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Chromatin compartment dynamics in a haploinsufficient model of cardiac laminopathy

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March 13, 2019

Re: JCB manuscript #201902117

Prof. Charles E Murry
University of Washington
Institute for Stem Cell and Regenerative Medicine
850 Republican Street
Seattle, WA 98109

Dear Prof. Murry,

Thank you for submitting your manuscript entitled "Chromatin compartment dynamics in a haploinsufficient model of cardiac laminopathy" to JCB. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

We're pleased to share that all reviewers appreciated the importance of testing, through genetically controlled studies in cardiomyocytes, the impact of lamin mutations on genome organization and compartmentalization and test links to gene expression that could contribute to our understanding of laminopathies and their pathogenesis. We greatly appreciated the reviewers' expert assessments of the work. Although they are largely supportive of the work, they raised some points that in our view should be addressed as follows to strengthen these important (and somewhat surprising) conclusions.

-- Rev#3's major concern relates to the possibility that changes in differentiation in the mutant cardiomyocytes contribute to gene expression changes. We agree with Rev#3 that this is a valid and significant concern that should be addressed to support the core conclusions of the work.

-- In addition, both Both Revs#2 and #3 ask that you document B type Lamin levels, which we agree would be an important addition (Rev#2 #1, Rev#3 minor #1; see also Rev#2's requests for stainings in #3). We agree with the reviewers that a more thorough characterization of the lamins is needed and will be informative to better understand the complex interplay between these proteins, regardless of the outcome of these experiments.

-- Rev#2 suggests documenting changes in ion channel amounts (i.e., CACNA1A) at the protein level (#4). We agree that this additional experiment would help support claims about functional changes. However, we also appreciate that the core claims are related to transcription (hence your adequate studies of mRNA levels) and that the inhibitor studies also support your functional claims. Therefore, we would suggest that you consider this point and try these experiments if technically straightforward with available antibodies. Data addressing this point would not be absolutely required for publication.

-- Rev#2 has interesting questions about the reprogrammed lines and whether the reprogramming could have impacted transcription (#5-6) and possible off-targets effects (#7). Please consider these three comments seriously and address them to the best of your ability in the time allowed, both by potentially performing additional tests and by discussing some of these caveats in the

manuscript (e.g. "comment #6 - needs to be highlighted as a study limitation").

-- Lastly, Reviewer #2 raised two broader issues. A broader comment from Rev#2 is what determines whether genes are sensitive to B compartmentalization in a manner that depends on WT lamins. We agree that this is a valid and fascinating question but one we feel would be beyond the scope of the work to address substantively. Please do answer this question to the best of your ability through discussions without producing more data. Rev#2 additionally pointed out (comment #2) that, to rule out that lamin A/C mutations may impact gene expression after mechanical stress, you should go further and carry out RNAseq and HiC analyses in the same cells but undergoing electrophysiological contractile stimulation. This is a crucial limit to the analyses presented; however, this is also a major undertaking and we greatly appreciate that you did note this important limit in the manuscript text already. We hope that your study will inspire investigators to tackle this point in future work. Data addressing these broader questions will not be needed for publication, but we look forward to reading your thoughts on these important and interesting questions for the field.

Please let us know if you have any questions or anticipate any issues addressing these points. We would be happy to discuss the revision process as needed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ana Pombo, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is a complete and well carried out manuscript. While results are somewhat negative, this should be of interest to many. I have no comments to improve the manuscript.

Reviewer #2 (Comments to the Authors (Required)):

Bertero A et al. report chromatin compartment dynamics in cardiomyocytes derived from human iPS cells carrying the haploinsufficient LMNA mutation R225X and from two gene-edited, isogenic control lines. Several electrophysiological and contractility assays were performed showing substantial disruption of such properties in mutant cardiomyocytes. The authors correlated the 3D chromatin architecture and its A/B compartmentalisation to gene expression dysregulation. Although the 3D organisation appears to be partially affected, the A/B compartmentalisation does not seem to play a direct role in modulating gene expression-related pathogenesis.

The manuscript is technically and conceptually very solid; it is also remarkably well written, with a thorough Discussion section. One key question however remains unanswered: what confers "lamin A/C-sensitivity" to the genes that escape B compartmentalization in mutant cells? Are these genomic regions characterized by a peculiar epigenetic profile or a specific spatial localization (i.e. nuclear interior vs periphery)? Any additional data in this direction would strengthen the manuscript significantly.

There are also some important points that have not been investigated and that might clarify why the hypothesis has not been proven:
1. Fig 1: the role of B-type lamins has been neglected. Although the authors state that other A-type lamins isoforms were not detectable, there is no report about lamin B1 or B2 presence. Although A- and B-type lamins form two separate meshworks, they do have points of contact, compensatory roles and both participate in the chromatin organisation (Shimi T et al. 2008, Dechat T et al. 2008).

2. Fig. 4-5: the authors investigate chromatin architecture changes and compartmentalisation disruption as a direct cause of electrophysiological and contractility abnormalities. The point is that

the mechanical forces triggered by spontaneous and, more importantly, electrical stimulation can induce nuclear dysmorphisms (Siu et al. 2012) that can further affect chromatin structure and LADs, thus impacting gene expression. Therefore, ideally Hi-c compartmentalisation and RNA-seq analysis should have been performed also in the very same cells undergoing electrophysiological/contractility assays. Additional analyses to clarify this point would be important to support the authors' conclusions.

3. In the introduction, the authors hypothesised that the low expression of lamin A and C "would lead to functional alterations of A/B compartmentalisation leading to aberrant gene expression". Nevertheless, the localization of both lamin A/C and lamin B1 has not been shown. Due to assembly alterations, the two nuclear lamins can be mislocalized (Wiesel N et al. 2008; Bhattacharjee P et al. 2013; Steele-Stallard H et al. 2018) which, in turn, could potentially affect chromatin organisation. Therefore, an immunostaining looking at these proteins' positioning would be desirable.

4. Given that most of the restored electrophysiological defects, upon genetic correction, are imputed to changes in the expression of genes encoding crucial subunits of ion channels, it would be important to show evidence that this is reflected at the protein level.

5. Could some of the transcriptional findings be linked to insertional mutagenesis events in the reprogramming to pluripotency of the original LMNA-mutant iPSCs (which were indeed generated with lentiviral vectors)? This could still be possible if those iPSCs were not clonal in the first place. It would also be useful if the authors could confirm silencing of the exogenous reprogramming factors in their CMs, as lentiviruses do not tend to silence the reprogramming transgenes as good as retroviruses. Any clarifications or experiments to address this point would be appreciated.

6. Related to the previous point, it is not clear why no sub-clones of the LMNA-mutant line have been used throughout the paper. The fact that this is only one patient and no multi clonal analysis has been performed to assess variability needs to be highlighted as a study limitation.

7. Off target events in the edited lines: it is mentioned that all efforts have been made to prospectively minimize them, but have they been studied/tested retrospectively?

