nature portfolio

Peer Review File

Disorganized chromatin hierarchy and stem cell aging in a male patient of atypical laminopathy-based progeria mandibuloacral dysplasia type A

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Jin et al study the pathomechanism driving mandibulacral dysplasia type A (MAD), an poorly characterized atypical progeroid disorder caused the p.R527C mutation in lamin A/C. Unlike typical HGPS, this pathology is not caused altered lamin A processing and so it is highly important to see if pathology arises from a similar or distinct mechanism.

The authors start by meticulously characterizing the impact of p.R527C by deriving inducible pluripotent stem cells (iPSCs) from a MAD patient, finding reprogramming ameliorates senescence-linked phenotypes and that this is reversed upon differentiation. The authors then employ genomics to investigate the origins of these p.R527C phenotypes in iPSC-derived mesenchymal stem cells (MSCs). First, RNA-seq and comparisons to published datasets revealed that MAD-MSCs enter a senescence-like state. Second, the authors use lamin A and B1 ChIP-seq to identify alterations to lamina-association which they show are partially-linked to the senescence-like gene expression changes. Finally, they deploy ATAC-seq, ChIP-seq and HiC to detect partially-correlating alterations to histone modifications, TADs and compartments. Overall, Jin et al conclude that p.R527C disrupts multiple levels of genome organisation and function to cause MAD.

The strength of the manuscript is the meticulous generation, characterization and differentiation of MAD iPSCs as well as the quality of genomics data produced from them. However, I am concerned that the authors consistently overstate their claims of causality and directness of mechanism. Specifically, the senescence-like state which MAD-MSCs cells enter into is demonstrated to involve extensive changes to lamina-association, gene expression, chromatin state and chromatin structure. As such, the chromatin changes Jin et al observe could be an indirect consequence of p.R527C inducing a senescence state. Moreover, as lamina-association is itself closely linked to chromatin state and transcription, any changes to it could be only an indirect consequence of changes to the activity of loci (rather than altered binding of p.R527C lamin A itself).

In summary, I believe Jin et al have performed an excellent characterization of the chromatin changes found in p.R527C cells. However, they overstate which of these changes (lamina-association) drives the others and this significantly undermines the manuscript. I also believe the extent of effects is often overstated. I summarise my major and minor points below.

1. There are many claims of the causal nature of altered lamina-association and other chromatin features which I believe must be removed. A few (but by no means a complete list) are below with key words highlighted for clarity;

Title – “Disorganized Chromatin Hierarchy Drives Stem Cells Aging in Atypical Laminopathy-based Progeria Mandibuloacral dysplasia type A”

Line 44 – “Topologically associating domain (TADs), and chromosome compartmentalization, that in turn contribute to the dysregulated epigenetic modifications, cell fate determination, and

“Line 229 – “HDAC4…was significantly downregulated as a consequence of gain A-LADs in MAD-MSCs…”

Line 240 – “Collectively, these data revealed that dramatic LADs reorganization in MAD-MSCs results in the dysregulation of aging-associated genes and lineage specification related genes.”

Line 265 “TBX2….was repressed in MAD-MSCs due to gained lamina-chromatin….”

Line 275 “Taken together, these data demonstrated that apart from LADs reorganization, reposition of non-LAD lamina-chromatin binding peaks also participated in transcriptional regulation that contributes to MAD-MSCs senescence and inevitably the progeroid pathogenesis.”

2. Figure 1. The authors show losses in expression for multiple proteins in MAD fibroblasts. However, as only one Wt fibroblast control is shown, it is unclear how consistent this affect is (i.e. it could simply be caused by the variance between individuals, interpedently of MAD). Likewise, the extent of reduction is unclear, particular for WRN and Ku70. Quantification of the western blot with error bars to indicate variance between replicates and multiple controls would resolve this.

Likewise, can the authors clarify if two wt-iPSC lines come from one donor or two?

3. More significantly, the nature of the replicates for all MSC experiments is unclear. Do replicates in the differentiation, RNA-seq, ChIP-seq, ATAC-seq, Hi-C experiments represent biological replicates of MSCs derived from each of the 2 wt and MAD iPSC lines? If not, how can the authors demonstrate that the observed effects are not just the product of variation between different individuals rather than the effects of MAD? Similarly, how similar are sample replicates to each other?

