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Nuclear Lamins are Not Required for Lamina Associated Domain Organization in Mouse Embryonic Stem Cells

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(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.)

Editor: Esther Schnapp

1st Editorial Decision 12 November 2014

Thank you for the submission of your manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, all referees acknowledge that the findings are potentially interesting, however, they also point out that important aspects of the study need to be strengthened and clarified. Both referees 1 and 3 remark that emerin has been reported to be cytoplasmic in the absence of lamin A, and that the staining pattern of lamina A-Dam fusion in dKO ES cells should be shown. Referee 2 further agrees in her/his cross-comments with referee 3 that the concerns regarding the lamin A antibody and the differentiation status of the cells should be addressed. Referee 3 also told us that s/he agrees with all the comments by referee 1, who mentions that LBR localization in dKO cells and the effects of LBR knockdown on LADs should be examined.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the 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.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if

you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

The manuscript addresses the question whether lamins are required for association of LADs to the nuclear lamina (nuclear periphery) and for gene repression? The authors apply their previously developed DamID technique using emerin-Dam fusion proteins in dKO ES cells, in which both LMNB1 and LMNB2 are knocked out and LMNA is naturally expressed at very low level.
Using this assay, they found no difference in emerin-associated cLADs and fLADs between WT and dKO ES cells and the profile was similar to the previously identified lamin B associated LADs in WT cells. In addition, genes within LADs in dKO cells were still mostly repressed. Expression of lamin A-Dam in dKO cells did not alter LADs nor did sh-RNA-mediated downregulation of lamin A.
While this is a well done study the novelty is limited, as based on studies by Solovei et al. showing two redundant pathways for heterochromatin attachment at the periphery (mediated by lamin A and LBR), it is not surprising that in the absence of lamins, LBR may be the main component involved in peripheral chromatin tethering. To provide novel insights into the mechanism of tethering it would be important to show LBR expression and localization in dKO cells and to test whether LBR knockdown affects LAD association.
A second major concern is the use of emerin-Dam in this assay. It has been shown by several labs that emerin depends on lamin A for its correct localization at the INM. Images in Fig S1B indicate significant staining of the ER, raising the possibility that the NE staining is mainly ONM. Emerin-Dam expression in dKO ES cells would even increase diffusion of Emerin out of the nucleus, assuming that emerin binding sites in the nucleus are limiting. Do the authors have any evidence how much of the emerin fusion protein localizes to the INM? Based on this, I find it surprising that LAD patterns are identical to those previously derived with Lamin B. The authors should at least discuss this aspect and provide potential explanations for this result.

Minor points:
In view of point 2 above, experimental details for DamID should be described. Particularly, how long was emerin-Dam expressed in cells before analysis compared to previous lamin B-Dam experiments? Does one have to express emerin-Dam longer compared to lamin B-Dam in order to obtain similar signals? If so how often do the cells go through mitosis during that time period?
Fig. 3: The authors assume a patchy localization of lamin A-Dam in dKO ES cells based on the expression of GFP-Lamin A fusion proteins. Given that the fusion part can affect lamin A assembly (particularly in the absence of lamin B) it would be more convincing to show the staining pattern of the lamin A-Dam fusion.
Fig. 5: The microscopic analysis is not very convincing due to the lack of a positive control. I doubt that heterochromatin association can be seen by intensity of DAPI staining. If so, DAPI staining in wt cells should show a clear rim staining, which is usually not observed.

Referee #2:

This study reports the absence of any considerable effect of loss of lamins on the association of lamina-associated domains (LADs) with the nuclear periphery in mouse ES cells. The authors use DamID of lamin B1/B2 knockout cells and using the nuclear envelope protein emerin as a bait to show that loss of B-type lamins does not affect interaction of LADs with emerin in ES cells. Loss of B-type lamins also does not affect gene expression. Loss of B-type lamins did also not affect interaction of lamin A with LADs. Knockdown of lamin A in a B-type lamin null environment had no effect on LAD formation. Finally, no correlation between lamin A patches and DNA localization to the periphery was observed. The authors conclude that lamins are not required for genome organization.

These are straightforward results based on technically sound experiments. They will be of interest to the specialist in the field. There are a number of points that should be clarified and/or corrected:

The use of the term "genome organization" in the title of the paper is misleading. The authors only look at LAD association with a particular protein (emerin). No other aspects of genome organization are investigated and it can not be ruled out that other features of genome organization do change when B-type lamins are lost. The title and several overly broad statements in throughout the text should be corrected.

The authors imply and discuss throughout the manuscript, including in the abstract, that they assess the "peripheral" localization of LADs. No data is shown to test whether the position of LADs is affected by B-type lamins (although a fair assumption). The presented data shows that interaction with emerin is not altered, but localization of the LADs is not tested. The authors should be more cautious in their interpretation and discussion of their data and in the absence of data, it would seem prudent to refrain from making statements about the localization of LADs and the role of B-type lamins in localizing them.