Reviewer #3 (Comments to the Authors (Required)):

Bertero et al. analyse the contribution of a haploinsufficient LMNA mutation on cardiomyocyte gene expression and function and address the role of chromatin organization into active and inactive compartments in the observed defects. They generate iPS cells from a haploinsufficient patient, correct the mutation using scarless CRISPR and differentiated two corrected clones and the parental patient cells into cardiomyocytes. Mutant cells are subsequently shown to contain electrophysiological defects, consistent with the disease phenotype. Comparing differential gene expression and HiC data the authors discover both differences in gene expression as well as alterations between active and inactive compartments. Surprisingly, however, the correlation between the two is relatively minor. The authors finally focus on a candidate gene that is dysregulated both on the gene expression levels and in transitioning to the inactive chromatin compartment and show that the ectopic expression of this calcium channel in the mutant cells contributes to the electrophysiological defects.
Overall this is a thoughtful and extensive manuscript that sheds light to the mechanisms underlying the pathophysiological mechanisms of laminopathies. The poor correlation between gene

expression and chromatin compartmentalization defects seems to rule out the so called 'chromatin hypothesis' of laminopathy ethiology. To justify this strong conclusion, the authors should more rigorously exclude a minor differentiation defect/delay in the mutant cardiomyocytes, which could explain a significant proportion of the gene expression differences between the mutant and corrected cells and thereby lead to the loss of correlation between chromatin architecture and gene expression.

Major point
The authors use PC analysis to show that there is no global defect or delay in the differentiation of the mutant iPSCs to cardiomyocytes. Although the result on first sight seems clear, looking at the mutant replicates, it seems that they higher degree of variation among themselves than from the corrected cells and they do not cluster together away from the corrected cells. The same is true for the PC analyses of the hiC data in Fig.6 where the mutants are farther away from the d14 differentiated cells with regard to PC1 that explains majority of the variance. This would imply that the PC analysis does not have the resolution to exclude variation/heterogeneity in the differentiation process as a significant underlying cause of the gene expression changes. This is of relevance as the many the differentially regulated genes are genes associated with cardiomyocyte development or fate (illustrated by the GO term analysis that ranks cardiac muscle fibre as the most significantly downregulated GO term group in the mutant and the variation in key marker expression in Supp. Fig, 2A between mutant and corrected). Therefore, the differentiation process and in particular the heterogeneity of the differentiated cells should be analysed in more detail. The key differentially expressed genes should be analysed not only in differentiated cardiomyocytes but also from mutant and corrected iPSC cells and intermediates (as in Supp Fig. 2A) to exclude that differences do not arise in a small delay in differentiation. Even mor eimportantly, the authors should address cell-to cell variation and heterogeneity of both mutation-specific gene expression and differentiation by performing in situs and/or immunofluorescence analysis of key differentiation markers and mutant

Minor points
1. Lamin B expression should be analysed and shown in panel 1F to address compensatory upregulation

2. It is not clear why the authors refer to Myh9, ACTA2 and CTGF as non-cardiomyocyte genes (p10 line 288) as they are also expressed in cardiomyocytes

Point by point answer to the Reviewers

Chromatin compartment dynamics in a haploinsufficient model of cardiac laminopathy

Bertero et al.

We thank the Reviewers for their helpful and encouraging comments. In our revised manuscript we have followed the Editors' recommendations to address the key concerns raised by the Reviewers by performing additional experiments and by modifying the text based on their suggestions. Towards that end, we have markedly improved the characterization of the differentiation dynamics of lamin A/C mutant hiPSC-CMs, confirming that they are remarkably similar to those of corrected controls. We have also performed additional genomic quality controls of CRISPR/Cas9 gene edited hiPSCs, which excluded off-target mutations and verified that lentiviral transgene expression was silenced. Furthermore, we have investigated the expression and localization of B-type lamins, which excluded a compensatory upregulation due to lamin A/C haploinsufficiency. Finally, we have performed extensive validation of our key findings from the Hi-C analysis by using 3D DNA FISH combined with immunofluorescence, which confirmed our hypothesis that, as a result of lamin A/C haploinsufficiency, specific lamin A/C-sensitive B compartments are not properly segregated to the nuclear periphery during hPSC-CM differentiation. Collectively, these experiments further validate our genetic model to test the effects of a pathogenic lamin A/C haploinsufficient mutation on genome topology, and they support our original findings that alterations in chromatin compartmentalization appear to be the exception rather than the general rule in the pathogenesis of cardiac laminopathy.

You will find below our detailed point by point answer to all of the Reviewers' comments, with detailed references to the new data and text changes. Furthermore, all text changes are indicated in magenta in the revised Manuscript and Supplemental Information.

Reviewer 1

Reviewer 1 general considerations:

"This is a complete and well carried out manuscript. While results are somewhat negative, this should be of interest to many. I have no comments to improve the manuscript."

We are delighted to hear such positive feedback from the Reviewer, and we thank them for having contributed to the peer-review of our work.

Reviewer 2

Reviewer 2 general considerations:

"Bertero A et al. report chromatin compartment dynamics in cardiomyocytes derived from human iPS cells carrying the haploinsufficient LMNA mutation R225X and from two gene-edited, isogenic control lines. Several electrophysiological and contractility assays were performed showing substantial disruption of such properties in mutant cardiomyocytes. The authors correlated the 3D chromatin architecture and its A/B compartmentalisation to gene expression dysregulation. Although the 3D organisation appears to be partially affected, the A/B compartmentalisation does not seem to play a direct role in modulating gene expression-related pathogenesis.

The manuscript is technically and conceptually very solid; it is also remarkably well written, with a thorough Discussion section. One key question however remains unanswered: what confers "lamin A/C-sensitivity" to the genes that escape B compartmentalization in mutant cells? Are these genomic regions characterized by a peculiar epigenetic profile or a specific spatial localization (i.e. nuclear interior vs periphery)? Any additional data in this direction would strengthen the manuscript significantly."