4. The extent of alteration to lamina-association observed in Figure 3, 4, and 6 seems limited and overstated to me. By eye, both the EDD and peak callers seems to be overly sensitive and calling differences in LADs that reflect minor (if any) differences in ChIP-seq signal (e.g. Fig3a and 4a). In the case of Figure 6J, I do not see any LAD changes at all.

Such subtle differences could easily be due to technical variation due to the variable nature of profiling Lamin-chromatin interactions. This extent of this variance is impossible to judge without a comparison of replicate variability. How similar are the replicates? Was peak calling performed on a merged replicate profile or separately on each replicate after which only consensus peaks were called?

Regardless, even if these minimal differences were not technical noise, I would argue the claim of "profound" LAD re-organisation is overstated. Indeed far more extensive LAD reorganization have been observed previously during, for example, differentiation (Peric Hupkes et al, 2010) and senescence (Chandra et al, 2015). What's more, this seems to directly contradict the claim made by the EM from a single nucleus that there is massive loss of peripheral heterochromatin. As such, I find myself very skeptical of the reported altered lamin-association and its extent.

5. Figure 6J and 7d. The authors claim the Setdb1 TAD is split in the MAD MSCs. However, again, this feels like an excessive overreach. It is hard to say with the dotted line added, but it seems like TAD structure is only weakly effected (if at all). Combined with the very minimal change to lamina-association, this makes me question if this cherry-picked example represents the extent of changes reported in panel k, for example. Similarly, for fig 7d, while the altered enhancer-promoter contact for Tgfb2 seems convincing, I cannot say the same for Cbx7 and Kdm6a.

6. In general, the manuscript while well written is very dense and has complex figures. I feel the main messages would be more accessible if some panels were removed and the text shortened. As an example, Figure 5 and its associated main text was very dense and hard to get through.

Minor points

1. Figure 1D. I may have misunderstood but I could not see HP1 in the western to show it is down regulated in MAD fibroblasts.

2. Figure 1e-g, S4 and S5. The authors claim that no differences in differentiation efficiency were observed for iPSCs and MSCs but it is hard to estimate this from images alone as there is no quantification. I believe adding this quantification would better highlight the authors beautiful and careful characterization of the cells.

3. Lines 165-170 – The authors state chromatin is "budding off from nuclei". However, are these not nuclear envelope ruptures and micronuclei? I believe it would be worth stating as such as this is an interesting finding that matches similar effects seen in HGPS.

4. Figures 6j and 7d. Could the authors remove the annotations from the HiC map and add a TAD caller track below (Fig 6j)? For figure 7d could they also add a zoom in of the contact they highlight as altered? It very hard currently to see the differences that the authors claim.

Reviewer #2 (Remarks to the Author):

This manuscript investigates the role of chromatin and gene regulation alterations in an understudied disease system: the atypical laminopathy Mandibuloacral dysplasia type A (MAD). The study uses iPSCs generated from a MAD patient with a homozygous lamin mutation and examines the phenotypes of mutant MSCs derived from these iPSCs. The study proposes that the mutant lamin leads to alterations in lamin associated domains, chromatin structure, and therefore gene misregulation of key genes, leading to mesenchymal stem cell senescence and therefore the phenotypes observed. While the scope explored in this work is admirable, throughout the paper there are numerous problems that make it difficult to draw conclusions from the presented results. These problems overall include 1) a lack of clarity about exactly how experiments were done (for example, what passage of MSCs were used?) 2) poor resolution figures and lack of labeling which makes the results hard to interpret, 3) discrepancies between figures (for example, one showing no progerin expressed in MAD and another showing high progerin levels in MAD), 4) problems in data analysis that make conclusions difficult, and 5) lack of sufficient replicates or sequencing depth which makes interpreting the genomic data very difficult as presented. Overall, while the goals of this study, the system they set up, and the scope of experiments are all very interesting, the data as presented are not sufficient to enable clear conclusions and move the field forward.

