EMBO reports

The nucleoporin ELYS regulates nuclear size by controlling NPC number and nuclear import capacity

Predrag Jevtić, Andria C. Schibler, Chase C. Wesley, Gianluca Pegoraro, Tom Misteli, Daniel L. Levy

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11th Dec 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees think the manuscript is of interest, but requires a major revision before publication in EMBO reports. As the reports are below, and I think all points need to be addressed in a revised manuscript and/or in a detailed rebuttal letter, I will not further detail them here. However, I think that in particular the two major points of referee #2 needs to be addressed with additional data (depletion of Nup153, characterize aggregates more and show their functional relevance).

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

REFEREE REPORTS:

Referee #1:

In this manuscript, the authors identified ELYS a nucleoporin required for postmitotic nuclear pore complex (NPC) assembly, as a determinant of nuclear size in mammals using a high-throughput imaging RNAi screening. They also showed that knockdown of ELYS resulted in a lower density of NPC and decreased nuclear import. In addition, overexpression of importin α or ELYS increased nuclear size, suggesting the importance of the nuclear import capacity for mammalian nuclear size determination. Overall, this manuscript contains novel and interesting data. However, there are some critical points that have not been addressed in the current manuscript.

(1) It is known that there exist nucleoporins, other than ELYS, whose knockdown causes the decrease of NPC numbers (ex: Nup107 (Doucet et al. 2010 Cell)): however, in this study, the reduction of nuclear size was not observed by the knockdown of these Nups. Authors should address this discrepancy.

(2) ELYS is most likely a multifunctional protein. ELYS is known to bind to chromatin and/or nucleosomes (Rasala et al. MBOC 2008, Zierhut et al. NSMB 2014), and associates with enhancer/promoter regions (Pascual-Garcia et al. 2017 Mol Cell). Thus, the function of ELYS on chromatin/gene regulation, not NPC formation, could affect the regulation of nuclear size. In addition, the authors identified various chromatin or epigenetic regulators in their screening. These points should be clearly discussed.

(3) The authors concluded that the decrease of NPC density only affects the nuclear import process, but not the nuclear export. Why is the nuclear import more sensitive than the nuclear export to the decrease of NPC? Are there any specific reasons?

These points should be properly addressed before publication.

Other specific points:

1) Fig. 1B: The size of nuclei is particularly heterogeneous in ELYS knocked down cells, compared to others. Is this related to the cell viability? (Also, the error bar for ELYS in Fig. 1C seems smaller than expected.)

2) Fig. 5: The expression level of overexpressed importin α should be examined (as compared with endogenous one).

3) Fig. S2: Have the authors examined the expression levels of ELYS and SEC13 in the cell lines used in this study? These nucleoporins may be highly expressed in MCF7, as compared with normal cell lines.

Referee #2:

The manuscript by Jevtić et al addresses the control of nuclear size. Previous studies in several model organisms have revealed regulators, repeatedly components of the nuclear transport system but also others including constituents of the nuclear lamina. Nuclear size regulation in humans is less understood. The current work reveals a role for the nucleoporin ELYS, a component of the NPC scaffold. The authors show that ELYS knockdown leads to smaller nuclei in several human cell lines. ELYS depletion reduces NPC density and alters transport capacity, which can be counteracted by overexpression of importins. Consistently they find that importin depletion reduces nuclear size while inhibiting exportin 1 results in larger nuclei.

Despite being perhaps not enormously surprising in the light of previous studies in other organisms, the main conclusions of the manuscript are clear and well supported by the presented data. They highlight a clear functional relationship between NPC density, nuclear import and ultimately the size of the organelle, which is interesting. Thus the reviewer recommends publication given that two

major points are sufficiently addressed.

1. Since NPC number seems crucial, it is surprising that only two Nups (ELYS and Sec13) were identified as size regulators in the screen performed in this study. ELYS has an instrumental role for postmitotic NPC assembly. In contrast, Nup153 is essential to make NPCs during interphase. One would assume that its depletion should also result in decreased NPC density. The authors should elaborate on this and provide the nuclear size and NPC density data for depletion of Nup153.

2. ELYS knockdown induces also cytoplasmic Lamin aggregates, presumably by their failed import into the nucleus. It is not clear to the reviewer why the authors focus so much on these aggregates, since they do not have to have a functional relevance for ELYS mediated nuclear size control (which is the issue in this work). In fact the authors show that removal of those aggregates by inhibiting PP1CA (a Lamin phosphatase) does not rescue nuclear size defects (Figure S3). They also show that interfering with lamins does not alter the larger nuclear size caused by decrease nuclear export (Figure S4). Thus the cytoplasmic Lamin aggregates seem more a marker for ELYS depletion than to have a functional relevance for what the paper actually is pointing at. Thus the authors should give it less importance. For example all 6 movies focus on that issue. In addition these movies are really redundant, since all show the emergence/fate of cytoplasmic Lamin aggregates. Some should be removed.

Alternatively, if the authors think these aggregates are that important, they should characterize them in more detail. They only show that, surprisingly however, they are distinct from NPCs at annulate lamellae (which are known to emerge upon ELYS depletion (Franz et al, 2007). For example do these Lamin aggregate contain membranes? In the movies mCherry-Lamin appears like ER.

The reviewer has also a comment on the general readability of the manuscript: Often the conclusions stated in the text and where they are supported by the respective display item are disconnected from each other. This significantly decreases the readability of the otherwise very clear and concise manuscript. For example, Figure S1 is discussed early in the text, while readers have to wait until the end of the results section that they learn about the effects of XPO1 depletion, which are however displayed in Figure S1A. This is just one example of many. The authors should try to improve this.