The possibility that these results are specific to ES cells, which have more plastic nuclei than differentiated cells, should be discussed in more detail.

The microscopy data in figure 5 is very rough and not very insightful. It should either be expanded on or deleted.

A more detailed discussion of reported effects of loss of lamins (A- and B-type) on genome organization and nuclear features should be included.

Referee #3:

The article entitled "Nuclear Lamins are Not Required for Genome Organization in Mouse Embryonic Stem Cells" by Mario Amendola and Bas van Steensel aims to answer the important question whether the main lamin isoforms Lamin B1, Lamin B2 and Lamin A/C are essential for the overall genome organization, as detected by the NL-Dam ID technique. The answer to that question is indeed crucial for the understanding of the mechanism of the genome organization since many assumptions have been made about this. This manuscript intends to provide an answer using a straightforward experimental strategy.

However, several important points are missing in order to draw accurate conclusions in this manuscript.

• The main criticism would be that in that paper, two key references are cited several times and consistency of the results are mentioned when appropriate, but important discrepancies with the same papers are omitted in both Results and Discussion sections.

• The first point concerns the lamin B1/B2 Double KO mouse embryonic stem (mES) cells used in that study: these are the ones that were produced and described in (Kim e al., 2011). Those cells were shown to be pluripotent (Oct-4, Nanog markers and more) and to lack Lamin B1 and B2 obviously, and to lack Lamin A/C by RT-PCR and by Western blot (Kim et al., 2011 Fig 3 and FigS5). In the present manuscript one of the antibodies used to show that Lamin A/C was present (in contrast to expectation) is the same as used in Kim et al., 2011, and even if the authors used the same cells and the same antibody they get a very important contradicting result concerning the absence of lamin A/C. This is not even discussed ! Additionally, in all the attempts to visualize the very low amounts of lamin A/C that ES cells apparently have, only the antibody from Active Motif used in Eckersley-Maslin et al., 2013, and in Guo et al., 2014 was sensitive enough to detect it convincingly. The second antibody detecting Lamin A in this manuscript is not this mentioned antibody.

One possible explanation would be that those WT and DKO ES cells have differentiated partially during the culture (maybe because of the lentiviral vector expressing the DamID), since detectable amount of lamin A/C by common antibodies as used in this study can be found in differentiated cells or partially reprogrammed iPS cells (that exhibit lamin A/C and certain pluripotent markers), but not in fully reprogrammed or fully pluripotent cells (Mattout et al., 2011, Zuo et al., 2012).
Another point that can support this possibility is the fact that all the IF shown from ES cells do not have a colony morphology that the cells should have (as in Kim et al, 2011, Guo et al., 2014 and many more...) if they are ES or ES-like cells after one day of growth(cf material and methods; and a minor point: the scale bars are also missing in IF figures).
¬ One indispensable control will be to characterize those ES/ or differentiating cells just prior to their use for creating the DamID profiles, and draw conclusions concerning the cell type or cell state that will be strongly supported by data. This characterization should include several pluripotent markers, and compare the levels of these pluripotent markers in the cells used in this study after LV transduction to the levels in the young passaged ES cells used in Kim et al., 2011 that showed differentiation potential. Alternatively the authors could try to derive ACs from the cells used in this study to prove their pluripotency, and the characterization should also include several differentiation markers. Also please show in WB or at least in the RNA-seq plots the absence of lamin B1 and B2, which is only shown in the RT-PCR, and this is the main point of the paper.
Even if the cells have slightly differentiated, the main conclusion of this paper could still be valid, yet an accurate description of the cell state is crucial to avoid misinterpretation in future studies. And if the cells are still fully pluripotent, then the authors need to mention and discuss the discrepancy between Kim et al and the ability to detect Lamin A.

• A second point concerns the Emd-DamID performed in the triple KO Lamin B1, B2 and shRNA lamin A/C, from which they conclude that the entire lamin meshwork is not required for genome organization. The authors cited many times Guo et al., 2014 for several aspects of the study, but do not mention that Emerin localization to the nuclear periphery was shown to be dependent on Lamins and more particularly on Lamin A (Guo et al., 2014, & more). Indeed, Emerin is cytoplasmic in the absence of lamin A.
¬ This point must be discussed, and explanations should be proposed. An important additional control in Supp Figure 4 is to show the emerin staining co-stained with lamin A/C in cells processed for Emd-DamID, to see if the nuclear periphery emerin positive cells are indeed lamin A negative, or could be cells with a residual lamin A or some few cells with normal lamin A levels. An additional control, that is probably out of the scope of this study but still would be nice to see, would be a negative control for comparison: i.e. DamID profiling of a nuclear protein that is not at the nuclear periphery, for which differences should be scored.