Specific concerns, issues and suggestions:

1) More carefully defining the issue with the lamin protein in these cells:

a. The authors show the R527C mutation, but did they sequence the entire LaminA gene to ensure that this was the only mutation?

b. To demonstrate that this mutation is purely an amino acid change rather than any splicing issue (like HGPS), it would be helpful if the authors could use their RNA-seq data to show the full transcript across the LMNA gene.

c. It is very confusing that Fig. S3b shows detectable progerin levels in MAD fibroblasts, when the authors earlier claim that progerin is not expressed in this condition, shown in the Western blot in Fig. S1. Finally, What does the Lamin A/C western blot look like in MAD-MSCs? Is the progerin form expressed?

d. What is the localization of the mutant Lamin A/C in the nucleus? To examine this, it will be necessary to show single central slice through the nucleus from confocal imaging. Currently, it is not clear at all what mode of imaging is used (that must be clarified in figure legends and methods), but it looks like WT LaminB and Lamin A/C are more localized to the periphery (as in Figure 1c, 2g) while mutant LaminA is not peripheral, but instead all throughout the nucleus (Figure 2g). But if these images are only epifluorescent, that is not sufficient to quantify localization. If indeed the MAD-MSCs do not properly localize their lamin A/C to the periphery, this might contribute to the extreme blebbing of their nuclei. This Lamin A/C localization should be further investigated and carefully examined with confocal microscopy Z-stacks.

2) Concerns about Western blot presentation

a. Original gels should be provided in the supplement for all Western blots (Fig1d, S2b, etc.)

b. The blots use a single loading control (B-actin) for all proteins, but these must have come from separate gels. A corresponding loading control should be shown for each separate blot.

c. The Lamin A/C panel for Figure 1d looks very underexposed compared to other blots, making it difficult to judge whether any Lamin A/C is actually expressed in the iPSCs.

3) Lack of proper data to enable comparisons between MAD fibroblasts and iPSCs

a. In lines 115-121, many protein levels are discussed as being "restored" in iPSCs, but not all show appropriate comparisons to MAD-fibroblasts. (For example, there is no indication of what HP1a or Ki67 look like in fibroblasts before reprogramming, so there is no way to judge whether the levels are altered in iPSCs).

b. The claim in line 120 that the premature senescence in MAD fibroblasts is rejuvenated in the pluripotent state only makes sense if you have first shown that MAD fibroblasts are prematurely senescent. This is not explicitly shown. Only wrinkled nuclear morphology and the downregulation of some proteins are shown as defects in MAD fibroblasts.

4) Discrepancies between images and quantification or claims

a. It is odd that Figure 1e shows 90% nuclear deformation in the quantification of VSMCs, but the image shown looks like only half the nuclei have deformations and only few of the nuclei have deformations in Fig S4f.

b. Figure S6b left panels are very saturated in the green channel making the patterns hard to observe.

c. It is really hard to judge from this one image in FigureS6a what would count as a "more diffused centromere". Could you quantify the number of puncta per nucleus and therefore include more than 3 example nuclei?

d. The authors claim that there is no change in LAP2 in MAD-MSCs, which may be true from simple average intensity, but from the images in Fig 2g, it is striking that LAP2 is focused in a few very bright small foci in MAD-MSCs rather than spread out as in WT.

e. The one EM example in figure 2f is not sufficient to show whether this loss of heterochromatin at the periphery is consistent across many nuclei or just seen for the most abnormal ones.

f. Figure S6C: p marker is significant value and is marker for senescence, so how would this be corroborated to P9 not showing a senescence phenotype?

5) Lack of details about MSC passages in the experiments shown:

a. In all the figures showing phenotypic quantifiers of the MAD-MSCs, a passage number needs to be specified. Senesence staining and DNA damage would be expected to be higher at later passage, for example.

b. Also, with the results presented in the paper, it seems as if only a single passage number is used to do the study, how are the targets mentioned changing or not changing over passages?

