorylation sites (including Tyr74/Tyr95), again resulting in faster emerin mobility and oligomerization. Our observation that excessive nuclear accumulation of actin after XPO6 knock-down leads to a reduced oligomerization of emerin, appears to be consistent with this second possibility. Those are exciting questions that we are currently pursuing.

7) Figs.6 & 7: interpretation of these data is complicate, especially because it is based not only on the new data reported by the authors, but also on the contradictory results found in the literature and obtained through very different experimental set up. Many arguments were unclear for this reviewer. Interpretation of Q133H: the distribution of oligomers displayed in Fig 6c seems close to the WT distribution, however the mobility of this variant at the INM is increased, how can these two observations can be consistently interpreted?

Indeed, the spatial distribution of Q133H monomers and oligomers in Fig. 6c is close to that of wild-type emerin, with emerin monomers being distributed over length scales identical to those of wild-type emerin at the INM (213±62 *vs*. 236±30 nm). However, oligomer densities are 50% higher for Q133H, as quantified in Fig. 6b. It indicates that Q133H emerin tends to over-oligomerizes at the INM compared to wild-type emerin. The increased diffusion of both Q133H monomers and oligomers at the INM is attributed to their inability to bind nuclear actin, as described in the manuscript.

As we proposed, the formation of emerin oligomers is promoted via a transient unbinding of monomers from nuclear actin and BAF. Yet, as our data show and as mentioned in manuscript, both nuclear actin and BAF also modulate the behavior (diffusion and organization) of emerin oligomers. This suggests that some emerin within oligomers can still bind/unbind nuclear actin and BAF. It is therefore not surprising that oligomers of the non-actin binding mutant Q133H display increased diffusion if they do not dynamically bind nuclear actin at self-assembly sites.
This brings the interesting question of the function of nuclear actin at lamin A/C- and SUN1-stabilized oligomerization sites. Lamin A has been shown to interact with actin and can bind and bundle F-actin *in vitro* (**Sasseville *et al.*, FEBS Letters 1998; Simon *et al.*, Nucleus 2010**). Other reports have indicated that lamin A/C can indeed regulate nuclear actin polymerization in cells (**Ho *et al.*, Nature 2013**; **Dopie *et al.*, Journal of Cell Science 2015**) and that LINC complex components, lamin A/C and emerin participate in transient nuclear actin polymerization upon cell spreading (**Plessner *et al.*, Journal of Biological Chemistry 2015**). It is thus conceivable that, in addition to being stabilized by direct lamin A/C binding, emerin oligomers also interact with lamin A/C-anchored nuclear actin at these sites. Such interactions would explain why wild-type emerin oligomers in nuclei depleted for nuclear actin (IPO9 siRNA) and oligomers of the non-actin binding Q133H display increased lateral mobility compared to wild type emerin. We note that if the mobility of emerin oligomers is normally maintained by both direct binding of lamin A/C and binding to lamin A/C-anchored nuclear actin, one would have expected lamin A/C siRNA to induce a faster mobility of wild-type emerin oligomers compared to nuclear actin depletion (IPO9 siRNA), notably since lamin A/C siRNA indirectly leads to decreased levels of nuclear actin (Supp Fig. S2), as we have noted. This is not what we have seen, but this apparent discrepancy is not unexpected. Indeed, emerin initially pertaining to oligomers does not efficiently self-assemble after lamin A/C siRNA. As we have discussed in response to question 4 of Reviewer 3 above, previously oligomerized emerin behave like monomers with regards to their dynamic interactions with BAF after lamin A/C depletion. As such, dynamic interactions of their LEM domain with BAF, and indirectly with chromatin, likely provide compensatory emerin/BAF binding that could counter-act the increase in mobility expected for emerin at oligomeric sites after lamin A/C depletion.
We have clearly indicated in the manuscript that nuclear actin (and BAF) modulates the diffusion of both emerin monomers and oligomers. It therefore not surprising that an emerin mutant defective in actin binding, such as Q133H, displays increase mobilities for both types of organization compared to wild-type emerin.
We also would like to comment that an increased mobility of emerin within oligomeric nanodomains (as observed during stress), could facilitate its self-assembly and the regulation of nanodomain sizes and molecular densities. Indeed, local increase in membrane protein diffusion coefficients can promote collective movements of membrane protein clusters, and can locally modify the size and molecule occupancy of such clusters, as previously described to explain the plasticity of protein nanodomains in postsynaptic membranes (**Haselwandter *et al.*, Physical Review E 2015**).