We thank the Reviewer for their positive and constructive feedback. We share the Reviewer's curiosity regarding the nature of lamin A/C-sensitive B compartments. The Editors also commented that **"*this is a valid and fascinating question*"**, while they acknowledged that it is **"*one we feel would be beyond the scope of the work to address substantively*".** Nevertheless, in order to at least partially explore this aspect, we have performed extensive analysis by immunoFISH to probe whether lamin A/C-sensitive B compartments are characterized by a peculiar spatial localization within the nucleus (**Fig. 7 and lines 322-339**). For this, we performed 3D DNA FISH for genes contained within the three key lamin A/C-sensitive B compartment hotspots on 19p13.13, 19q13.33, and 5q31.3 (*CACNA1A*, *LRRC4B*, and *PCDHGB4*, respectively). We also characterized two control loci found on 19p and 19q (*VAV1* and *LGALS14*, respectively), which transition appropriately from the A to B compartment both in mutant and corrected hiPSC-CMs (lamin A/C-insensitive; **Fig. 7C**). To monitor the spatial localization of these loci in relationship to the nuclear lamina, we combined such 3D DNA FISH experiments with immunofluorescence for Lamin B1, and quantified the distance between each locus and the nuclear lamina boundary (**Fig. 7A-B**). These results demonstrated that for all genes except *PCDHGB4*, transition from the A to B compartment during hiPSC-CM differentiation correlates with increased proximity to the nuclear lamina. However, in mutant hiPSC-CM this transition is impaired specifically for genes found in lamin A/C-sensitive compartments (*CACNA1A* and *LRRC4B*). Of note, in corrected control hiPSCs and hiPSC-CMs the position of *CACNA1A* and *LRRC4B* with respect to the nuclear periphery was similar to that of *VAV1* and *LGALS14*, indicating that lamin A/C-sensitive B compartments are not characterized by an obviously peculiar localization. Interestingly, we noted that *PCDHGB4* is already closely associated with the nuclear lamina in hiPSCs (despite being in the A compartment), and its localization does not change any further neither during hiPSC-CM differentiation nor due to lamin A/C haploinsufficiency. Thus, aberrant compartmentalization of this chromatin region in mutant hiPSC-CMs must reflect some mechanism other than marked changes in association to the nuclear lamina. Collectively, these results indicate that lamin A/C-sensitivity in B compartments cannot be simply predicted as a function of their localization within the nuclear space.

Considering these findings, we speculate that the local epigenetic state is likely dictating lamin A/C-sensitivity in selective B compartments, as suggested by the Reviewer. Whilst comprehensive epigenetic profiling of histone marks and/or DNA modifications and accessibility in mutant and corrected cells was beyond the scope of the current study, this is an important area of investigation that we plan to pursue in the future.

"There are also some important points that have not been investigated and that might clarify why the hypothesis has not been proven:"

We appreciate the insightful suggestions presented by the Reviewer, which helped strengthening our manuscript as described in detail below.

Reviewer 1 specific comments:

Comment 1: ***"Fig 1: the role of B-type lamins has been neglected. Although the authors state that other A-type lamins isoforms were not detectable, there is no report about lamin B1 or B2 presence. Although A- and B-type lamins form two separate meshworks, they do have points of contact, compensatory roles and both participate in the chromatin organisation (Shimi T et al. 2008, Dechat T et al. 2008)."***

We thank the Reviewer for pointing out this important aspect. First, we have included the RT-qPCR data for non-canonical A-type lamins, which we previously mentioned as "data not shown" (**Figure S3C**). We confirm lack of expression of A-type lamins other than A and C in both mutant and corrected cells at any time point during hiPSC-CM differentiation. Moreover, we have profiled expression of B-type lamins by RT-qPCR (**Fig. S3E**), western blot (**Fig. 1F**), and immunofluorescence (**Fig. S3D**; **lines 174-176**). These analyses indicate that levels of B-type lamins inversely correlate with those of A-type lamins, as both lamin B1 and lamin B2 are downregulated during hiPSC-CM differentiation. Importantly, lamin B1 and lamin B2 are expressed at comparable levels in mutant and corrected hiPSC-CM, and show comparable localization. Overall, these findings exclude a possible compensatory upregulation of non-canonical A-type lamins and/or of B-type lamins in mutant cells due to lamin A/C haploinsufficiency.

Comment 2: "*Fig. 4-5: the authors investigate chromatin architecture changes and compartmentalisation disruption as a direct cause of electrophysiological and contractility abnormalities. The point is that the mechanical forces triggered by spontaneous and, more importantly, electrical stimulation can induce nuclear dysmorphisms (Siu et al. 2012) that can further affect chromatin structure and LADs, thus impacting gene expression. Therefore, ideally Hi-c compartmentalisation and RNA-seq analysis should have been performed also in the very same cells undergoing electrophysiological/contractility assays. Additional analyses to clarify this point would be important to support the authors' conclusions.*"

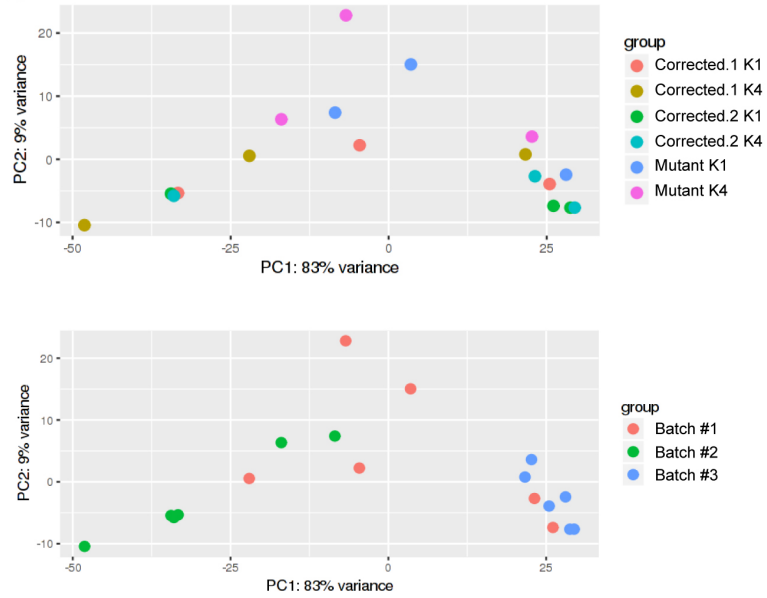
We would like to clarify that our analyses of electrophysiological and contractile properties were performed either in the absence of external electrical pacing (MEA experiments, as we wished to probe the spontaneous activity of mutant cells to determine beat irregularity) or after short-term electrical pacing just during the course of the measurement (calcium and contractility assays, as these aspects are strongly dependent on the beat rate). Therefore, our experiments differ from those performed by Siu and colleagues in that we did not use chronic electrical stimulation as a stimulus during hiPSC-CM culture. Since the alterations in electrophysiological and contractile properties that we observed in lamin A/C haploinsufficient cells arise in cells not subjected to electrical stress, our Hi-C and RNA-seq analyses on un-stressed hiPSC-CM are therefore representative of the cell state that we most extensively phenotyped.

The exception to this was of course the use of 3D engineered heart tissues (3D-EHTs): while these were also cultured in the absence of chronic electrical stimulation, 3D-EHTs experience more substantial mechanical forces during contraction, as a result of increased preload (resistance during diastole) and afterload (resistance during systole). We used 3D-EHTs to provide additional validation of the cellular phenotype due to lamin A/C haploinsufficiency, as it is well-established that by promoting hiPSC-CM maturation 3D-EHTs represent a more physiological model to assess cardiac physiology. However, given the technical challenges that we had foreseen (see below), our study was not designed with the aim of evaluating the combined effect of lamin A/C mutations and mechanical stress on chromatin organization. The Editors commented that: "***this is a crucial limit to the analyses presented*"**, but they also acknowledged that **"*this is also a major undertaking and we greatly appreciate that you did note this important limit in the manuscript text already. We hope that your study will inspire investigators to tackle this point in future work*".** We completely share the Reviewer and Editors' interest regarding the possible effect of mechanical forces on the chromatin organization in laminopathic cells, and accordingly, we had already begun experiments that we expected to contribute to a follow up study.