Referee #3:

In this manuscript, Jevtić, Schibler et al. study the role of nucleoporin ELYS as a determinant of nuclear size in mammalian cells. The authors perform a high throughput RNAi screen of 867 genes implicated in NE function, chromatin structure and epigenetic mechanisms. They carry out measurements of nuclear cross-sectional area and select the genes that are in charge of a significant nuclear size decrease. The authors find 19 genes that can cause nuclear size decrease. Among them, they select ELYS protein because it is causing cytosolic lamin puncta and has an assembly role of the nuclear pore complex. Further, they characterize the effect of both knocking down and overexpressing ELYS. Interestingly, they find a relation between ELYS KD, active transport though nucleopores, and nuclear size. The relationship found between nuclear import and size is interesting and worth publishing. However, there are important flaws in the rationale of the paper that need to be resolved before considering it for publication.

1. Importantly, the results of figure 1 are impossible to interpret without a quantification of knock down levels. Indeed, the effect of the different sIRNAs could be largely due to the knock down level of the different siRNAs, rather than a specific effect of the protein itself. This is made even more confusing by the fact that the authors do not even use the results of this figure to choose their main molecular focus. Rather, they pick ELYS (number 7 in their screen) due to its previously described role as a required element for NPC assembly.

2. Thus, the authors could dispense with figure 1 altogether, and simply state that they will focus on ELYS due to its previously described role. However, since ELYS is known to be required for nuclear growth, that its knock down results in smaller nuclei is somewhat to be expected and not entirely novel. To circumvent this problem, the authors state that their work is "the first demonstration that NPC numbers can modulate nuclear size and that nuclear transport can tune nuclear size in mammalian cells". However, the data shown with ELYS alone are not sufficient, in

my view, to support this statement. Indeed, it is unclear whether ELYS knock-down merely results in decreased NPC numbers, or transport through NPCs is also impaired, and how. To support this statement, the authors should alter NPC numbers through some other mechanism, and demonstrate that the effects are the same.

3. Authors should give more details on how they calculate the z-core. For example, they should clarify if they use the mean of the control sample or the mean of the whole screening.

4. I think that the representation would be more robust if the median z-score instead of the maximum z-score were used in Fig. 1C.

5. The term correlation is used in the text, but there is no correlation study. It would be more useful if correlations were really shown. For example, Fig. 2D would be more valuable if for each data point the normalized ELYS staining intensity vs. cytoplasmic Lamin intensity was shown, and then a correlation study was carried out.

6. Fig. S2 A: siELYS merge does not correspond to the 3 previous images.

7. Authors mention and show that ELYS siRNA knock down resulted in formation of cytoplasmic lamin puncta, containing both lamin A and B. However, then they only quantify the link between ELYS intensity decrease and lamin type B1. Does this link also exist for lamin B2 and A/C?

8. Scalebar, time and what each channel represents should be in each video.

9. " Knockdown of PPP2R4 had no impact on the appearance of lamin puncta (data not shown)". Please show.

10. The quantifications of Fig. 4E-F would be better understood if stainings were shown.

11. The Western Blot of siRNA ELYS+EGFP ELYS is missing.

12. Why do authors sometimes calculate cell area (Fig. 6 C) and other times use z-score (Fig. S2)?

1st Revision - authors' response 7th Mar 2019

Please see next page.

Point-by-point response to previous reviews

The comments of the referees are copied here in italics. Our responses are just below each point made by the referee. Our major additions and changes to the revised manuscript are in blue font. Other small changes were made throughout the text but are not explicitly mentioned here.

Referee #1:

In this manuscript, the authors identified ELYS a nucleoporin required for postmitotic nuclear pore complex (NPC) assembly, as a determinant of nuclear size in mammals using a high-throughput imaging RNAi screening. They also showed that knockdown of ELYS resulted in a lower density of NPC and decreased nuclear import. In addition, overexpression of importin α or ELYS increased nuclear size, suggesting the importance of the nuclear import capacity for mammalian nuclear size determination. Overall, this manuscript contains novel and interesting data. However, there are some critical points that have not been addressed in the current manuscript.

We appreciate the support and have now addressed the points raised by the referee.

(1) It is known that there exist nucleoporins, other than ELYS, whose knockdown causes the decrease of NPC numbers (ex: Nup107 (Doucet et al. 2010 Cell)): however, in this study, the reduction of nuclear size was not observed by the knockdown of these Nups. Authors should address this discrepancy.

We have repeated the Nup107 siRNA treatment and find that while nuclear size is not affected, consistent with our screen results, there is a modest reduction in NPC number. Nup107 is a scaffold Nup known to have a particularly long half-life, likely explaining why Nup107 siRNA did not reduce NPC numbers to the same extent as ELYS

knockdown. These new results are presented in Fig. EV3D-E and in the Results section as follows:

Lines 136-140: The observed effects of SEC13 and ELYS are specific and not a general property of Nups since, out of 33 Nups tested in the screen, siRNA oligos against only these two Nups decreased nuclear size. The reason for this may be because some Nups are particularly long-lived and/or because of differences in post-mitotic versus interphase NPC assembly (see Discussion).

Lines 183-186: Consistent with results from the screen, knockdown of Nup153 and the longer-lived Nup107 did not affect nuclear size, likely because NPC numbers were not reduced to the same extent as upon knockdown of ELYS (Fig. EV3D-E, see Discussion).