Also, the authors write: "Q133H mutation disrupts emerin binding to actin but does not impede interactions with lamin A/C, SUN1 or BAF". I would be more cautious because these results have rarely been obtained on purified proteins. These are often more indications than clear demonstrations of (a lack of) binding. Other studies also report opposite results, as Herrada et al. (2015) concerning emerin binding to lamin A/C. Also, the authors write: "The increased lateral diffusion of Q133H, only at the INM, therefore indicates that it does not bind nuclear actin" > it could be that it does not bind lamin A/C as well.

We agree that caution is required regarding reported binding of Q133H to other partners. As recommended by Reviewer 3 we have now modified the sentence as follows:

"Previous in vitro studies indicated that the Q133H mutation disrupts emerin binding to actin (Holaska et al., 2004) but does not impede interactions with lamin A/C (Holt et al., 2001), SUN1(Haque et al., 2010) or BAF (Bengtsson and Wilson, 2004). The increased INM diffusion of Q133H, therefore suggests that it doesnot bind nuclear actin. (**lines 337-341**).

We are aware of contradictory results concerning the binding of Q133H to lamin A/C. For instance, using a quantitative surface plasmon resonance (Biacore) affinity binding assay, Holt *et al.* (**Biochemical and Biophysical Research Communications 2001**) have shown that there is no apparent difference in the binding of wild-type and Q133H emerin to lamin A *in vitro*. However,

based on an antibody proximity ligation assay (PLA) in cells, Herrada *et al.* (**ACS Chemical Biology 2015**) have found the Q133H is less frequently close to lamin A/C than wild-type emerin in Hela cells. Herrada *et al.* have proposed that the diminished ability of this mutant to self-assemble *in vitro* reduces its interaction with lamin A/C in cells. It is our understanding that this interpretation is based, in part, on previous results from the same lab indicating that lamin A/C only directly binds oligomerized emerin (**Samson *et al.*, Nucleic Acids Research 2018**). Those are the only two studies published to date that looked at the interaction/proximity of Q133Hemerin with lamin A/C.
Our own results show that Q133H efficiently forms oligomers at the INM (based on both diffusion and super-resolution data) and could, in principle, bind lamin A/C as per the results of Samson *et al.* (**Nucleic Acids Research 2018**). As we have also shown that lamin A/C binding is required for the stabilization of emerin oligomers (lamin A/C siRNA and Δ95-99 emerin mutant), our data argue against the idea that Q133H does not bind lamin A/C. To ensure that this interpretation is correct, we have now additionally studied the influence of lamin A/C siRNA on the organization of Q133H. As shown in **Fig. R13**, depletion of lamin A/C induces a loss of Q133H oligomers, as previously seen for wild-type emerin. Q133H monomers also distribute over larger INM areas, as already observed for wild-type emerin following interference with lamin A/C expression. This indicates that, like for wild-type emerin, the organization of