• A third experimental point that concerns the LmA-DamID profile in the DKO cells. Since the authors want to test whether the LADs from the LmA-DamID would change if the Lamin A/C is not uniformly peripheral but patchy because of the lack of Lamin B1, to my opinion the confirmation of the patchy localization of Lamin A, which is known to be dependent on the amount (Guo et al., 2014), in the cells sent to LmA-DamID should have been shown by simple IF in those cells and not by an additional fusion protein (that may behave aberrantly).
¬ So, instead of panel E and G in Supp Figure 3 I would have been more convinced by the results if I could see a patchy LmA and /or a LmA/C staining in dKO cells previously transduced with a LV encoding for the LmA-DamID fusion protein.

• A final, general point concerns the interpretation of the results of the NL-DamID profiles obtained in the different conditions. The general conclusion which is important and consistent with the results is that the major lamin proteins are dispensable for the overall genome organization (if indeed the controls confirm the absence of lamin B1 B2 and Lamin A in the triple, as well as a nuclear peripheral localization of Emerin in most of the cells). The subtle changes they find are not sufficiently discussed nor interpreted in my opinion. For instance, with 94 genes downregulated in the DKO vs WT - I would not call this a highly similar pattern- and 100-200 different LADs were identified between conditions in this study. Between ESCs and NPCs or ACs the approximate number of LADs that changed were also around 200 (Peric-Hupkes et al., 2010) ! So could it be that lamins affect the facultative/developmental LADs. A comparison of facultative LADs and the probes shown in Figures 1, 3 and 4, is needed. It would be nice to see some discussion about this

point in the manuscript, in order to get the authors point of view.

Minor points:
In the supplementary figures and Figure 5 many typos or missing letters in the legend.
No reference to Suppl Fig 5 in the text of the manuscript

1st Revision - authors' response 21 January 2015

Common responses to the main reviewer comments as highlighted by the editor:

1. *"As you will see, all referees acknowledge that the findings are potentially interesting, however, they also point out that important aspects of the study need to be strengthened and clarified. Both referees 1 and 3 remark that emerin has been reported to be cytoplasmic in the absence of lamin A…"*

There are indeed reports showing that emerin depends on lamin A for its correct localization at the nuclear envelope in differentiated cells. However, several papers reported that *in mES cells* emerin is present at the NE also in the absence of B-type or A- and B-type lamins (Kim, Science, 2011; Kim, Cell Res., 2013; Guo, Mol Biol Cell, 2014). Guo et al. showed that emerin gets mislocalized in the absence on lamin A/C *only when mES cells are fully differentiated into fibroblasts*.

To address this issue in our mES cell cultures, **we now included immunofluorescence staining data to visualize emerin localization in the presence or absence of B- and A-type lamins**:

- mES cells wt, where lamin A/C is localized at the nuclear rim (**Sup Fig 1B, top panel**)
- mES cells dKO (lacking B-type lamins), where lamin A/C is localized at the nuclear rim in patches (**Sup Fig 1B, bottom panel**)
- mES cells tKO (lacking B- and A-type lamins), where lamin A/C is absent (**Sup Fig 4B**)

In all conditions we observed the same emerin staining, which is characterized by a clearly defined nuclear rim staining plus some staining in the cytoplasm, which may be endoplasmatic reticulum staining. Therefore, we conclude that also in absence of lamin A/C emerin localizes at least partially at the NE.

Is this emerin perhaps only located at the outer nuclear membrane? DamID would not work in that case, as there would be no contact with the genome; yet, the yield of adenine-methylated DNA is similar between wildtype and mutant cells. **We now show this in Sup Fig 1E and 4F and mention the result on page 4 and 7**.

2. *"and that the staining pattern of lamina A-Dam fusion in dKO ES cells should be shown"*

For DamID mapping the Dam-fusion proteins are always expressed at extremely low levels using the slightly leaky activity of a *Drosophila* heat-shock promoter, in the absence of heat-shock. It is therefore highly unlikely that the Dam-fusion protein affects the endogenous protein pool. **We now mention this in the Results, page 4**. Unfortunately, at these low expression levels it is very difficult to visualize the Dam-LmA protein by IF microscopy. This is why we used for our microscopy studies a different vector that expresses LmA tagged with GFP at higher levels; note that Dam and GFP have virtually the same size and are tagged both at the N-terminus of LmA.

To address the reviewers' request, we went back to the cells with the very low Dam-LmA expression, and found a few cells in which the expression level was high enough for detection, albeit with difficulty. We now added an image of one of those cells, showing that Dam-LmA exhibits, as expected, a homogeneous or patchy rim staining in wt or dKO cells,

similarly to the endogenous LmA. **The low expression level and this result are now shown in Sup Fig 3I and discussed on page 6**.