We have also added the following text to the Discussion to speculate as to why knockdown of ELYS, and not other nucleoporins, led to reduced nuclear size:

Lines 238-261: Why did knockdown of only these two Nups decrease nuclear size in our screen? We found that ELYS knockdown decreased NPC numbers to a much greater extent than knockdown of other Nups, including Nup153 and Nup107. One possibility is that some long-lived scaffold Nups, like Nup107, were not efficiently depleted over the two-day siRNA treatment typically used in our experiments [79-81]. In line with this interpretation, twelve continuous days of Nup107 knockdown were required to reduce Nup107 protein levels to ~30% [80], while we found that ELYS levels were reduced to ~40% after only two days of depletion and SEC13 is known to turn over rapidly [79]. Another possibility is that the effect of Nup depletion on nuclear size might depend on whether the Nup is involved in post-mitotic versus interphase NPC assembly [80,82]. ELYS is critical for post-mitotic NPC assembly, while nuclear import of Nup153 and subsequent recruitment of the Nup107-160 complex is required for interphase, but not post-mitotic, NPC assembly [82,83]. Post-mitotic NPC assembly occurs within minutes of nuclear formation while interphase NPC assembly is more sporadic and much slower, on the order of an hour [84-87]. Reducing post-mitotic NPC assembly by ELYS depletion might influence nuclear size more strongly because those are the pores that will drive early nuclear growth. On the other hand, reducing interphase NPC assembly by Nup153 depletion might have less of an effect on NPC numbers and nuclear size, especially if post-mitotic NPCs primarily drive the acquisition of a steady-state size. In support of this hypothesis, blocking interphase NPC insertion had no effect on nuclear volume [88]. Lastly, depletion of some Nups may have resulted in a modest reduction in nuclear size, but because we defined hits as having a z-score less than -1.5 we naturally focused on the stronger hits. In that sense, our screen was not saturating.

(2) ELYS is most likely a multifunctional protein. ELYS is known to bind to chromatin and/or nucleosomes (Rasala et al. MBOC 2008, Zierhut et al. NSMB 2014), and associates with enhancer/promoter regions (Pascual-Garcia et al. 2017 Mol Cell). Thus, the function of ELYS on chromatin/gene regulation, not NPC formation, could affect the

regulation of nuclear size. In addition, the authors identified various chromatin or epigenetic regulators in their screening. These points should be clearly discussed.

These points are now discussed in the manuscript as follows:

Lines 285-293: ELYS is a multifunctional protein as it has been shown to interact with chromatin, enhancers, and promoters [53,104,105]. While we cannot eliminate the possibility that ELYS depletion affects transcription, the fact that reduced nuclear import and size resulting from ELYS depletion were rescued by importin α overexpression strongly argues that the nuclear size effects are import-mediated. ELYS knockdown decreased nuclear lamin B2 import and nuclear size while ELYS overexpression gave the opposite result. It seems unlikely that these reciprocal effects reflect ELYS-mediated changes in chromatin or gene regulation. At least some of the nuclear size effects we observe must result from ELYS-mediated effects on NPC number and import.

Lines 261-264: It is worth noting that chromatin and epigenetic regulators were also identified as hits in our screen, and future work will focus on how changes in transcription and chromatin structure affect nuclear size.

(3) The authors concluded that the decrease of NPC density only affects the nuclear import process, but not the nuclear export. Why is the nuclear import more sensitive than the nuclear export to the decrease of NPC? Are there any specific reasons?

It is indeed possible that export is affected in ELYS knockdown cells. However, the fact that ELYS knockdown resulted in decreased nuclear lamin B2 and reduced bulk import measured with GFP-NLS strongly implicates nuclear import. In support, increasing bulk import by overexpressing importin α rescued nuclear size and lamin B2 nuclear import in ELYS knockdown cells. We mention this in the manuscript as follows:

Lines 191-202: ELYS knockdown reduced NPC density, potentially affecting nucleocytoplasmic transport. While both nuclear import and export could be affected, reduced nuclear lamin B2 in ELYS knockdown cells suggested an import defect. Indeed, increasing bulk import in ELYS knockdown cells by importin α overexpression resulted in nuclear sizes comparable to control cells and a reduction in the percentage of cells with cytoplasmic lamins (Fig. 4A-C and EV3F). These data indicate that small nuclear size and the formation of lamin aggregates in ELYS knockdown cells are due to limited nuclear import capacity. Consistent with this notion, importin α overexpression increased nuclear levels of both lamin B2 and GFP-3x SV40 NLS, a reporter of importin α/β-mediated nuclear import (Fig. 4D). We also observed that importin α overexpression alone resulted in a 40% increase in nuclear cross-sectional area compared to control cells (Fig. 4A-B).

Other specific points:

1) Fig. 1B: The size of nuclei is particularly heterogeneous in ELYS knocked down cells, compared to others. Is this related to the cell viability?

Our cell cycle analysis suggested that cell viability was not affected in ELYS knockdown cells. We also measured viable cell numbers during the screen, and these data also showed that ELYS knockdown does not affect cell viability. These data are included in the manuscript in Fig. EV1F and mentioned as follows:

Lines 141-144: While SEC13 and ELYS knockdown might be expected to have pleiotropic effects, there was no pronounced change in the cell cycle profiles and cell numbers were not affected (Fig. EV1F), suggesting that observed nuclear size reductions were not indirectly due to altered cell proliferation or cell cycle progression.