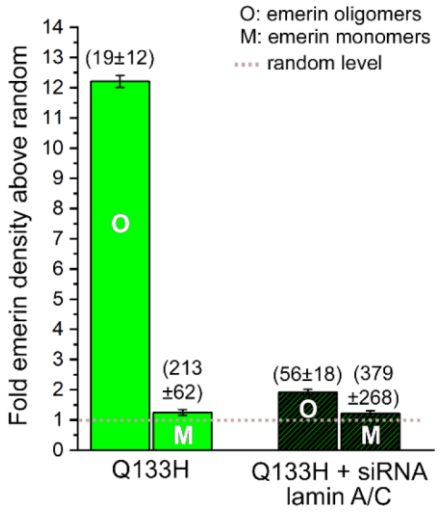

Fig. R13: **Effects of lamin A/C siRNA on Q133H emerin organization.** Molecular densities above random (± s.e.m.) for Q133H emerin oligomers (O) and monomers (M) in untreated cells (149340 localizations, 6 nuclei) or after siRNA of lamin A/C (60748 localizations, 4 nuclei). Values in parenthesis represent the length scale (± s.e.m.) of each domain in nanometers.

Q133H emerin at the INM, and in particular that of oligomers, requires stabilization by lamin A/C. These data strongly imply that Q133H binds lamin A/C, as we have argued above. As such, based on the direct binding measurements of Holt *et al.*, our data, and previous observations by Samson *et al.* that lamin A/C can bind oligomerized emerin, we have concluded that Q133H binds lamin A/C, both *in vitro* and in cells. We are unclear as to why Herrada *et al.* detected low proximity between Q133H and lamin A/C in their PLA measurements. We note that, using similar PLA measurements to probe emerin-emerin interactions, Herrada *et al.* (**ACS Chemical Biology 2015**) also did not detect an over-oligomerization of Q133H compared to wild-type emerin in cells. It is likely that the "sandwich" approach of PLA measurements (primary and secondary antibodies, combined size of ~20-30 nm) cannot clearly differentiate oligomerized emerin (~20 nm nanodomains) from monomeric emerin (~200 nm domains) at the NE. Effectively, emerin/emerin interaction PLA data reported for wild-type and Q133H emerin by Herrada *et al.* appear very similar, consistent with our observation that monomers for both types of emerin have the same overall spatial distribution at the INM. On the other hand, their PLA data for Δ95-99 show significantly reduced emerin/emerin proximity, consistent with the wider INM distribution of Δ95-99 monomers (~420nm).
As we have not, ourselves, made PLA measurements in the lab, we do not know how reliable/reproducible the technique might be. Others have reported that interpretation of some *in situ* PLA assays can be difficult (**Alsemarz *et al.*, bioRxiv 2018**). Considering the potential technical limitations of PLA assays and the fact that surface plasmon resonance remains one of the "gold" standards to measure in vitro biomolecular interactions, we feel more comfortable relying

on current data showing that Q133H binds lamin A/C directly at wild-type levels (**Holt *et al.*, Biochemical and Biophysical Research Communications 2001**).

Also, depletion of nuclear actin increases the mobility of the outer membrane fraction of emerin (Fig. 2a) whereas depletion of lamin A/C does not (Fig. 2a also). So why strongly correlating an increased lateral diffusion of Q133H to a lack of binding to nuclear actin?

We did not solely rely on a comparison of Q133H diffusion data with lamin A/C and IPO9 siRNA to conclude that the increased lateral mobility of Q133H is linked to its inability to bind nuclear actin. As discussed above, our conclusion is based on an ensemble of diffusion and nanoscale organization data for emerin under various conditions (lamin A/C siRNA, IPO9 siRNA, Δ95-99 emerin mutant, etc…) and on previous observations that: (i) Q133H does not bind actin (**Holaska *et al.*, PLoS Biology 2004**), (ii) Q133H is predicted to bind BAF via its conserved LEM domain (**Berk *et al.*, Nucleus 2013**), (iii) Q133H binds lamin A/C at wild-type levels (**Holt *et al.*, Biochemical and Biophysical Research Communications 2001**) and Q133H binds SUN1 at wild-type levels (**Haque et al., Journal of Biological Chemistry 2010**). We maintain that the simplest explanation (Occam's razor) for the faster INM mobility of Q133H is that it does not bind nuclear actin.
We understand that Reviewer 3 is concerned with the fact that the diffusion of Q133H resembles more our lamin A/C siRNA diffusion data than our IPO9 siRNA diffusion data, because 10% of ONM emerin behave differently. We would argue, however, that the apparent molecular organization of Q133H at the INM, where 90% of the emerin pool is located resemble more our IPO9 siRNA super-resolution data than our lamin A/C siRNA super-resolution data or than our super-resolution data for the Δ95-99 emerin, which does not bind lamin A/C, as shown in **Fig. R14** below.