3. *"Referee 2 further agrees in her/his cross-comments with referee 3 that the concerns regarding the lamin A antibody and the differentiation status of the cells should be addressed"*

We included two new analyses to address the Lamin A antibody issue:

- We performed new immunofluorescence analyses on WT and dKO cells to check that mES cells positively identified as undifferentiated (based on Oct4 staining) express lamin A/C (**Sup Fig 3D**).
- We compared the lamin A/C expression of our cells to the previously published microarray expression data from the same cells (Kim, Science, 2011): in both datasets lamin A/C is expressed to a similar extent (**Sup Fig 6E-F**). This observation indicates that lamin A/C expression was present also in the mES cells of Kim et al 2011, although they were unable to detect it by western blot.

Please note that Kim et al (Cell Res., 2013) and Guo et al (Mol Biol Cell, 2014) (i.e., the same lab studying the same cells) performed a RT-PCR (Sup Fig 1B) and a western blot (Sup Fig 3B) for wt and dKO mES cells and now they observed lamin A/C expression. We feel that it is not up to us to explain a particular negative result in (Kim et al, Science, 2011) that is now considered even by the authors to be incorrect.

So far already 4 antibodies other than the antibody from Active Motif have been successfully used to detect lamin A or A/C expression in mES cells. In particular, Eckersley-Maslin et al. (Nucleus, 2013) used three different antibodies to stain mES cells of different origins and they always managed to detect lamin A/C protein. Guo et al. 2014 detect lamin A in mES cells using the Active Motif antibody as well as a rabbit anti-lamin A from Santa Cruz Biotechnology. Thus, lamin A/C protein detection in mES cells does not strictly require the use of the Active Motif antibody. **We now mention on page 5 (bottom) that lamin A/C has been detected with several antibodies and cite the relevant papers**.

We further assessed the differentiation state of our mES cells in two complementary ways:

- We assessed the expression of 4 mES cell specific proteins: flow cytometry to evaluate E-cadherin 1 and SSEA-1 expression and IF microscopy to evaluate Oct4 and Nanog. (**Sup Fig 4D and Sup Fig 6C-D**). The vast majority of our cells express these markers.
- We compared our mRNA-seq data to the previously published microarray expression data from the same cell lines (Kim, Science, 2011) and we found that the overall expression pattern, as well as that of a series of specific mES cell markers, are very similar between the two datasets both for wt and dKO mES cells (**Sup Fig 6 E-F**), again indicating that our mES cells are pluripotent – at least to a similar degree as in Kim et al (Science, 2011).

Regarding the possible impact of lentiviral vectors on mES cell state, there are already several publications reporting the use of lentiviral vectors similar to the ones used in this study and no evidence of stem cell differentiation was reported (Kim, Science, 2011; Peric-hupkes, Mol Cell, 2010; Meuleman, Genome res, 2013). In addition, to our best knowledge, there is no report suggesting that

lentiviral vector transduction *per se* can influence mES cells pluripotency. For example, lentiviral vectors are commonly used in mES cells to generate transgenic animals with high efficiency (Pfeifer, PNAS, 2002; Pfeifer, Curr Gene Ther, 2006, Lois, Science, 2002, and many more), to generate induced pluripotent stem cells (Brambrink, Cell Stem Cell 2008; Sommer, Stem Cells, 2009, and many more) and to identify and isolate embryonic stem cells (Hotta, 2009, Nat Methods; Warlich, 2011, Mol Ther).

Finally, we note that differentiation of mES cells was previously reported to cause changes in NL interactions of >1,400 genes (Peric-Hupkes, Mol Cell 2010). If our cells had differentiated significantly, we should have seen some changes in NL–gene interactions. **We now analyzed this statistically, and found 0 genes to be significantly altered in their emerin interactions (Results, page 2nd paragraph; and page 7, 2nd paragraph)**.

4. "Referee 3 also told us that s/he agrees with all the comments by referee 1, who mentions that LBR localization in dKO cells and the effects of LBR knockdown on LADs should be examined."
We have now included expression analysis and immunostaining of LBR in dKO and tKO cells (Sup Fig 5) and mention this in the Discussion. We feel that an additional study of the genome-wide effects of Lbr knockout or knockdown is really beyond the scope of this manuscript, and it would take us many months of additional work. "*EMBO Reports publishes short-format papers that communicate a single major finding*" [Aim & Scope online]; our single major finding is that lamins do not contribute detectably to the genome-wide LAD pattern in mES cells. This is very surprising, given that lamins are the major component of the lamina and that several studies have claimed that lamins bind to DNA and chromatin.

Referee #1:

1. *"While this is a well done study the novelty is limited, as based on studies by Solovei et al. showing two redundant pathways for heterochromatin attachment at the periphery (mediated by lamin A and LBR), it is not surprising that in the absence of lamins, LBR may be the main component involved in peripheral chromatin tethering.*
   We respectfully disagree. The work by Solovei et al. (Solovei , Cell, 2013) while extremely interesting – was strictly microscopy based, and primarily studied chromocenter (dominated by pericentromeric DNA) positioning. They did not specifically track any of the ~1,000 non-pericentromeric LADs, so it was not known whether these LADs depend on lamins.