Lines 1075-1077: Median cell number z-scores were 0.35 (p-value 0.62) and 0.41 (p-value 0.34) for ELYS and SEC13 knockdown, respectively, indicating no significant effect on cell numbers.

The likely explanation for the size heterogeneity is that the amount of ELYS knockdown varied significantly from cell-to-cell (Fig. EV1C-D). Cells with greater ELYS depletion had particularly small nuclei with cytoplasmic lamin aggregates. In cells where ELYS was less depleted, nuclear size was less reduced and cytoplasmic lamin aggregates were not apparent (Fig. EV1D-E). This is mentioned in the manuscript as follows:

Lines 1063-1064: ELYS knockdown was greater in cells with lamin aggregates.

Lines 1066-1068: While not all ELYS and SEC13 knockdown cells exhibited cytoplasmic lamins, those that did had smaller nuclei compared to knockdown cells without cytoplasmic lamin accumulations.

(Also, the error bar for ELYS in Fig. 1C seems smaller than expected.)

The error bars in Fig. 1C represent the variability between biological replicates and not the variability in nuclear size. This has now been clarified in the figure legend as follows:

Line 922: Error bars represent the SEM for biological replicates.

2) Fig. 5: The expression level of overexpressed importin α should be examined (as compared with endogenous one).

We have measured the level of importin α overexpression by western blot and show that it is at 81% of endogenous importin α levels. These data are presented in Fig. EV3F and in the manuscript as follows:

Lines 1144-1146: Ectopically expressed mCherry-importin $\alpha 2$ was expressed at 81% ± 35% (average ± SD) of endogenous importin α levels.

3) Fig. S2: Have the authors examined the expression levels of ELYS and SEC13 in the cell lines used in this study? These nucleoporins may be highly expressed in MCF7, as compared with normal cell lines.

We have now measured ELYS levels in the various cell lines we tested. These data are presented in Fig. EV4F and discussed in the manuscript as follows:

Lines 308-310: In particular, ELYS and SEC13 knockdown significantly reduced nuclear size in three roughly normal cell lines but minimally affected nuclear size in MCF7 breast cancer cells in which ELYS expression was the lowest (Fig. EV4).

Lines 1163-1166: Notably, ELYS levels are lowest in MCF7 cells and ELYS was efficiently knocked down by siRNA treatment, eliminating high ELYS expression or poor ELYS knockdown as reasons for why ELYS knockdown minimally affected nuclear size in MCF7 cells.

Referee #2:

The manuscript by Jevtić et al addresses the control of nuclear size. Previous studies in several model organisms have revealed regulators, repeatedly components of the nuclear transport system but also others including constituents of the nuclear lamina. Nuclear size regulation in humans is less understood. The current work reveals a role for the nucleoporin ELYS, a component of the NPC scaffold. The authors show that ELYS knockdown leads to smaller nuclei in several human cell lines. ELYS depletion reduces NPC density and alters transport capacity, which can be counteracted by overexpression of importins. Consistently they find that importin depletion reduces nuclear size while inhibiting exportin 1 results in larger nuclei.

Despite being perhaps not enormously surprising in the light of previous studies in other organisms, the main conclusions of the manuscript are clear and well supported by the presented data. They highlight a clear functional relationship between NPC density, nuclear import and ultimately the size of the organelle, which is interesting. Thus the reviewer recommends publication given that two major points are sufficiently addressed.

We appreciate the support and have now addressed the points raised by the referee.

1. Since NPC number seems crucial, it is surprising that only two Nups (ELYS and Sec13) were identified as size regulators in the screen performed in this study. ELYS has an instrumental role for postmitotic NPC assembly. In contrast, Nup153 is essential to make NPCs during interphase. One would assume that its depletion should also result in decreased NPC density. The authors should elaborate on this and provide the nuclear size and NPC density data for depletion of Nup153.

We have repeated the Nup153 siRNA treatment and find that while nuclear size is not affected, consistent with our screen results, there is a modest reduction in NPC number. These new results are presented in Fig. EV3D-E and in the Results section as follows:

Lines 136-140: The observed effects of SEC13 and ELYS are specific and not a general property of Nups since, out of 33 Nups tested in the screen, siRNA oligos against only these two Nups decreased nuclear size. The reason for this may be because some Nups are particularly long-lived and/or because of differences in post-mitotic versus interphase NPC assembly (see Discussion).

Lines 183-186: Consistent with results from the screen, knockdown of Nup153 and the longer-lived Nup107 did not affect nuclear size, likely because NPC numbers were not reduced to the same extent as upon knockdown of ELYS (Fig. EV3D-E, see Discussion).

We have also added the following text to the Discussion to speculate as to why knockdown of ELYS, and not other nucleoporins, led to reduced nuclear size:

Lines 238-261: Why did knockdown of only these two Nups decrease nuclear size in our screen? We found that ELYS knockdown decreased NPC numbers to a much greater extent than knockdown of other Nups, including Nup153 and Nup107. One possibility is that some long-lived scaffold Nups, like Nup107, were not efficiently depleted over the two-day siRNA treatment typically used in our experiments [79-81]. In line with this interpretation, twelve continuous days of Nup107 knockdown were required to reduce Nup107 protein levels to ~30% [80], while we found that ELYS levels were reduced to ~40% after only two days of depletion and SEC13 is known to turn over rapidly [79]. Another possibility is that the effect of Nup depletion on nuclear size might depend on whether the Nup is involved in post-mitotic versus interphase NPC assembly [80,82]. ELYS is critical for post-mitotic NPC assembly, while nuclear import of Nup153 and subsequent recruitment of the Nup107-160 complex is required for interphase, but not post-mitotic, NPC assembly [82,83]. Post-mitotic NPC assembly occurs within minutes of nuclear formation while interphase NPC assembly is more sporadic and much slower, on the order of an hour [84-87]. Reducing post-mitotic NPC assembly by ELYS depletion might influence nuclear size more strongly because those are the pores that will drive early nuclear growth. On the other hand, reducing interphase NPC assembly by Nup153 depletion might have less of an effect on NPC numbers and nuclear size, especially if post-mitotic NPCs primarily drive the acquisition of a steady-state size. In support of this hypothesis, blocking interphase NPC insertion had no effect on nuclear volume [88]. Lastly, depletion of some Nups may have resulted in a modest reduction in nuclear size, but because we defined hits as having a z-score less than -1.5 we naturally focused on the stronger hits. In that sense, our screen was not saturating.

2. ELYS knockdown induces also cytoplasmic Lamin aggregates, presumably by their failed import into the nucleus. It is not clear to the reviewer why the authors focus so

much on these aggregates, since they do not have to have a functional relevance for ELYS mediated nuclear size control (which is the issue in this work). In fact the authors show that removal of those aggregates by inhibiting PP1CA (a Lamin phosphatase) does not rescue nuclear size defects (Figure S3). They also show that interfering with lamins does not alter the larger nuclear size caused by decrease nuclear export (Figure S4). Thus the cytoplasmic Lamin aggregates seem more a marker for ELYS depletion than to have a functional relevance for what the paper actually is pointing at. Thus the authors should give it less importance. For example all 6 movies focus on that issue. In addition these movies are really redundant, since all show the emergence/fate of cytoplasmic Lamin aggregates. Some should be removed.

Alternatively, if the authors think these aggregates are that important, they should characterize them in more detail. They only show that, surprisingly however, they are distinct from NPCs at annulate lamellae (which are known to emerge upon ELYS depletion (Franz et al, 2007). For example do these Lamin aggregate contain membranes? In the movies mCherry-Lamin appears like ER.

We agree that we focused too much on cytoplasmic lamin aggregates in our original manuscript. As correctly noted by the referee, our data indicate that lamin aggregates are not the underlying cause of reduced nuclear size in ELYS depleted cells. We have now moved most of the lamin aggregate data to Supplementary Information, greatly reduced our description of lamin aggregates in Results, eliminated a whole paragraph about lamin aggregates from the Discussion, and removed three movies. We thank the referee for this suggestion as it helps to focus our manuscript on the more important data relating to how ELYS controls nuclear size through NPC number and nuclear import.

As a potential point of interest, removal of lamin aggregates by PPP1CA knockdown did not rescue the nuclear size defect likely because import of those now solubilized lamins was still defective. In addition, other INM proteins that may contribute to nuclear growth are probably also mislocalized in ELYS knockdown cells (PMID 16950114, 27802161, 22555603). The fact that lamin A and B1 siRNA only partially reduced nuclear size in XPO1 knockdown cells may be due to incomplete lamin depletion and the fact that other non-lamin nuclear proteins likely contribute to nuclear growth.

The reviewer has also a comment on the general readability of the manuscript: Often the conclusions stated in the text and where they are supported by the respective display item are disconnected from each other. This significantly decreases the readability of the otherwise very clear and concise manuscript. For example, Figure S1 is discussed early in the text, while readers have to wait until the end of the results section that they learn about the effects of XPO1 depletion, which are however displayed in Figure S1A. This is just one example of many. The authors should try to improve this.

Figure panels have been rearranged to correspond linearly to when they are mentioned

in the manuscript.

Referee #3:

In this manuscript, Jevtić, Schibler et al. study the role of nucleoporin ELYS as a determinant of nuclear size in mammalian cells. The authors perform a high throughput RNAi screen of 867 genes implicated in NE function, chromatin structure and epigenetic mechanisms. They carry out measurements of nuclear cross-sectional area and select the genes that are in charge of a significant nuclear size decrease. The authors find 19 genes that can cause nuclear size decrease. Among them, they select ELYS protein because it is causing cytosolic lamin puncta and has an assembly role of the nuclear pore complex. Further, they characterize the effect of both knocking down and overexpressing ELYS. Interestingly, they find a relation between ELYS KD, active transport though nucleopores, and nuclear size. The relationship found between nuclear import and size is interesting and worth publishing. However, there are important flaws in the rationale of the paper that need to be resolved before considering it for publication.

We appreciate the support and have now addressed the points raised by the referee.

1. Importantly, the results of figure 1 are impossible to interpret without a quantification of knock down levels. Indeed, the effect of the different sIRNAs could be largely due to the knock down level of the different siRNAs, rather than a specific effect of the protein itself. This is made even more confusing by the fact that the authors do not even use the results of this figure to choose their main molecular focus. Rather, they pick ELYS (number 7 in their screen) due to its previously described role as a required element for NPC assembly.