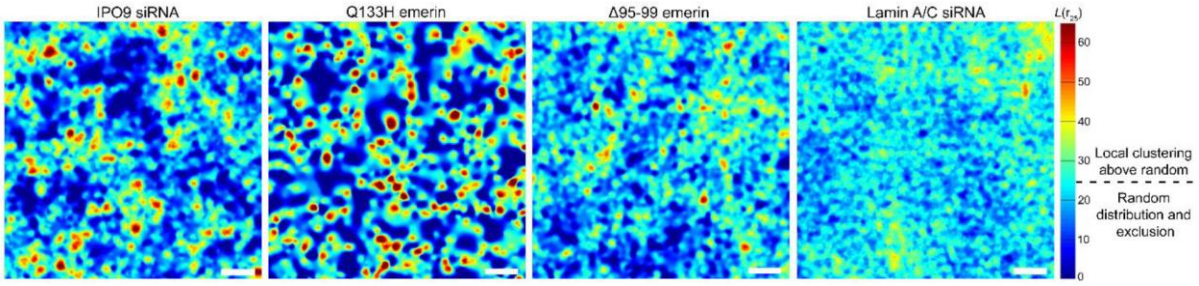

Fig R14: Comparison of emerin cluster maps between wild-type emerin after nuclear actin depletion by IPO9 siRNA, Q133H emerin mutant, Δ95-99 emerin mutant and wild-type emerin after lamin A/C depletion by lamin A/C siRNA. Scales: 250 nm.

Again, this argues for Q133H being capable of binding lamin A/C and the conclusion that this mutant has an increased INM mobility most likely because it does not bind nuclear actin and not because it does not bind lamin A/C.

8) Figs. 6& 7: in the case of P183H, it is not clear for this reviewer how different the behavior of this variant is, when compared to del95-99. And what are the green bars on Fig. 6g compared to the red bars ? Also, why is the study of the behavior of del95-99 under stress (Fig. 7) not shown also for P183H?

The green bars on Fig. 6g report on the diffusion coefficients and the populations detected when we perform CALM imaging with the P183H emerin mutant fused to split-GFP fragments. As discussed in the manuscript, these data show that P183H has a strong tendency to dimerize. This behavior, which was predicted by Herrada *et al.* (**ACS Chemical Biology 2015**) based on their observations that P183H emerin has high propensity to self-assemble in vitro, is also consistent with reports that P183 is positioned in the 168-186 emerin region required to limit emerin-emerin self-association (**Berk *et al.* Journal of Cell Science, 2014**).
In terms of lateral mobility (sptPALM data), both P183H and Δ95-99 are indeed quite similar. They both show a decrease mobility of emerin monomers and an apparent immobilization of emerin in oligomerization nanodomains. We have attributed these slower mobilities to increased interaction frequencies of both mutants with BAF.

(i) In the case of P183H, dimerization likely induces more frequent interactions with BAF

because LEM domain binding sites along the IDR might not be accessible in P183H emerin dimers. Note that the effect of BAF binding (slow diffusion) is probably amplified because two LEM domains in a P183H dimer could potentially interact simultaneously with BAF, and indirectly with lamin A/C and chromatin. As an alternative explanation, we have also mentioned that P183H has been reported to have a stronger lamin A/C binding capacity than wild-type emerin.

(ii) In the case of Δ95-99, the mutation is positioned in the 48-118 region of emerin, which has been proposed to act as a binding site for the LEM domain and for emerin/emerin bridging interactions. The potentially reduced flexibility of the 48-118 region in Δ95-99 emerin and the limited ability of this mutant to self-assemble could indeed result in its LEM domain interacting more frequently with BAF, as for P183H dimers. This likely explains why both mutants have relatively similar diffusive behaviors.