For this, we took advantage of a system that we recently developed to control the degree of afterload in 3D-EHTs by modulating the flexibility of one of the posts used for casting (Leonard et al., 2018). We generated 3D-EHTs from mutant and corrected control hiPSC-CMs, and subjected them to low or high afterload (K1 and K4, respectively). We note that K1 and K4 impose a degree of afterload that is ~10 fold lower and ~10 fold higher,

respectively, than what induced by the older post system used in our manuscript. We then subjected individual 3D-EHTs from three separate experiments to RNA-seq, and made the following observations (**Reviewer's Figure 1**, below): (1) the variability between 3D-EHT batches dominates the variance in global gene expression; (2) batch-controlled differential gene expression analysis using DEseq2 identifies a modest number dysregulated genes in mutant hiPSCs, while only a handful of differences are observed among the two corrected controls; (3) while gene dysregulation appears modestly stronger in K4 compared to K1, the number of genes consistently dysregulated in mutant and both corrected controls is relatively low and comparable in both conditions (50 and 55, respectively); (4) of these genes, ~60% (31 genes) are dysregulated both in K1 and K4 (97% of which show consistent up- or down-regulation in all comparisons); (5) among genes consistently dysregulated in mutant cells both in K1 and K4, ~50% (16 genes) had been also identified as dysregulated in RNA-seq from day 14 hiPSC-CM. Collectively, these findings suggest that culture on 3D-EHTs in either low or high afterload does not greatly exacerbate gene expression dysregulation due to lamin A/C haploinsufficiency. On the contrary, gene expression differences do not seem much different from those observed in monolayer cultures. However, we note that we might have underestimated the number of differential expressed genes in these experiments due to the large batch-to-batch variability.

A



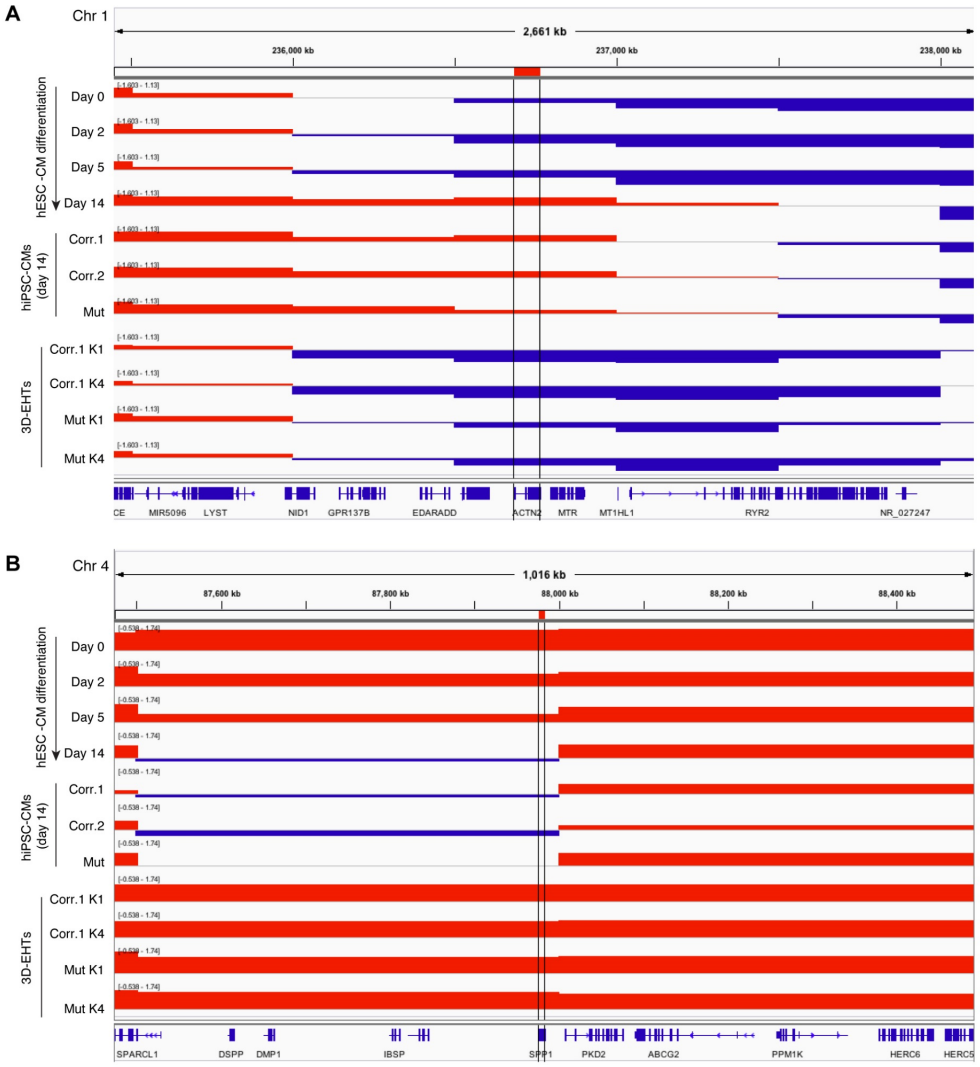
B

Comparison	Differential genes
K1 conditions	
Mut vs Corr.1	94
Mut vs Corr.2	105
Corr.1 vs Corr.2	8
Mut vs Corr.1 & Corr.2	50
Upregulated	31 (62%)
Downregulated	19 (38%)
K4 conditions	
Mut vs Corr.1	248
Mut vs Corr.2	134
Corr.1 vs Corr.2	22
Mut vs Corr.1 & Corr.2	55
Upregulated	35 (64%)
Downregulated	20 (36%)
K1 & K4 conditions	
Mut vs Corr.1. & Corr.2	32
Concordant?	31 (97%)
Also in day 14 hiPSC-CM	16/32 (50%)

Reviewer's Figure 1. RNA-seq analysis of 3D-EHTs from mutant and corrected hiPSC-CM. (**A**) Linear dimensionality reduction by principal component (PC) analysis of RNA-seq data from all expressed genes in 3D-EHTs from mutant and corrected hiPSC-CM. Samples were analyzed after 4 weeks of culture in conditions of low (K1) and high (K4) afterload. Three independent hiPSC-CM differentiations were used for as many independent 3D-EHT casting experiments (batches #1-3) The first PC, which explains the vast majority of the variance across the datasets, captures batch-to-batch variability. The second PC, which explains ~10% of the variance, captures the effect of lamin A/C haploinsufficiency. Samples are color-coded based on the experimental condition (top) or the batch number (bottom) (**B**) Results of batch-controlled differential gene expression analysis using DESeq 2.