   To provide novel insights into the mechanism of tethering it would be important to show LBR expression and localization in dKO cells and to test whether LBR knockdown affects LAD association."
   Please see common response to reviewers, point 4.

2. *"A second major concern is the use of emerin-Dam in this assay. It has been shown by several labs that emerin depends on lamin A for its correct localization at the INM. Images in Fig S1B indicate significant staining of the ER, raising the possibility that the NE staining is mainly ONM. Emerin-Dam expression in dKO ES cells would even increase diffusion of Emerin out of the nucleus, assuming that emerin binding sites in the nucleus are limiting. Do the authors have any evidence how much of the emerin*

fusion protein localizes to the INM? Based on this, I find it surprising that LAD patterns are identical to those previously derived with Lamin B. The authors should at least discuss this aspect and provide potential explanations for this result."
Please see common response to reviewers, point 1.

3. *Minor points:*
In view of point 2 above, experimental details for DamID should be described. Particularly, how long was emerin-Dam expressed in cells before analysis compared to previous lamin B-Dam experiments? Does one have to express emerin-Dam longer compared to lamin B-Dam in order to obtain similar signals? If so how often do the cells go through mitosis during that time period?
We thank the referee for the useful comment. DamID for Emd was performed as for Lamin B1. We harvest the cells 2 days after transduction. **We have now added these details in the revised manuscript.**

4. *"Fig. 3: The authors assume a patchy localization of lamin A-Dam in dKO ES cells based on the expression of GFP-Lamin A fusion proteins. Given that the fusion part can affect lamin A assembly (particularly in the absence of lamin B) it would be more convincing to show the staining pattern of the lamin A-Dam fusion."*
Please see common response to reviewers, point 2.

5. *"Fig. 5: The microscopic analysis is not very convincing due to the lack of a positive control. I doubt that heterochromatin association can be seen by intensity of DAPI staining. If so, DAPI staining in wt cells should show a clear rim staining, which is usually not observed."*
Actually, close inspection of these nuclei does show a patchy rim staining with DAPI. To further substantiate this, we have now added a quantitative analysis that illustrates the variance in DAPI signals along the nuclear rim (**Fig 5C**). We generally see a 2-4 fold dynamic range in DAPI intensities along each nuclear rim, which is far from homogenous. These observations led us to ask whether the patches of relatively DAPI-dense chromatin are preferentially associated with the patches of LmA, which one might expect if LmA plays a prominent role in chromatin anchoring. We have modified the text and the cartoon to explain the logic better. We note that this analysis is conceptually not so different from Solovei et al, Cell 2013, who visually scored relocalization of DAPI-dense patches.

Referee #2:

1. *This study reports the absence of any considerable effect of loss of lamins on the association of lamina-associated domains (LADs) with the nuclear periphery in mouse ES cells. The authors use DamID of lamin B1/B2 knockout cells and using the nuclear envelope protein emerin as a bait to show that loss of B-type lamins does not affect interaction of LADs with emerin in ES cells. Loss of B-type lamins also does not affect gene expression. Loss of B-type lamins did also not affect interaction of lamin A with LADs. Knockdown of lamin A in a B-type lamin null environment had no effect on LAD formation. Finally, no correlation between lamin A patches and DNA localization to the periphery was observed. The authors conclude that lamins are not required for genome organization.*
These are straightforward results based on technically sound experiments. They will be of interest to the specialist in the field. There are a number of points that should be clarified and/or corrected:
The use of the term "genome organization" in the title of the paper is misleading. The authors only look at LAD association with a particular protein (emerin). No other

aspects of genome organization are investigated and it can not be ruled out that other features of genome organization do change when B-type lamins are lost. The title and several overly broad statements in throughout the text should be corrected.
We now changed the title and text according to the suggestion.

2. *The authors imply and discuss throughout the manuscript, including in the abstract, that they assess the "peripheral" localization of LADs. No data is shown to test whether the position of LADs is affected by B-type lamins (although a fair assumption). The presented data shows that interaction with emerin is not altered, but localization of the LADs is not tested. The authors should be more cautious in their interpretation and discussion of their data and in the absence of data, it would seem prudent to refrain from making statements about the localization of LADs and the role of B-type lamins in localizing them.*
We modified the text accordingly. We refer to the observed DamID patterns as "LAD organization", by which we mean the genome-wide domain pattern of DamID signals. In the revised Discussion we do however write that it is likely that this reflects peripheral positioning – backed up by earlier validation work in several papers, and by the observation that the Emd profile in wildtype cells is indistinguishable from the Lamin A (our manuscript) and Lamin B1 (Meuleman, Genome Res, 2013) profile. We hope that the way we re-phrased this issue is acceptable to the reviewer.