In terms of structural organization (dSTORM data), P183H and Δ95-99 display differences.

(i) P183H emerin organizes as oligomers with reduced molecular density (only 2-fold above random) compared to wild-type emerin (8-fold above random). As we have proposed, the tendency of P183H to dimerize and its slow diffusion likely result in its reduced capacity to form higher order oligomers at the INM, because access of the LEM domain to LEM binding sites on other P183H emerin (for emerin self-association) is probably hindered by IDR/IDR interactions between dimers. The ability of P183H to bind lamin A/C, likely explains why residual oligomerization is observed compared to Δ95-99 in non-patterned cells.

(ii) In contrast, Δ95-99 emerin primarily displays a random distribution (1.3-fold above random for oligomer sites). As we have proposed, the drastically reduced oligomerization of Δ95-99 likely stems from its slow diffusion and from its inability to bind lamin A/C, which would prevent a stabilization of the few oligomers that might form.

Herrada *et al.* (**ACS Chemical Biology 2015**) have shown that P183H emerin has a strong capacity to self- assemble in vitro, while Δ95-99 emerin has reduced self-assembly properties. Our data in emerin-null dermal fibroblasts confirm the in vitro observations that Δ95-99 emerin fails at forming oligomers. Yet, we did not observe an increased oligomerization of P183H emerin at the NE. As we have explained above, the tendency of P183H to dimerize and its reduced mobility at the INM likely influence its ability to form oligomers at wild-type levels in cells, leading to an organization that resemble more that of Δ95-99 emerin than that of wild-type emerin. We believe that we have clearly presented these explanations in the main text.

We have not yet studied the behavior of P183H in micropatterned cells. Our choice to study Δ95-99 emerin under mechanical stress, as opposed to other emerin mutants, was based on the expectation that this mutant would provide the clearest effects for a need to properly modulated emerin oligomer formation during nuclear response to force, as it is the mutant that showed the largest disruption in oligomer formation in non-patterned cells.

Only the discussion is sometimes difficult to follow, because it is based on a large number of (sometimes contradictory) experimental data previously published on emerin. Summarizing these previous data at the beginning of the discussion in a clear and condensed manner would have helped to follow the interpretation of the new data provided by this study.

We would gladly provide a summary of the previously published data we discussed at the beginning of the discussion section. However, length limits for research articles in the Journal of Cell Science (8000 words) makes it difficult to integrate such a discussion, as the manuscript is already above this word limit. If Reviewer 3 and the editor agree, we think that some of the responses and discussion above, if satisfactory, could be provided as part of the peer review history published together with the manuscript.

Second decision letter

MS ID#: JOCES/2021/258969

MS TITLE: Emerin self-assembly and nucleoskeletal coupling regulate nuclear envelope mechanics against stress

AUTHORS: Anthony Fernandez, Markville Bautista, Liying Wu, and Fabien Pinaud
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, one of the reviewers (#2) still raises some critical points that will require amendments to your manuscript. Please address them as best you can I because I would like to be able to accept your paper, depending on further comments from this reviewer as well as my own assessment regarding the need for particular experiments.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewer in the 'Response to Reviewers' box. Please attend to all of this reviewer's comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

I thank the authors for responding so rigorously to all my comments. All necessary corrections have been made, and the new data added is useful and helpful. I enjoyed the thorough discussion of various aspects (e.g., on dynamic localization accuracy), I found it very helpful. In fact, I would like to say that this is probably one of the best revisions I have ever read. Knowing how much time this takes, I can only thank the authors again for their motivation and effort.
I recommend that the revised manuscript be published as is.

Comments for the author

I recommend that the revised manuscript be published as is.

Reviewer 2

Advance summary and potential significance to field

Overall, the authors have addressed the majority of the concerns that I raised in my review of their original manuscript. However, I still have several issues that I would like the authors to tackle

before I am willing to accept their revised manuscript for publication. These issues are listed below.