Despite the challenges in interpreting these RNA-seq data, we decided to also attempt DNase Hi-C experiments in the same conditions with the hypothesis that changes at the level of chromatin compartmentalization might suffer from less variability across different 3D-EHT batches. Thus, we analyzed 3D-EHTs from two batches (#1 and #2) for mutant and one corrected control line (Corr.1), pooling 4 to 5 EHTs for each condition to obtain sufficient material for the analysis. Before presenting these results we would like to point out that 3D-EHTs are casted by mixing hiPSC-CM and stromal cells (the immortalized human HS27a bone marrow stromal cell line) at a 10:1 ratio. However, during the subsequent 4 weeks of culture HS27a proliferate more rapidly than hiPSC-

CMs and finally account for ~30% of the total cell population in mature 3D-EHTs. We hypothesized that this degree of heterogeneity would not substantially affect Hi-C-determined A/B compartmentalization: if so, we expected that a majority of cardiac genes would be found in the expected compartment (A), while non-cardiac genes would be segregated in the opposite compartment (B). However, we were disappointed to note that this was not the case (**Reviewer's Figure 2**, below): among many other examples, the cardiac gene *ACTN2*, which encodes for the sarcomeric protein α-actinin 2 and transitions from B to A during hPSC-CM differentiation, was found in the B compartment in all 3D-EHT samples. On the other hand, the stromal gene *SPP1*, which encodes for osteopontin and transitions from A to B during hPSC-CM differentiation, was found in the A compartment in all 3D-EHT samples. We concluded that DNase Hi-C data from 3D-EHTs is too heavily confounded by the contamination of stromal cells to be accurately interpreted. We speculate that HS27a nuclei are oversampled during the nuclear extraction step required for the generation of DNase Hi-C library (as nuclei are notoriously hard to isolate from sarcomere-rich hiPSC-CM), which further aggravates the degree of contamination.



Reviewer's Figure 2. DNase Hi-C analysis of 3D-EHTs from mutant and corrected hiPSC-CM. (**A**) Genomic tracks of chromatin compartmentalization for the genomic locus containing the *ACTN2* gene (highlighted). Positive and negative Hi-C matrix PC1 scores are shown in red and blue, and indicate 500 Kb genomic bins in the A and B compartments, respectively. (**B**) As in A, but for the gene *SPP1*.

Overall, we are sharing these results with the Reviewer to showcase the substantial technical challenges involved in reliably measuring gene expression and chromatin organization dynamics in a heterogeneous and highly variable sample type such as 3D-EHTs. In the future, we plan to optimize single-cell approaches for RNA-seq and Hi-C to 3D-EHTs to circumvent these limitations.

Comment 3: "*In the introduction, the authors hypothesised that the low expression of lamin A and C "would lead to functional alterations of A/B compartmentalisation leading to aberrant gene expression". Nevertheless, the localization of both lamin A/C and lamin B1 has not been shown. Due to assembly alterations, the two nuclear lamins can be mislocalized (Wiesel N et al. 2008; Bhattacharjee P et al. 2013; Steele-Stallard H et al. 2018) which, in turn, could potentially affect chromatin organisation. Therefore, an immunostaining looking at these proteins' positioning would be desirable.*"

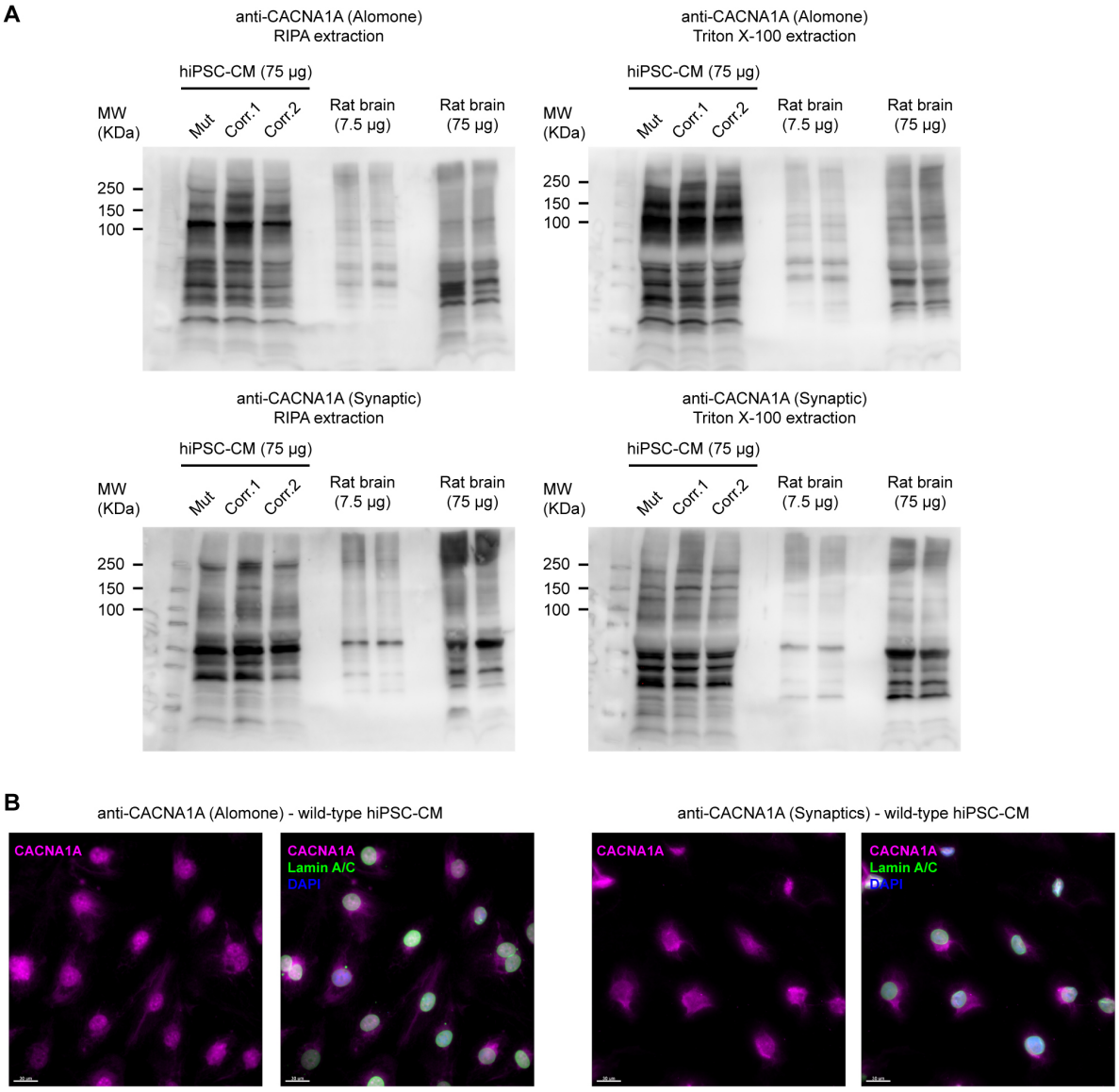
We appreciate the importance of the point raised by the Reviewer, and we have performed these immunostainings as he/she recommended (**Fig. S3D**; also see the ImmunoFISH in **Fig. 7A**). These analyses confirmed that both lamin A/C and lamin B1 show the expected localization with strong enrichment at the nuclear periphery in both mutant and corrected hiPSC-CM.