The screen was critical in allowing us to identify gene knockdowns that reduce nuclear size and focus on authentic nuclear size effectors. It was not previously known that ELYS levels affect nuclear size, and we would not have initiated work on ELYS's role in nuclear size determination had it not been a prominent hit in the screen. Any of the 867 genes that we screened were potential candidate nuclear size effectors, and we could not have chosen ELYS for our follow-up studies without the screen. The fact that ELYS and SEC13 were top hits prompted these studies. In addition, the screen was useful in identifying promising factors and eliminating others, such as some of the other Nups. Indeed, we verified that Nup153 and Nup107 siRNA failed to affect nuclear size, and these data are now presented in Fig. EV3D-E. While SEC13 was the top hit in the screen, the reviewer questions why we focused on ELYS that was the sixth hit. Please note that we were potentially interested in all hits with a z-score <-1.5. Our rationale for selecting ELYS from the screen results was perhaps not clearly articulated in the original version of our manuscript. We now clarify this issue as follows:

Lines 126-140: Out of 867 genes screened, knockdown of 19 resulted in decreased nuclear size with median z-scores <-1.5 (Appendix Tables S1-S2, Fig. 1B-C). The hit rate of 2.2% indicates high specificity of the screen. Interestingly, two related Nups, SEC13 and ELYS, were the top and sixth hits with median z-scores of -2.7 and -2.0, respectively. SEC13 and ELYS are components of the Nup107-160 complex that has known roles in NPC assembly [56-63]. We were intrigued to further investigate these proteins because their expression levels had not previously been implicated in nuclear size control. While nuclear transport factors are known to regulate nuclear size, less is known about how Nups might affect nuclear size. In addition, siRNA knockdown of these Nups not only induced smaller nuclei but also resulted in formation of cytoplasmic lamin puncta containing both A- and B-type lamins (Fig. EV1A-E). The observed effects of SEC13 and ELYS are specific and not a general property of Nups since, out of 33 Nups tested in the screen, siRNA oligos against only these two Nups decreased nuclear size. The reason for this may be because some Nups are particularly long-lived and/or because of differences in post-mitotic versus interphase NPC assembly (see Discussion).

As to the issue of knockdown levels, this is a confounding issue in all RNAi screens regardless of whether they are imaging based or not. It is simply not feasible to perform qPCR or western blots for all siRNA oligos in a screening library to validate RNAi knockdown efficiency. The approach used in most screening strategies to circumvent this problem is to use multiple siRNA oligos targeting the same gene. In our case, we used the standard approach of using three independent siRNA oligos per gene. Furthermore, following standard screening procedures, validation was done using two additional independent siRNA oligos different in sequence and chemistry from the ones used in the screen, followed by western blot analysis. Relevant details are provided in Materials and Methods. For hits identified in the screen, we can assume that knockdown was successful because nuclear size was altered. We acknowledge that the screen is not saturating given that not all siRNA oligos will be equally efficient or specific, however again the use of multiple siRNA oligos per gene minimizes these concerns. This caveat is now acknowledged in the manuscript as follows:

Lines 114-115: To minimize the frequency of false negatives, we used the standard approach of employing three independent siRNA oligo sequences per target gene.

2. Thus, the authors could dispense with figure 1 altogether, and simply state that they will focus on ELYS due to its previously described role. However, since ELYS is known to be required for nuclear growth, that its knock down results in smaller nuclei is somewhat to be expected and not entirely novel. To circumvent this problem, the authors state that their work is "the first demonstration that NPC numbers can modulate nuclear size and that nuclear transport can tune nuclear size in mammalian cells". However, the data shown with ELYS alone are not sufficient, in my view, to support this statement. Indeed, it is unclear whether ELYS knock-down merely results in decreased NPC numbers, or transport through NPCs is also impaired, and how. To support this statement, the authors should alter NPC numbers through some other mechanism, and

demonstrate that the effects are the same.

The referee is correct that ELYS is required for post-mitotic NPC assembly. We agree that in the complete absence of ELYS, there would be no NPCs, no nuclear transport, and no nuclear growth. However, this does not necessarily mean that a partial reduction in ELYS levels and NPC numbers will result in reduced nuclear import and smaller nuclei. In principle, NPCs might not be limiting for nuclear import and growth, so eliminating half the NPCs might have had no effect on nuclear size. We show in Figs. 2F, 3H, and 4D that reducing ELYS levels results in fewer NPCs and a concomitant reduction in nuclear import capacity. The question as to whether "transport through NPCs is also impaired" was explicitly answered in our study by direct measurement of nuclear import (Fig. 4D) and is part of our model, and the fact that importin α overexpression rescued nuclear import and size shows that the NPCs are still functional. This issue is now more clearly articulated in the manuscript as follows:

Lines 97-102: Previous work demonstrated that nuclei assembled in *X. laevis* egg extract failed to assemble NPCs when ELYS was immunodepleted or upon addition of a dominant negative fragment of ELYS and, as expected for import-deficient nuclei, no nuclear growth was observed [53,54]. Here we demonstrate that NPC densities are sensitive to ELYS protein levels in cultured mammalian cells. In turn, nuclear import capacity and nuclear size scale as a function of ELYS expression.

Lines 268-269: These data suggest that NPC number can limit nuclear import, thereby scaling nuclear size.

While we appreciate the suggestion to manipulate NPC number "though some other mechanism", we are not aware of any experimental methods to do so other than eliminating Nups. We have repeated the Nup153 and Nup107 siRNA treatments and find that while nuclear size is not affected, consistent with our screen results, there is a modest reduction in NPC number. These new results are presented in Fig. EV3D-E and in the Results section as follows:

Lines 183-186: Consistent with results from the screen, knockdown of Nup153 and the longer-lived Nup107 did not affect nuclear size, likely because NPC numbers were not reduced to the same extent as upon knockdown of ELYS (Fig. EV3D-E, see Discussion).