Comments for the author

1) I appreciate that the authors have struggled to verify by Western blotting and Immunofluorescence that they have successfully depleted IPO9 and XPO6. While I agree that the authors' ability to demonstrate that nuclear actin levels change as expected in their IPO6- or XPO6-depleted cells, I still would like to see some verification that they are actually decreasing the levels of IPO6 or XPO6 in their RNAi-treated cells. Since Western blotting has been problematic, perhaps the authors could use quantitative RT-PCR to demonstrate that they are depleting the levels of IPO6 or XPO6 mRNA in their RNAi-treated cells? That being said, it does appear that the levels of IPO6 are being significantly decreased in their shRNA-treated cells as shown in Figure R3. Can the authors quantify these immunofluorescence results?

2) I still have issues with the authors not having demonstrated that they can rescue the effects of knocking down BAF, IPO9, or XPO6 on emerin oligomerization. Since they are only using one siRNA duplex per protein their ability to rescue the effect of depleting these proteins on emerin oligomerization is essential to be able to rule out any potential off-target effects that may or may not be influencing their results. While the authors state that they "already assessed potential off-target or side effects of (their) siRNAs by immunoblotting and confocal imaging as described in Supplementary Figs. S2-3, this is not entirely accurate; the authors only tested the impact of depleting BAF, IPO9, and XPO6 on the levels and subcellular localization of lamin A/C nuclear actin, BAF, SUN1, and H2B. To truly rule out off-target effects in RNAi experiments the gold standard to demonstrate that the phenotype observed in cells depleted of a given protein can be rescued by the re-
expression of the depleted protein. In addition, it is common to use more than one siRNA/shRNA per protein of interest as an alternative method for ruling out off-target effects.

3) I am a little concerned by the less-than impressive rescue of emerin oligomerization observed in SUN1-depleted cells that are expressing EGFP-SUN1 as demonstrated in Figure 3F. This suggests to me that there is something off about the EGFP-SUN1 construct being used in this work. Perhaps the particular SUN1 isoform that is encoded in this EGFP-SUN1 construct is missing parts of the SUN1 nucleoplasmic domain that are important for emerin oligomerization? Alternatively, the presence of EGFP on the N-terminus of SUN1 may be problematic for SUN1's function in controlling the oligomerization of emerin? Since the authors were able to observe impressive rescue of emerin oligomerization in their lamin A/C-depleted cells that expressed unlabeled lamin A/C, perhaps the authors could try to see if a similar approach might work for SUN1?
Alternatively, they could test the ability of a SUN1-EGFP construct with EGFP fused to the SUN1 C-terminus to rescue emerin oligomerization in their SUN1-depleted cells.

4) I appreciate that the authors were able to show that over-expression of DN-KASH inhibited emerin oligomerization in their cells. However, there are two important controls that the authors need to perform in order to properly interpret these results. First, it is common to delete the luminal KASH peptide of the DN-KASH construct and then test the impact of over-expressing this construct (DN-KASHΔL), which cannot interact with SUN proteins, on the phenotype of interest. Second, it is important that the authors demonstrate that the expression of DN-KASH, but not the DN-KASHΔL construct, results in the displacement of endogenous nesprin proteins from the nuclear envelope. It would be very useful for the authors to perform these control experiments and include their results in their revised manuscript.

5) I am happy that that authors were able to use Utr230-EN to better assess the impact of depleting IPO9 or XPO6 on nuclear actin organization. However, I would like the authors to provide some representative images of Utr230-EN in their IPO9- or XPO6-depleted cells in addition to the images of this construct expressed in control cells, as shown in Figure S3.

6) In the abstract (lines 21-22), the authors state, "We show that emerin monomers form localized oligomeric nanoclusters stabilized by both lamin A/C and SUN1 at the LINC complex". A similar statement is made in lines 267-268. I do not believe that these statements are very accurate, as the authors' results show that emerin oligomerization is dependent on A-type lamins and SUN1-containing LINC complexes. The results do not show that emerin oligomerization is occurring at LINC complexes. The authors need to tone-down this over-interpretation of their results.