Comment 4: "*Given that most of the restored electrophysiological defects, upon genetic correction, are imputed to changes in the expression of genes encoding crucial subunits of ion channels, it would be important to show evidence that this is reflected at the protein level.*"

We anticipated substantial challenges in performing this experiment due to: (1) the notorious difficulties in identifying specific antibodies for ion channels as members of the same family often share extensive homology; (2) technical challenges in detecting large ion channels by western blot due to their tendency to aggregate and precipitate as a result of possessing multiple hydrophobic transmembrane domains; (3) the low expression levels of *CACNA1A* mRNA in mutant hiPSC-CM, which is detected at a cycle threshold of ~27. Nevertheless, we attempted both western blot and immunofluorescence analyses for the *CACNA1A* protein product (**Reviewer Figure 3** below).

In order to maximize our chances for success, we obtained two distinct antibodies against *CACNA1A*: one from Alomone Labs, cat #ACC-001; the second from Synaptic Systems, cat #152-103. Both antibodies were validated for western blot and immunocytochemistry analysis, and have been cited in numerous publications. Finally, the antibody from Alomone Labs has been validated using a knockout model (Dorgans et al., 2017; Jung et al., 2016). However, we note that both antibodies were generated against rat CACNA1A, and while reactivity for human CACNA1A is predicted by the suppliers based on homology of the epitope used for immunization, this has not been experimentally validated. Unfortunately, we could not identify any antibody specifically raised against human CACNA1A that has been validated in the literature. We performed western blot using two protocols to generate protein lysates: (1) lysis using conventional RIPA buffer followed by denaturation by boiling (as described in our Materials and Methods); (2) lysis using a 1% Triton X-100-based isotonic buffer followed by mild heating at 37 °C for 20 min before direct loading on the gel (no freeze-thaw). This second protocol has been recently optimized in our lab for analysis of cardiac ion channels, and aims to reduce their aggregation and precipitation by avoiding strong ionic detergents during lysis and by preventing excessive heat-induced protein denaturation. We used a large amount of protein lysate for hiPSC-CMs (75 µg) to improve the chances of detecting a signal, and also generated protein lysates from rat brain as positive controls. Blotting was performed overnight at medium voltage, and resulted in near-complete transfer of high molecular weight proteins (as indicated by Comassie blue staining of the 4-20% acrylamide gel used for electrophoresis). Unfortunately, however, despite numerous attempts to optimize antibody concentrations and wash conditions, neither antibody nor lysis condition resulted in a specific signal at the expected molecular weight (150-200 KDa) in the positive

controls (**Reviewer's Figure 3A**, below). We do not know the reason for this failure, but speculate that it might be at least partially due to lot-to-lot variability in the antibodies, which are both rabbit polyclonal. Moreover, we noticed that the both antibodies resulted in widespread non-specific staining in hiPSC-CMs (regardless of the genotype), indicating that they recognize several cardiac-specific proteins. Accordingly, immunofluorescence with both antibodies resulted in a strong signal in wild-type hiPSC-CMs with notable enrichment in the nucleus, which is unexpected for a ion channel that should be predominantly localized at the plasma membrane (**Reviewer's Figure 3B**, below).



Reviewer's Figure 3. Test of anti-CACNA1A antibodies. (**A**) Representative western blots from protein lysates obtained according to the indicated lysis methods. anti-CACNA1A from Alomone Labs (cat #ACC-001) was used at 1:200 dilution (0.4 µg/ml); anti-CACNA1A from Synaptic Systems (cat #152-103) was used at 1:500 dilution (0.2 µg/ml). (**B**) Representative immunofluorescences. anti-CACNA1A from Alomone Labs (cat #ACC-001) was used at 1:100 dilution (0.8 µg/ml); anti-CACNA1A from Synaptic Systems (cat #152-103) was used at 1:250 dilution (0.4 µg/ml). Nuclei counterstained with DAPI; scale bars: 30 µm.

Thus, while we agree that validation of CACNA1A upregulation at the protein level would have been elegant and useful, technical difficulties prevented us to address this point satisfactorily. We note that the Editors stated that:

"***we also appreciate that the core claims are related to transcription (hence your adequate studies of mRNA levels) and that the inhibitor studies also support your functional claims. Therefore, we would suggest that you consider this point and try these experiments if technically straightforward with available antibodies***". We respectfully posit that this data is not required to support our key conclusions.

Comment 5: "*Could some of the transcriptional findings be linked to insertional mutagenesis events in the reprogramming to pluripotency of the original LMNA-mutant iPSCs (which were indeed generated with lentiviral vectors)? This could still be possible if those iPSCs were not clonal in the first place. It would also be useful if the authors could confirm silencing of the exogenous reprogramming factors in their CMs, as lentiviruses do not tend to silence the reprogramming transgenes as good as retroviruses. Any clarifications or experiments to address this point would be appreciated*".

We understand the Reviewer's concern about the potential transcriptional influence of insertional mutagenesis events during hiPSC generation using lentivirus, but we submit that our experimental design is as robust to this unlikely eventuality as it is realistically possible. Indeed, we decided to generate and analyze two independent CRISPR/Cas9-corrected clones specifically to be able to control for biological variability between clonal sublines. In this, we went beyond what is currently considered the "gold standard" in the field of disease modeling using hiPSCs, namely the generation of a single isogenic control. Notably, the two corrected controls we generated proved remarkably similar in virtually every molecular and phenotypic assay that we performed (including in their genome-wide gene expression). This gives us substantial confidence that the differences observed compared to the parental mutant line are specifically due to the correction of *LMNA* R225X heterozygous mutation. Thus, we respectfully argue that additional experiments designed to strengthen this conclusion are not essential.

Regarding the second aspect raised by the Reviewer, silencing of exogenous reprogramming factors in the R225X mutant hiPSC line was previously confirmed by the authors that originally generated it (Fig. S1C in Siu et al., 2012). Because we had not considered the possibility that the corrected subclones might have arisen from a rare hiPSC that had not fully silenced the reprogramming factors, we repeated the RT-qPCR analyses described by Siu and colleagues, which rely on a primer specific to the 5' UTR generated upon transcription from the lentiviral EF1α promoter in order to detect the exogenous transgenes (**Fig. S1F**). We are glad to confirm that exogenous reprogramming factors could not be detected even after 40 cycles of PCR in either the mutant or corrected lines, indicating robust silencing. We also confirmed that such transgenes do not get unexpectedly reactivated upon hiPSC-CM differentiation, as the cycle threshold remains undetectable (data not shown).

Comment 6: "*Related to the previous point, it is not clear why no sub-clones of the LMNA-mutant line have been used throughout the paper. The fact that this is only one patient and no multi clonal analysis has been performed to assess variability needs to be highlighted as a study limitation*".