We have also added the following text to the Discussion to speculate as to why knockdown of ELYS, and not other nucleoporins, led to reduced nuclear size:

Lines 238-261: Why did knockdown of only these two Nups decrease nuclear size in our screen? We found that ELYS knockdown decreased NPC numbers to a much greater extent than knockdown of other Nups, including Nup153 and Nup107. One possibility is that some long-lived scaffold Nups, like Nup107, were not efficiently depleted over the two-day siRNA treatment typically used in our experiments [79-81]. In line with this interpretation, twelve continuous days of Nup107 knockdown were required to reduce

Nup107 protein levels to ~30% [80], while we found that ELYS levels were reduced to ~40% after only two days of depletion and SEC13 is known to turn over rapidly [79]. Another possibility is that the effect of Nup depletion on nuclear size might depend on whether the Nup is involved in post-mitotic versus interphase NPC assembly [80,82]. ELYS is critical for post-mitotic NPC assembly, while nuclear import of Nup153 and subsequent recruitment of the Nup107-160 complex is required for interphase, but not post-mitotic, NPC assembly [82,83]. Post-mitotic NPC assembly occurs within minutes of nuclear formation while interphase NPC assembly is more sporadic and much slower, on the order of an hour [84-87]. Reducing post-mitotic NPC assembly by ELYS depletion might influence nuclear size more strongly because those are the pores that will drive early nuclear growth. On the other hand, reducing interphase NPC assembly by Nup153 depletion might have less of an effect on NPC numbers and nuclear size, especially if post-mitotic NPCs primarily drive the acquisition of a steady-state size. In support of this hypothesis, blocking interphase NPC insertion had no effect on nuclear volume [88]. Lastly, depletion of some Nups may have resulted in a modest reduction in nuclear size, but because we defined hits as having a z-score less than -1.5 we naturally focused on the stronger hits. In that sense, our screen was not saturating.

It is worth noting that ELYS overexpression had the opposite effect to depletion. While the referee states that “ELYS is known to be required for nuclear growth” and so the knockdown phenotype is “somewhat to be expected,” it is not obvious that ELYS overexpression would increase NPC number, nuclear import capacity, and nuclear size. Thus the ELYS overexpression phenotype is consistent with NPC number affecting nuclear size. All of that being said, we have edited the text throughout the manuscript to indicate that our data “suggest” that NPC numbers affect nuclear import and size.

3. Authors should give more details on how they calculate the z-score. For example, they should clarify if they use the mean of the control sample or the mean of the whole screening.

We clarified in the Materials and Methods that we used the distribution of the samples for the z-score calculation of the primary screen. The relevant text follows:

Lines 477-487: The statistical analysis was performed using R (v 3.3.2) and the cellHTS2 R package (v 2.36.0) [124]. Per well results were normalized on a per plate basis using the B-score method (Calculation based on the siRNA oligo library samples) in the cellHTS2 package. Normalized values for each biological replicate were then scored across all the different screen plates by taking the z-score of the B-scores distribution for the siRNA oligo library samples. The biological replicates z-score values were then aggregated by calculating their mean, which is the value reported for each siRNA oligo. Putative positive hits in the RNAi screen were defined as genes that showed a z-score value of < -1.5 for at least 2 out of the 3 targeting siRNA oligos. Results for ELYS and SEC13 were validated by ordering 2 independent siRNA oligo sequences that were different from the ones used in the screen against these genes.

4. I think that the representation would be more robust if the median z-score instead of the maximum z-score were used in Fig. 1C.

Median z-scores are now plotted in Fig. 1C rather than maximum z-scores, and both sets of data are presented in Table S1.

5. The term correlation is used in the text, but there is no correlation study. It would be more useful if correlations were really shown. For example, Fig. 2D would be more valuable if for each data point the normalized ELYS staining intensity vs. cytoplasmic Lamin intensity was shown, and then a correlation study was carried out.

In this figure panel, we are asking a binary question about whether or not cytoplasmic lamin aggregates are present and we then measure ELYS staining intensity for each of these conditions. We are not comparing the staining intensities of ELYS and cytoplasmic lamins to each other. We believe this is a more complete representation of the data since only ~40% of ELYS knockdown cells exhibit cytoplasmic lamin aggregates. If we plotted ELYS staining intensity versus cytoplasmic lamin staining intensity, we would be excluding ~60% of the cells. To clarify, we have removed the term “correlation” from our manuscript so as not to give the impression that correlation studies were carried out. Upon recommendation by referee #2, we now also de-emphasize the significance of the lamin aggregates. These data have been moved to Supplementary Information and are now only briefly mentioned.

6. Fig. S2 A: siELYS merge does not correspond to the 3 previous images.

This has been fixed.

7. Authors mention and show that ELYS siRNA knock down resulted in formation of cytoplasmic lamin puncta, containing both lamin A and B. However, then they only quantify the link between ELYS intensity decrease and lamin type B1. Does this link also exist for lamin B2 and A/C?

This comment refers to the same figure panel mentioned in point 5 above. Our data show that all three lamin types co-localize in cytoplasmic lamin puncta (Figs. 2A-B and EV1A). As mentioned above, in this panel we are comparing ELYS intensity with whether or not cytoplasmic lamin aggregates are present, not the intensity of cytoplasmic lamin staining. In this case, we use lamin B1 staining merely to identify cytoplasmic lamin puncta, knowing that all three lamin types are present in those puncta and that the staining pattern is similar for all three. Again, lamin aggregates have been greatly de-emphasized in the revised manuscript, and these data have been moved to Supplementary Information. With respect to the levels of nuclear lamins in ELYS depleted cells, data for all three lamin types are shown in Fig. 2C.