6) Description of and data regarding MSC differentiation into osteo/adipo/chondro lineages is lacking

a. Details need to be included in the Methods section about how the adipogenesis, osteogenesis, or chondrogenesis was induced, what passage of MSCs were used, and at what day after induction the stains were performed.

b. Figure S5b needs a paired negative control (these same stains on uninduced populations of MSCs).

c. Conceptually, it is overall very surprising that with the degree of DNA damage, senescence, and nuclear deformation that the MAD-MSCs show, they apparently differentiate equally efficiently as WT into these different lineages? HGPS MSCs are impaired in their differentiation into these lineages. The very limited amount of basic staining shown is not enough to prove that the differentiation is “fine” in MAD-MSCs. The expression of key marker genes during the timecourse of differentiation should be tracked. If the authors do not want to emphasize this angle, that is fine, but they should then remove this off-handed but not well supported comment about how these MSCs differentiate.

7) Figure 2c-e labeling and representation problems:

a. There is no scalebar for WT-MSCs in c and d and the length represented by the scalebar is not specified in the legend. Because of this scalebar issue, it is not clear whether MAD-MSC nuclei are truly larger than WT as they appear, or if that is just a zoom issue. From Figure S5, it appears that it really is true that the MAD nuclei are often 2 times larger than WT. Is this true? This should be commented on.

b. What kind of replicates are the quantifications representing? Different biological experiments or different fields from the same experiment?

c. The senescence representative images are taken at such different focal planes (one with very rounded appearance, the other more in the middle of the cells) that it is hard to judge the staining. I

see some blue in the WT but it is hard to tell the gal staining from the already bluish tinge of the image.

8) Other figure labeling and description problems:

a. Figure S6c: what do error bars represent? What internal control gene was used to normalize the qPCR? (this is particularly important since almost all the genes are increased, which could also just be more RNA in the reaction).

b. Figure S7b, what does the colorbar represent? Z score of RPKM?

c. Plots in Figure S8b and c are not described at all in terms of what they are showing (what are the connected vertical lines? What is on the x axis?)

d. Figure 3f claims to show "all expressed genes" but no genes are plotted between the -1 to 1 log2FC range. These "unchanged' genes should be plotted as well for context of how many are differentially regulated.

e. Figure S17 shows CTCF and ChIP-seq histone modification data that is so blurry as to be completely unreadable.

f. Figure S5a is not high resolution enough for readers to be able to read the percentages on the gates shown.

9) Vastly inconsistent gene expression analyses in comparison datasets make interpretation impossible

a. Comparing MAD-MSC gene expression to other disease models is a very good idea, but the datasets are processed in all kinds of different ways, which makes the comparison hard to evaluate. Some differentially expressed genes are calculated by p-value, others by q-value, others with log2 fold change >2, others >0.58. Most confusingly, DMSO_HGPS reports the top 100 average RPKM genes. This does not indicate differential expression, just highly expressed genes. It is also not clear what "GAtreat" means. Likely owing to these very different processing methods, WS_DMSO does not correlate well with WRN_KO and HGPS does not correlate well with HGPS_DMSO. Given these discrepancies, it is not clear what we can learn from the MAD profile being similar to some and not others. In fact, MAD is similar both to accelerated senescence conditions (HGPS) and alleviated (TertOE). It would be much clearer if all these datasets could be processed in the same way and analyzed together with batch correction.

b. As a control for co-enrichment in Fig 3g, the opposite from expected comparison should be done. That is, the Upregulated MAD genes should be checked for enrichment among "geroprotection" genes. And vice versa. It also seems an overstatement to call these "LAD mediated gene expression changes" when many go in the opposite direction that you would expect

(Gained LAD upregulated genes). Similarly, line 229 is overstating things to say that HDAC4 was downregulated “as a consequence of gained A-LADs”.

c. With the transcriptome data collected in this study, are there are any micro RNAs/long non coding RNAs enriched in the intronic regions along with the LADs?

10) Lamin association data needs replicates to be believable

a. Lamin ChIP can often have low/noisy signal, so the data appearance in Fig 3a is somewhat expected, but overall the differences are hard to evaluate. Some regions classified as “gained or lost” LADs look to be very minor differences in signal. A biological replicate would help clarify this.

b. The Gained A peaks and lost A peaks also are very hard to trust without clear replicates. In some cases, it is hard to see how the raw Lamin data even leads to these peaks. In Fig S13, the Myo6 figure, there is a gained A peak and a lost A peak under the same bin of LaminA signal, which shows little change between MAD and WT, so that doesn’t make sense.