Despite our request, we unfortunately only received a single *LMNA* R225X heterozygous mutant line from Dr. Hung Fat-Tse (to our knowledge this is the only clone that they characterized in any detail). We also did not receive dermal fibroblasts that could be used to generate new hiPSCs in house. While as discussed in the previous section our experimental design based on two isogenic corrected controls gives us strong confidence that the phenotype we determined within the genetic background of this patient is due to the R225X mutation, we acknowledge that use of a single lamin A/C haploinsufficient mutant line remains a limitation of the study. This was unrealistic to mitigate within the timeframe of this revision, but it is now clearly stated in the discussion (**lines 518-520**): "*Furthermore, replication of our findings in additional hiPSC lines with nonsense/haploinsufficient mutations will be important, as our study focused on only one such mutant line*".

Comment 7: "*Off target events in the edited lines: it is mentioned that all efforts have been made to prospectively minimize them, but have they been studied/tested retrospectively?*".

We share the Reviewer's concerns regarding the possibility of CRISPR/Cas9-induced off-target mutations. Indeed, we had decided to use two separate sgRNAs to generate the individual corrected control clones because it was extremely unlikely that these sgRNA (whose sequence overlap is only 10 bp) could induce off-target mutations in the same genes. This was confirmed by *in silico* analyses for putative off-targets for each of the two sgRNAs used, which showed that there is no overlap between the genes found closest to their respective potential off-target sites (**Table S1;** details described in the **Material and Methods** at **lines 657-676**). Nevertheless, we still examined experimentally a few putative off-target sites to validate the specificity of the enhanced-specificity SpCas9 that we had elected to use for the gene targeting experiments. For this, we focused on all putative off-target sites with only 2 mismatches to the sgRNAs (one for sgRNA 1 and two for sgRNA 2), as well as on 3 additional off-targets that while less likely to be cut (due to more mismatches and/or the inclusion of a bulge between the sgRNA and the target sequence) present a high risk given to their location within a gene expressed during hiPSC-CM differentiation (or within putative regulatory sequences for such a gene). Sanger sequencing of genomic PCR products excluded any such mutation in either of the corrected control hiPSCs (**Fig. S1E**).

Reviewer 3

Reviewer 3 general considerations:

"***Bertero et al. analyse the contribution of a haploinsufficient LMNA mutation on cardiomyocyte gene expression and function and address the role of chromatin organization into active and inactive compartments in the observed defects. They generate iPS cells from a haploinsufficient patient, correct the mutation using scarless CRISPR and differentiated two corrected clones and the parental patient cells into cardiomyocytes. Mutant cells are subsequently shown to contain electrophysiological defects, consistent with the disease phenotype. Comparing differential gene expression and HiC data the authors discover both differences in gene expression as well as alterations between active and inactive compartments. Surprisingly, however, the correlation between the two is relatively minor. The authors finally focus on a candidate gene that is dysregulated both on the gene expression levels and in transitioning to the inactive chromatin compartment and show that the ectopic expression of this calcium channel in the mutant cells contributes to the electrophysiological defects.***

Overall this is a thoughtful and extensive manuscript that sheds light to the mechanisms underlying the pathophysiological mechanisms of laminopathies. The poor correlation between gene expression and chromatin compartmentalization defects seems to rule out the so called 'chromatin hypothesis' of laminopathy ethiology. To justify this strong conclusion, the authors should more rigorously exclude a minor differentiation defect/delay in the mutant cardiomyocytes, which could explain a significant proportion of the gene expression differences between the mutant and corrected cells and thereby lead to the loss of correlation between chromatin architecture and gene expression."

We thank the Reviewer for their encouraging and constructive comments. As described in detail below, we have performed extensive new analyses of differentiating hiPSC-CM from mutant and corrected control cells, which strengthened our original conclusion that the differentiation kinetics of these cell lines is remarkably similar.

Reviewer 3 specific comments:

Major point

We elected to break down this detailed major comment into multiple sections to more clearly address the points contained within and to clarify any potential misunderstanding of our data.

Section 1: *"The authors use PC analysis to show that there is no global defect or delay in the differentiation of the mutant iPSCs to cardiomyocytes. Although the result on first sight seems clear, looking at the mutant replicates, it seems that they higher degree of variation among themselves than from the corrected cells and they do not cluster together away from the corrected cells*".

We would like to point out that based on PC1 (which explains the majority of the variance, 41%, and indicates the differentiation status) mutant and corrected hiPSC-CM are basically overlapping (**Fig 4E**). While there is some separation among samples along PC2, this explains only 14% of the variability and has no clear biological meaning (as hiPSC-CMs are in between samples from day 0 and day 5 of hESC-CM differentiation). Further, separation along PC2 does not correlate with genotype. If anything, PC2 may be capturing some minor technical variance due to batch-to-batch variability in hiPSC-CM differentiation (see the relative separation between samples from replicate 3 and those from replicates 1 and 2 in **Fig. 4E**; furthermore, corrected controls cluster by replicate in **Fig. 4B**). Thus, we stand by our conclusion that while some significant and substantial gene expression differences do exist between mutant and corrected controls (**Fig. 4B-D**), these are comparatively minor to the large degree of transcriptional rearrangement that characterizes hiPSC-CM differentiation. We also would like to stress that mutant hiPSC-CMs did not underperform in any of the physiological assays that we performed (action potential generation, calcium update, contractility), as it would be expected of hiPSC-CMs were they developmentally delayed. Thus, these data already provided substantial evidence that mutant hiPSCs acquire cardiac identify with comparable speed and efficiency to corrected control hiPSCs. We now provide even stronger evidence for this, as discussed in the reply to Section 3 below.

Section 2: "*The same is true for the PC analyses of the hiC data in Fig.6 where the mutants are farther away from the d14 differentiated cells with regard to PC1 that explains majority of the variance. This would imply that the PC analysis does not have the resolution to exclude variation/heterogeneity in the differentiation process as a significant underlying cause of the gene expression changes*".

We would like to clarify that the analysis of A/B compartmentalization by PCA in **Figure 6C** revealed that despite being very similarly matched based on their developmental gene expression profile (as just discussed), mutant hiPSC-CMs exhibit a clear developmental delay compared to corrected controls from the perspective of A/B compartmentalization. This discrepancy is actually one of the pieces of evidence that changes in chromatin compartmentalization and gene expression are largely independent. Indeed, as shown in our subsequent analyses in **Figure 8A** and **Supplemental Figure 5B**, only a small portion of dysregulated A/B compartments results in functional alterations of gene expression, while *vice versa,* barely any of the strong changes in gene expression is recapitulated by a compartment transition.