3. *The possibility that these results are specific to ES cells, which have more plastic nuclei than differentiated cells, should be discussed in more detail.*
We mention in the Discussion that mES cells have a more dynamic chromatin architecture, exhibit an extremely rapid cell cycle, and have a more plastic NL architecture in which LmB1 is less stably incorporated.

4. *The microscopy data in figure 5 is very rough and not very insightful. It should either be expanded on or deleted.*
We should have explained the rationale and experimental design better. Close inspection of these nuclei does show a patchy rim staining with DAPI. **To further substantiate this, we have now added a quantitative analysis that illustrates the variance in DAPI signals along the nuclear rim (new Fig. 5C).** We generally see a 2-4 fold dynamic range in DAPI intensities along each nuclear rim, which is far from homogenous. These observations led us to ask whether the patches of relatively DAPI-dense chromatin are preferentially associated with the patches of LmA, which one might expect if LmA plays a prominent role in chromatin anchoring. **We have modified the text and the cartoon to explain the logic better.**

5. *A more detailed discussion of reported effects of loss of lamins (A- and B-type) on genome organization and nuclear features should be included.*
This was previously not possible due to the word count limit. As the Editor has relaxed this limit we now extended the Discussion as suggested (page 8). Together with the papers cited in the Introduction we hope that we now have covered the topic sufficiently.

Referee #3:

1. *The article entitled "Nuclear Lamins are Not Required for Genome Organization in Mouse Embryonic Stem Cells" by Mario Amendola and Bas van Steensel aims to answer the important question whether the main lamin isoforms Lamin B1, Lamin B2 and Lamin A/C are essential for the overall genome organization, as detected by the NL-Dam ID technique. The answer to that question is indeed crucial for the*

understanding of the mechanism of the genome organization since many assumptions have been made about this. This manuscript intends to provide an answer using a straightforward experimental strategy.
However, several important points are missing in order to draw accurate conclusions in this manuscript.
• The main criticism would be that in that paper, two key references are cited several times and consistency of the results are mentioned when appropriate, but important discrepancies with the same papers are omitted in both Results and Discussion sections.
• The first point concerns the lamin B1/B2 Double KO mouse embryonic stem (mES) cells used in that study: these are the ones that were produced and described in (Kim e al., 2011). Those cells were shown to be pluripotent (Oct-4, Nanog markers and more) and to lack Lamin B1 and B2 obviously, and to lack Lamin A/C by RT-PCR and by Western blot (Kim et al., 2011 Fig 3 and FigS5). In the present manuscript one of the antibodies used to show that Lamin A/C was present (in contrast to expectation) is the same as used in Kim et al., 2011, and even if the authors used the same cells and the same antibody they get a very important contradicting result concerning the absence of lamin A/C. This is not even discussed ! Additionally, in all the attempts to visualize the very low amounts of lamin A/C that ES cells apparently have, only the antibody from Active Motif used in Eckersley-Maslin et al., 2013, and in Guo et al., 2014 was sensitive enough to detect it convincingly. The second antibody detecting Lamin A in this manuscript is not this mentioned antibody.
One possible explanation would be that those WT and DKO ES cells have differentiated partially during the culture (maybe because of the lentiviral vector expressing the DamID), since detectable amount of lamin A/C by common antibodies as used in this study can be found in differentiated cells or partially reprogrammed iPS cells (that exhibit lamin A/C and certain pluripotent markers), but not in fully reprogrammed or fully pluripotent cells (Mattout et al., 2011, Zuo et al., 2012).
Another point that can support this possibility is the fact that all the IF shown from ES cells do not have a colony morphology that the cells should have (as in Kim et al, 2011, Guo et al., 2014 and many more...) if they are ES or ES-like cells after one day of growth (cf material and methods; and a minor point: the scale bars are also missing in IF figures). One indispensable control will be to characterize those ES/ or differentiating cells just prior to their use for creating the DamID profiles, and draw conclusions concerning the cell type or cell state that will be strongly supported by data. This characterization should include several pluripotent markers, and compare the levels of these pluripotent markers in the cells used in this study after LV transduction to the levels in the young passaged ES cells used in Kim et al., 2011 that showed differentiation potential. Alternatively the authors could try to derive ACs from the cells used in this study to prove their pluripotency, and the characterization should also include several differentiation markers.
Please see our common response to the reviewers, point 3.

2. *Also please show in WB or at least in the RNA-seq plots the absence of lamin B1 and B2, which is only shown in the RT-PCR, and this is the main point of the paper.*
We have now added the RNA-seq data for the expression of lamin B1 and B2 in WT and dKO mES cells (Sup Fig 6E-F). This confirmation is mentioned in the Materials & Methods.