11) Insufficient Hi-C data sequencing to draw conclusions at the TAD or loop level

a. The sequencing depth for the Hi-C is very low, and certainly not enough valid pairs to accurately represent the data at 10 kb resolution. 14-16 million unique valid pairs per replicate is not enough to detect TADs and loops reliably at 10 kb resolution. To reliably gain information at this resolution, there should be at least 100 million unique valid pairs, and likely much more than that.

b. Additionally, TAD callers can be very sensitive to minor variations in the data, making metrics like TAD size and shifts in TAD boundaries hard to interpret. Before TAD and loop data can be interpreted reliably, more reads are needed and then the data should be analyzed by a continuous quantitative method such as insulation score that will allow the TAD boundary strength at given locations to be compared rather than a “yes/no” answer about whether a TAD boundary exists.

c. Finally, claims about “shorter TADs” (if validated by more data) would need to be discussed further in light of what is known about gene regulatory mechanisms and TADs.

12) Comparison to other literature needed:

a. Padhiar et al., BioRxiv 2022 deals with similar work and the field would benefit from a cross comparison of results from these studies which do or don’t match (in particular, this preprint suggests impaired MSC differentiation, unlike the work currently under consideration by Jin et al.) https://www.biorxiv.org/content/10.1101/2022.08.31.504639v1.abstract

b. Perepelina et al. Cells, 2019 also investigates MSCs with this same mutation and their osteogenic potential https://www.mdpi.com/2073-4409/8/3/266#B26-cells-08-00266 . This work should be cited and discussed.

c. The sentence in abstract: “revealed an essential role for Lamin A/C in the maintenance of chromatin architecture”. Is an overstatement that doesn’t acknowledge that this was already well known before…

d. In the discussion section, a study is cited which identifies PSG4 as a key locus-- how is that locus changing with respect to this disease model?

13) Other Minor issues:

a. While the gene name LMNA should be written in all caps, LAMIN is not usually capitalized (line 76) when just referring to the type of intermediate filament.

b. Line 73: “A different mechanism as TPS” should be “A different mechanism than TPS”

c. Supp Fig 1c y axis should either be labeled “percentage” and listed as whole numbers (20, 40 60 etc.) or “fraction” and listed as shown (0.2, 0.4). Otherwise, it looks like 0.7% of cells showed abnormalities.

d. Supplementary Table Legends are needed.

Reviewer #3 (Remarks to the Author):

In this study authors put together an impressive amounts of epigenomic datasets to describe nuclear and genome alterations in Mandibuloacral dysplasia type A (MAD).

They generated iPSCs from a MAD patient with LMNA p.R527C mutation. They performed different levels of epigenome analysis on mesenchymal stem cells (MSCs) generated from iPSCs. They started with RNA-seq and integrated with ChIP of lamins, histone marks and HiC. They found a subset of genes involved in geroprotection and cell fate determination affected by chromatin remodelling.

Chromatin remodelling is one of the hallmarks of the pathological premature aging and the identification/characterization of molecular mechanisms driving this aberrant process is of interest for the scientific community. I did not understand if all analysis were done on MSCs generated by a unique iPSCs clone, but this is an important point. Considering that genome reprogramming is accompanied by chromatin remodelling the authors should have performed the study starting from at least two different iPSCs clones, to be independent on putative aberrancies generated during the cell reprogramming.

I recommend a major revision prior the publication in Nature Communication.

Criticisms:

1. Introduction: the authors stated: “The difference between TPS and APS stems primarily from the production and accumulation of progerin or prelamin A which competes with LAMIN A/C for the interaction with lamina associated proteins, including DNA damage repair-associated proteins (DNAPKcs [7], PARP1[8], TRF2[9], SIRT1[10] and SIRT6[11]) and epigenetic modifiers (RBBP4/7[12], SUV39H1[13] and HDAC2[14]), while specifically impairing the mitochondrial fitness associated PGC1α[15] and anti-oxidation related NRF2[16].” However among the epigenetic regulators the authors should have included Polycomb as described in several works: Lin YR et al., Biochim Biophys Acta Mol Cell Res. 2021; Lionetti MC et al., Biophys J. 2020 and Sebestyén E et al., Nat Comm 2020 .