Section 3: "*This is of relevance as the many the differentially regulated genes are genes associated with cardiomyocyte development or fate (illustrated by the GO term analysis that ranks cardiac muscle fibre as the most significantly downregulated GO term group in the mutant and the variation in key marker expression in Supp. Fig, 2A between mutant and corrected). Therefore, the differentiation process and in particular the heterogeneity of the differentiated cells should be analysed in more detail. The key differentially expressed genes should be analysed not only in differentiated cardiomyocytes but also from mutant and corrected iPSC cells and intermediates (as in Supp Fig. 2A) to exclude that differences do not arise in a small delay in differentiation. Even more importantly, the authors should address cell-*

***to cell variation and heterogeneity of both mutation-specific gene expression and differentiation by performing in situs and/or immunofluorescence analysis of key differentiation markers and mutant*".**

We appreciate the Reviewer pointing out the need to more strongly substantiate our conclusion that changes in gene expression between mutant and corrected control hiPSC-CM are not merely the result of developmental delay and/or heterogeneity. Accordingly, we have examined more closely the degree of heterogeneity during hiPSC-CM differentiation of mutant and corrected control cells by profiling the expression of key differentiation markers by flow-cytometry and immunofluorescence (**Fig. 1D**, **Fig. S2B-H**, and **Fig. S3D**; results described in the text at **lines 154-163;** also refer to the figure legend at **lines 68-79 of the Supplemental Information** for additional details). To cover all of the key developmental stages we performed such analyses at the following time points: day 0 (pluripotent cells), day 2 (mesoderm), day 5 (cardiac mesoderm), day 10 (early cardiomyocytes), day 14 (cardiomyocytes), and day 30 (maturing cardiomyocytes). Flow cytometry confirmed the expected expression of well-established differentiation markers: POU5F1/OCT4 at day 0; T/Brachyury at day 2; PDGFRA and CD56 at day 5; PDGFRA and CD82 at day 10; TNNT2/cTnT, NKX2-5, TTN/titin, and ACTC1/cardiac α-actinin at day 14; TNNI3/cardiac troponin I at day 30. Most importantly, these markers were expressed with high homogeneity, and with remarkable similarity between mutant and corrected control cells. Finally, immunofluorescence in day 14 and day 30 cardiomyocyte confirmed lack of any obvious morphological difference between mutant and corrected hiPSC-CM, as both formed well-defined sarcomeres expressing the expected key markers (TTN/titin, ACTC1/cardiac α-actinin, and TNNI3/cardiac troponin I).

Other data also support our conclusion that the mutant cells are not developmentally delayed. We profiled the expression of genes contained within lamin A/C-sensitive B compartment in hiPSC-CM that had been matured for longer time *in vitro* or in 3D-EHTs (**Fig. 8C-D** and **Fig. S5D-E**). These results confirmed that most of the examined genes, most notably *CACNA1A*, were consistently upregulated in mutant cells across these multiple stages of cardiac maturation, providing clear evidence that these could not be simply attributed to a developmental delay of mutant hiPSC-CMs. Based on the Reviewer's recommendation, we further profiled the expression of these same genes at earlier intermediate stages of hPSC-CM specification (**Fig. S5F**). These new analyses indicated that nearly all genes tested are not substantially expressed during normal cardiogenesis (expression lower than 0.1% of the housekeeping gene *RPLP0*), and that they show strongest upregulation in mutant hiPSC-CMs. This was particularly clear for *CACNA1A*, which is significantly upregulated in mutant cells only in hiPSC-CMs. We note that for other genes, such as *LRRC4B* and *PCDHGB4* there was a modest but significant upregulation in mutant cells already at earlier stages of hPSC-CM differentiation during which lamin A/C levels are very low (**Fig. 1F**). We speculate that these genes are exquisitely sensitive to lamin A/C expression, which may explain why they are among the few genes found in lamin A/C-sensitive B compartments. Overall, these additional analyses confirm that no obvious developmental delay and/or substantial heterogeneity is observed during hPSC-CM differentiation of lamin A/C haploinsufficient hiPSCs.

Minor points

Comment 1: "*Lamin B expression should be analysed and shown in panel 1F to address compensatory upregulation*".

We thank the Reviewer for bring up this important point. As discussed in detail the reply to Comment 1 from Reviewer 2, who made a similar remark, we have examined the expression of B-type lamins by RT-qPCR, western blot, and immunofluorescence (**Fig. 1F, Fig. S3E**, and **Fig. S3D**), confirming the lack of any compensatory upregulation due to lamin A/C haploinsufficiency.

Comment 2.: "*It is not clear why the authors refer to ACTA2 and CTGF as non-cardiomyocyte genes (p10 line 288) as they are also expressed in cardiomyocytes*".

We apologize for the imprecise semantics that was used in this sentence. We now rephrased this to: "*genes preferentially expressed in fibroblast and smooth muscle*" (**lines 253-254**). We hope this more clearly reflects that while these genes are indeed expressed at low levels in cardiomyocytes, they are typical of other lineages where their expression is much more abundant. Accordingly, they appear in the ontology used for the analyses as "smooth muscle" and "fibroblast" genes.

References

Dorgans, K., J. Salvi, F. Bertaso, L. Bernard, P. Lory, F. Doussau, and A. Mezghrani. 2017. Characterization of the dominant inheritance mechanism of Episodic Ataxia type 2. *Neurobiol. Dis.* 106:110–123. doi:10.1016/j.nbd.2017.07.004.

Jung, D., Y.J. Hwang, H. Ryu, M. Kano, K. Sakimura, and J. Cho. 2016. Conditional Knockout of Cav2.1 Disrupts the Accuracy of Spatial Recognition of CA1 Place Cells and Spatial/Contextual Recognition Behavior. *Front. Behav. Neurosci.* 10:214. doi:10.3389/fnbeh.2016.00214.

Leonard, A., A. Bertero, J.D. Powers, K.M. Beussman, S. Bhandari, M. Regnier, C.E. Murry, and N.J. Sniadecki. 2018. Afterload promotes maturation of human induced pluripotent stem cell derived cardiomyocytes in engineered heart tissues. *J. Mol. Cell. Cardiol.* 118:147–158. doi:10.1016/j.yjmcc.2018.03.016.

Siu, C.W., Y.K. Lee, J.C.Y. Ho, W.H. Lai, Y.C. Chan, K.M. Ng, L.Y. Wong, K.W. Au, Y.M. Lau, J. Zhang, K.W. Lay, A. Colman, and H.F. Tse. 2012. Modeling of lamin A/C mutation premature cardiac aging using patient-specific induced pluripotent stem cells. *Aging*. 4:803–822. doi:10.18632/aging.100503.

July 4, 2019

RE: JCB Manuscript #201902117R

Prof. Charles E Murry
University of Washington
Institute for Stem Cell and Regenerative Medicine
850 Republican Street
Seattle, WA 98109

Dear Prof. Murry,

Thank you for submitting your revised manuscript entitled "Chromatin compartment dynamics in a haploinsufficient model of cardiac laminopathy". You will see that both returning reviewers - and we agree - felt that you and your colleagues provided thorough and interesting responses to the reviews and that the paper is now stronger. We would be happy to publish this solid and high-quality body of work in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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Ana Pombo, PhD
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Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

Bertero et al. have addressed most of my comments. I appreciate the technical difficulties related to some of the experiments performed. Overall the quality of the manuscript has improved and I am happy with this revised version.

Reviewer #3 (Comments to the Authors (Required)):

The authors have fully addressed all my concerns