3. *Even if the cells have slightly differentiated, the main conclusion of this paper could still be valid, yet an accurate description of the cell state is crucial to avoid misinterpretation in future studies. And if the cells are still fully pluripotent, then the authors need to mention and discuss the discrepancy between Kim et al and the ability to detect Lamin A.*

As explained in our common response to the reviewers, point 3, there is not really a discrepancy, because Kim et al (2013) and Guo et al (2014) reported that the cells express Lamin A after all. We now briefly mention this historical course of events on page 5, bottom.

4. *A second point concerns the Emd-DamID performed in the triple KO Lamin B1, B2 and shRNA lamin A/C, from which they conclude that the entire lamin meshwork is not required for genome organization. The authors cited many times Guo et al., 2014 for several aspects of the study, but do not mention that Emerin localization to the nuclear periphery was shown to be dependent on Lamins and more particularly on Lamin A (Guo et al., 2014, & more). Indeed, Emerin is cytoplasmic in the absence of lamin A. This point must be discussed, and explanations should be proposed. An important additional control in Supp Figure 4 is to show the emerin staining co-stained with lamin A/C in cells processed for Emd-DamID, to see if the nuclear periphery emerin positive cells are indeed lamin A negative, or could be cells with a residual lamin A or some few cells with normal lamin A levels. An additional control, that is probably out of the scope of this study but still would be nice to see, would be a negative control for comparison: i.e. DamID profiling of a nuclear protein that is not at the nuclear periphery, for which differences should be scored.*
   Please see common response to reviewers, point 1.

5. *A third experimental point that concerns the LmA-DamID profile in the DKO cells. Since the authors want to test whether the LADs from the LmA-DamID would change if the Lamin A/C is not uniformly peripheral but patchy because of the lack of Lamin B1, to my opinion the confirmation of the patchy localization of Lamin A, which is known to be dependent on the amount (Guo et al., 2014), in the cells sent to LmA-DamID should have been shown by simple IF in those cells and not by an additional fusion protein (that may behave aberrantly). ¬ So, instead of panel E and G in Supp Figure 3 I would have been more convinced by the results if I could see a patchy LmA and /or a LmA/C staining in dKO cells previously transduced with a LV encoding for the LmA-DamID fusion protein.*
   Please see our common response to the reviewers, point 2.

6. *A final, general point concerns the interpretation of the results of the NL-DamID profiles obtained in the different conditions. The general conclusion which is important and consistent with the results is that the major lamin proteins are dispensable for the overall genome organization (if indeed the controls confirm the absence of lamin B1 B2 and Lamin A in the triple, as well as a nuclear peripheral localization of Emerin in most of the cells). The subtle changes they find are not sufficiently discussed nor interpreted in my opinion. For instance, with 94 genes downregulated in the DKO vs WT - I would not call this a highly similar pattern- and 100-200 different LADs were identified between conditions in this study. Between ESCs and NPCs or ACs the approximate number of LADs that changed were also around 200 (Peric-Hupkes et al., 2010) ! So could it be that lamins affect the facultative/developmental LADs. A comparison of facultative LADs and the probes shown in Figures 1, 3 and 4, is needed. It would be nice to see some discussion about this point in the manuscript, in order to get the authors point of view.*
   Regarding the gene expression analysis: we now replaced the admittedly subjective "highly similar" with two objective values (i) a correlation coefficient of 0.99, and (ii) 94 genes changed out of 37,991 that were analyzed. Only 18 of these changed genes are located in LADs.
   We apologize for not elaborating on the difference in overall LAD numbers (e.g. Fig 1C), which – we now realize – could be misleading. Due to inevitable noise in the data, one cannot expect the number of LADs to be exactly identical even between

replicate experiments. This is because random noise tends to break up LADs: a random drop-out of signal over a few array probes inside a LAD will cause the segmentation algorithm to score it as two LADs. One therefore also needs to take into account several other analyses, which we now added and/or highlight more explicitly in this context:

- The total coverage of the genome by LADs is essentially the same between the wild-type and mutant cell lines, indicating that overall loss or gains of entire LADs is negligible. Total coverage numbers are now included in Fig 1C, 3D and 4C, and do not indicate a substantial loss of LADs.
- The scatterplots (Fig 1A, 3B and 4A) would show separate clouds of data points off the diagonal in case entire LADs were lost or gained. This is not the case.
- We added an additional statistical analysis that was specially designed to identify genes with significantly altered NL interactions. Not a single significant gene was found genome-wide, while the same analysis applied to differentiating mES cells found >1,400 significantly altered genes (Peric-Hupkes, Mol Cell 2010).

We now mention and integrate these results in the text parts discussing Fig 1, 3 and 4.

7. Minor points:
   In the supplementary figures and Figure 5 many typos or missing letters in the legend.
   No reference to Suppl Fig 5 in the text of the manuscript
   We thank the reviewer for pointing out the mistakes. We have now corrected the text.