7) I suggest that the authors change their statement “Emerin oligomers are stabilized by lamin A/C and SUN1 at LINC complexes” (line 203) to “Emerin oligomers are stabilized by lamin A/C and SUN1-containing LINC complexes”.
8) I recommend that the authors change “SUN1/nesprin interactions at LINC complexes” (line 264) to “SUN1-containing LINC complexes, as SUN/nesprin interactions result in the formation of LINC complexes.
9) I suggest that the authors change their statement “When SUN1 is depleted to destabilize LINC complexes” (line 239) to “When SUN1 is depleted to destabilize SUN1-containing LINC complexes”. This raises the question of what happens to emerin oligomerization in SUN2-depleted cells. Have the authors tested this specificity control?

Reviewer 3

Advance summary and potential significance to field

The authors answered in details to the reviewer comments and modified their manuscript accordingly. It would be interesting for the readers to have access to the answers to the reviewers, given that there are plenty of additional discussion in these answers.

Comments for the author

The authors answered in details to the reviewer comments and modified their manuscript accordingly. It would be interesting for the readers to have access to the answers to the reviewers, given that there are plenty of additional discussion in these answers.

Second revision

Author response to reviewers' comments

Reviewer 2: comments 6-9

(6) In the abstract (lines 21-22), the authors state, “We show that emerin monomers form localized oligomeric nanoclusters stabilized by both lamin A/C and SUN1 at the LINC complex”. A similar statement is made in lines 267-268. I do not believe that these statements are very accurate, as the authors’ results show that emerin oligomerization is dependent on A-type lamins and SUN1-containing LINC complexes. The results do not show that emerin oligomerization is occurring at LINC complexes. The authors need to tone-down this over-interpretation of their results.
The sentence has now been changed to “We show that emerin monomers form localized oligomeric nanoclusters stabilized by both lamin A/C and SUN1 LINC complex”. A change of a similar sentence has also been implemented on line 265-266 (equivalent to lines 267-268, mentioned by thereviewer).

(7) I suggest that the authors change their statement “Emerin oligomers are stabilized by lamin A/C and SUN1 at LINC complexes” (line 203) to “Emerin oligomers are stabilized by lamin A/C and SUN1-containing LINC complexes”.
The requested change has now been implemented and the sentence reads: “Emerin oligomers are stabilized by lamin A/C and SUN1-containing LINC complexes and are modulated by nuclearactin and BAF**” (lines 200-201, not 203)**

(8) I recommend that the authors change “SUN1/nesprin interactions at LINC complexes” (line 264) to “SUN1-containing LINC complexes, as SUN/nesprin interactions result in the formation of LINC complexes.
The sentence has been changed as follows: “In cells expressing mCherry-DN-KASH, emerin oligomers were disrupted at levels equivalent to those of SUN1 siRNA (Fig. 3C; Table S2), indicating that the oligomerization of emerin requires functional SUN/nesprin interactions and

a coupling of the nucleoskeleton and the cytoskeleton via SUN1-containing LINC complexes."

(9) I suggest that the authors change their statement "When SUN1 is depleted to destabilize LINC complexes" (line 239) to "When SUN1 is depleted to destabilize SUN1-containing LINC complexes". This raises the question of what happens to emerin oligomerization in SUN2-depleted cells. Have the authors tested this specificity control?
As requested, the sentence has now been changed to: "When SUN1 is depleted to destabilize SUN1-containing LINC complexes (Fig. S2), the formation of emerin oligomers is reduced to levels equivalent to lamin A/C depletion."

Third decision letter

MS ID#: JOCES/2021/258969

MS TITLE: Emerin self-assembly and nucleoskeletal coupling regulate nuclear envelope mechanics against stress

AUTHORS: Anthony Fernandez, Markville Bautista, Liying Wu, and Fabien Pinaud
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.