2. Figure 1d: Lamin B and Lap2 are overloaded in the western blot and, although it is clear that their levels are restored in MAD derived iPSCs, I cannot appreciate differences between wt and MAD derived iPSCs.

3. Figure 2g-h: this part of the work should be improved, also considering what the authors found by ChIP-seq experiments.

ChIP-seq of histone marks (H3K27ac, H3K27me3 and H3K9me3) precipitated more genomic regions in MAD compared with wt. However the histone levels are dropped. This is possible when there is a redistribution of the histone marks along the chromatin fiber or unspecific binding that render the IF signal more diffused. For this reason, the figure 2g-h need a more accurate quantification, by segmentation of intranuclear H3K9me3 and H3K27me3 bodies. Moreover, H3K27ac should be added in the IF analysis because the authors performed H3K27ac ChIPseq assay.

4. Figure 2g-h: it is strongly suggested to perform the IF analysis in both clones of iPSCs to see when the histone remodeling take place. A western blot done in parallel on iPSCs and MAD-MSCs at distinct point of differentiation will clarify if there is a global decrease in histone amounts along differentiation or a intranuclear redistribution.

5. Figure 2g-h: the authors stated: “Although loss of nuclear structure protein LAP2a and heterochromatin associated HP1a were observed in multiple senescence conditions and MAD fibroblasts (Figure 1d), change in neither protein were observed in MAD-MSCs at p9 (Figure 2g-h)”. However in the figure LAP2a and HP1alpha seems to be less in MAD-MSCs cells. Thus, images chosen by the authors are not representative.

6. Figure S6b: the H3K9me3 immunofluorescence is overexposed.

7. Figure 3a-f and Figure 4a-f: ChIP-seq of Lamin A is very informative, but the ChIP-seq assay is dependent on Ab-antigen binding that could be affected by the MAD mutation on Lamin A. The gained LADs, that also correspond to 10% of downregulated genes in Lamin A ChIP, could be dependent on the efficiency of immunoprecipitation. The authors should show the ab binding efficiency in wt and MAD-MSCs by IP followed by western blot.

8. The number of genes in gained and lost LADs should be indicated. I can understand that not all the genes belonging to a specific gained or lost LADs will behave the same, but this should be clearly commented in the text. For example in the region od HDAC4 what about the flanking genes in that gained LAD? The authors could show the transcription reads of the entire gained or lost domains to support the link of lamin binding/transcription. I would expect an higher correlation between gained LAD/transcriptional repression rather than lost LAD/transcriptional activation, because the activation requires additional steps of activator recruitments or DNA/DNA interactions.

9. One of the controls required when working with high coverage ChIP (as lamins) is to randomize the domains and to see how many DEGs fall inside random domains. This will unequivocally demonstrate that the selected genes were specific.

10. Figure 4a-f: the tool used to call out-of-LADs peaks is not indicated.

11. Figure 6a: although the lack of interactions is very clear in MAD-MSCs the eingen vector that generate compartment analysis is very similar between wt and MAD-MSCs. In fact they found only 20% of compartment switches. Moreover, despite the higher amounts of genomic regions bound by lamins they found more B to A than A to B switches. I would not expect that all different levels of epigenome analysis are in line, but the authors should comment in the text more about not expected data.

12. Figure 6g: ChIP of CTCF should be supported by quantification of CTCF protein amounts by western blot.

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Response: Thanks for the suggestions, we have revised these statements accordingly in our new manuscript.

2. Figure 1. The authors show losses in expression for multiple proteins in MAD fibroblasts. However, as only one WT fibroblast control is shown, it is unclear how consistent this affect is (i.e. it could simply be caused by the variance between individuals, interpedently of MAD). Likewise, the extent of reduction is unclear, particular for WRN and Ku70. Quantification of the western blot with error bars to indicate variance between replicates and multiple controls would resolve this. Likewise, can the authors clarify if two WT-iPSC lines come from one donor or two?