2nd Editorial Decision 04 February 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, both referees support publication of the study by EMBO reports now and only have 2 more minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

As suggested by the referees, please discuss your results more directly in light of the findings by Solovei et al, and please add the wild type samples to figures 4B and E.

Please also specify "n" in the legends for figures 1E, 3F, 4E and SF2B. Even if this information is mentioned in the manuscript text, the figure legends need to stand on their own and must include this information.

I also would like to suggest a few minor changes to the abstract, which needs to be written in present tense, as follows:

In mammals, the nuclear lamina interacts with hundreds of large genomic regions, termed lamina-associated domains (LADs) that are generally in a transcriptionally repressed state. Lamins form the major structural component of the lamina and have been reported to bind DNA and chromatin. Here we systematically evaluate whether lamins are necessary for LAD organization in murine embryonic stem cells. Surprisingly, removal of essentially all lamins does not have detectable effects on the genome-wide interaction pattern of chromatin with emerin, a marker of the inner nuclear membrane. This suggests that other components of the nuclear lamina mediate these interactions.

Please let me know whether you agree with these changes.

I look forward to seeing a final, revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

In the revised manuscript the authors have addressed most of my criticism and, as far as I can judge, also most of the concerns of the other reviewers.
The new data on emerin localization in dKO and tKO cells as well as those on lamin A localization and on the differentiation status of ES cells are convincing.
My only remaining concern is my previous point on LBR, which is directly linked to the novelty of the reported findings. In the author's view their results showing that lamin A/C and lamin B are not required for LAD-NL interaction are surprising and unexpected. Based on their new data that LBR is expressed in ES cells I am not surprised by these results and even think their data nicely support the previously proposed mechanism. Solovei et al (Cell, 2013) have clearly shown by microscopic techniques that lamin A and LBR are components of two redundant complexes linking heterochromatin to the NE. They also showed that B-type lamins are not sufficient for peripheral localization of heterochromatin in the absence of lamin A and LBR. As in the revised version the authors now show that lamin-deficient dKO ES cells express LBR at the NE, one can assume that this protein is sufficient to anchor LADs to the periphery also in ES cells.
I take the argument of the authors that the Solovei study was based solely on microscopic observation looking at DAPI-dense chromatin regions, like most other studies in mammalian cells reporting defects of chromatin organization upon loss of lamins. However, based on the patchy appearance of DAPI staining at the NE as reported in this study in Fig. 5, it is fair to assume that LADs are present in these regions. See also Clowney et al. Cell 151, 724-737 (2013) and Hirano et al. J Biol Chem 287, 42654-63 (2012) for the involvement of LBR in chromatin and gene localization
Although I tend to agree with the author's response that knockdown of LBR may go beyond this study, I still think these previous data could be mentioned more prominently in the current manuscript and/or the results could be better put within the context of these previous findings.

Referee #3:

The revised version of the article by Amendola and van Steensel addressed most of the issues raised in the previous revision step.
Two major important controls have been convincingly addressed by the authors:
- both through new experiments and discussion, the authors show that emerin DamID can be conveniently used to address genome-nuclear lamina interactions in the absence of lamins, at least in mES.
- improved evidence that the used mES are pluripotent, and comparable to the ones shown in previous studies such as Kim et al., 2011, is now provided, and this is an important point to correctly interpret and frame the study.

Additionally, the authors applied a previously used statistical analysis to detect significant changes in NL interactions (Peric-Hupkes 2010) and found no significant change in any condition, further strengthening their conclusions.
Also, I find the addition of total coverage numbers for LADs a helpful element in order to interpret the results correctly.

The added analysis showing the unperturbed expression and localization of LBR in dKO and tKO further suggests that this protein might be a critical determinant of LAD organization in mES, especially in light of the results by Solovei et al., 2013. Nonetheless, this study takes advantage for the first time of a powerful, genome-wide approach to accurately investigate genome-nuclear lamina interactions in conditions of lamins depletion and conveys a simple and clear message: the main components of the nuclear lamina do not play a major role in LAD organization (both constitutive and facultative) in mES. This result constitutes an important starting point for future research in the field, strengthening the rationale for focusing on non-lamin components of the nuclear lamina for example, and therefore I find it suitable for publication in EMBO reports.

Minor point:
- In Figure 4B and 4E it would be useful to have a side by side comparison with wt as it was done in the other figures.

2nd Revision - authors' response 16 February 2015

Please find included what should be the final version of our manuscript. We have made the following modifications and additions:

1) added the 'n=' to the figure legends (figure 1, 2,3, 4, SF2)

2) added wt data for comparison to figures 4B and E

3) changed past tense to present tense in the Abstract

4) discussed results more directly in light of the findings by Solovei et al (Cell 2013), Clowney et al (Cell 2013) and Hirano et al (J Biol Chem
2012) for the involvement of LBR in chromatin and gene localization....

Please let me know if anything needs further work from our end.

3rd Editorial Decision 16 February 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