8. Scalebar, time and what each channel represents should be in each video.

Scale bars were added to each movie. Times and channels are described in the Movie Legends.

9. " Knockdown of PPP2R4 had no impact on the appearance of lamin puncta (data not shown)". Please show.

These data have been added to Fig. EV2B-D.

10. The quantifications of Fig. 4E-F would be better understood if stainings were shown.

The Ran and NTF2 immunofluorescence images are now shown in Fig. EV3B-C.

11. The Western Blot of siRNA ELYS+EGFP ELYS is missing.

The western blots are now included in Fig. EV3A.

12. Why do authors sometimes calculate cell area (Fig. 6 C) and other times use z-score (Fig. S2)?

Z-scores are reported for data obtained using the high-throughput screening approach, as Z-scores are a convenient way to normalize these data. Nuclear area measurements are reported for all of the follow-up experiments. We briefly clarify this in the manuscript as follows:

Lines 490-492: Data obtained using the high-throughput screening approach are reported as z-scores, a convenient way to normalize these data. For follow-up experiments, nuclear area measurements are reported.

2nd Editorial Decision 5th Apr 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study (you will find below). As you will see, the referees now support the publication of your manuscript in EMBO reports. However, referee #3 has a remaining point, we ask you to address in a final revised version of your manuscript. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

Further, I have these editorial requests, which I ask you to also address in the final revised version of the manuscript:

REFEREE REPORTS:

Referee #1:

The authors have adequately addressed my concerns in this revised manuscript.

Referee #2:

In the revised manuscript, Jevtić et al have accordingly addressed all the points for which I have raised concern in my comments. They have reinvestigated the potential effect of impaired NPC interphase assembly and they have focused the general message of the work by less focusing on the Lamin aggregates. They also revised the paper in a way that it improved readability. The manuscript should be published in its current version.

Referee #3:

In my view, the authors have largely addressed my comments, and the manuscript is ready for publication pending only a remaining minor comment. There is an issue that is still not resolved, perhaps I admit due to a lack of clarity on my side in my previous review. In my previous comments, I asked to discriminate whether the effects of ELYS were due to reduced NPC numbers, or reduced import. What I meant was to discriminate whether the reduced nuclear import was due simply to a reduction in NPC numbers, or also due to a reduced import capacity of each individual NPC. in my view, this issue is not resolved in the current manuscript: Fig 4D, which is used to assess import, could be explained by either mechanism. In fact the finding that depletion of other nups lead to reduced NPC number, but no change in nuclear size, could potentially also be due to different effects of each depletion in NPC number versus the import capacity of each NPC. Whereas I agree that this question is difficult to assess in the current manuscript, the authors should acknowledge this in the discussion or results.

2nd Revision - authors' response 9th Apr 2019

Referee #3: In my view, the authors have largely addressed my comments, and the manuscript is ready for publication pending only a remaining minor comment. There is an issue that is still not resolved, perhaps I admit due to a lack of clarity on my side in my previous review. In my previous comments, I asked to discriminate whether the effects of ELYS were due to reduced NPC numbers, or reduced import. What I meant was to discriminate whether the reduced nuclear import was due simply to a reduction in NPC numbers, or also due to a reduced import capacity of each individual NPC. in my view, this issue is not resolved in the current manuscript: Fig 4D, which is used to assess import, could be explained by either mechanism. In fact the finding that depletion of other nups lead to reduced NPC number, but no change in nuclear size, could potentially also be due to different effects of each depletion in NPC number versus the import capacity of each NPC. Whereas I agree that this question is difficult to assess in the current manuscript, the authors should acknowledge this in the discussion or results.

We understand this comment to mean that ELYS depletion might result in pores with reduced import capacity, for instance resulting from reduced numbers of ELYS molecules or ELYS associated proteins within the pore. While we have no evidence that pore composition is altered upon ELYS depletion, we agree that our data do not formally exclude this possibility, so we now acknowledge this caveat in the Discussion as follows:

Lines2 80-282:Although we cannot formally exclude the possibility that ELYS levels affect the import capacity of individual NPCs, our data suggest that NPC number can limit nuclear import, thereby scaling nuclear size.

The authors performed all requested editorial changes.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Daniel Levy and Tom Misteli
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-47283V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were chosen based on previous experience and common practice in the field. For high throughput microscopy, > 650 cells were analyzed per siRNA per experiment. Three siRNA oligo sequences were used per gene and two biological replicates were performed. The effect size was not pre-specified as this is not common practice in cell biology. In general, 2-3 biological replicates were performed for all experiments, which was sufficient to draw statistically significant conclusions. See Materials and Methods for further details about our statistical analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	In cell biology, samples are virtually identical before treatment, hence randomization is not a major concern.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Most quantifications were performed in an automated fashion, which minimizes subjective bias. The screen was performed in a blinded fashion, as the identities of knocked down genes that altered nuclear size were not determined until after the analysis. Different investigators were responsible for setting up the screen and for analyzing the data. For quantifying nuclear sizes and staining intensities, immunofluorescence slide regions/fields were randomly selected. Group allocation was randomized in each experiment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Statistical tests are stated in the Figure Legends.

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Is the variance similar between the groups that are being statistically compared?	Yes. Statistical tests are stated in the Figure Legends.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers for all antibodies used in this study are included in Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The MCF-10AT1k.cl2 and MCF-10A cell lines were obtained from the Barbara Ann Karmanos Cancer Institute. The MCF7 cell line was obtained from ATCC. Commercial cell lines were provided mycoplasma-free.

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8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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