Response: Thank you for the question. The declining expression levels of detected proteins (LMNB1, WRN and Ku70) are aging-associated. Fibroblasts derived from a normal individual (WT) and MAD patient express LMNB1 at the similar level cross different passages, without obvious change up to passage 8. However, at passage 15, LMNB1 expression dropped significantly in MAD fibroblasts (**Figure 1a-b**, below). We also quantified the expression of

WRN and Ku70 proteins in fibroblast and iPSCs (**Figure 1**, below).

The reprogramming of WT and MAD fibroblast were performed from one normal individual and one patient simultaneously. At least two clones iPSCs from each donor were generated and data were collected from both clones.

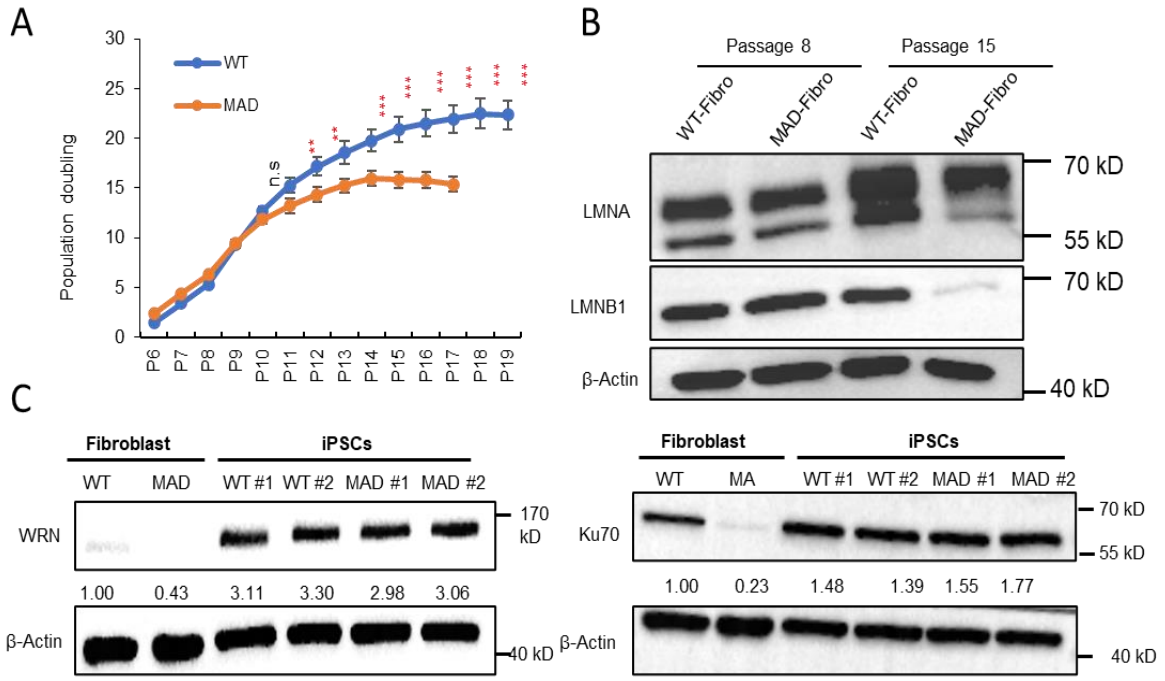

Figure 1: A, Growth curve of WT and MAD fibroblasts. Data represent the mean ± s.e.m.; n=3 (unpaired t-test). B, Accelerated loss of senescence biomarker LMNB1 in MAD fibroblasts. C, Quantification of WRN and Ku70 in MAD fibroblast and iPSCs.

3. More significantly, the nature of the replicates for all MSC experiments is unclear. Do replicates in the differentiation, RNA-seq, ChIP-seq, ATAC-seq, Hi-C experiments represent biological replicates of MSCs derived from each of the 2 WT and MAD iPSC lines? If not, how can the authors demonstrate that the observed effects are not just the product of variation between different individuals rather than the effects of MAD? Similarly, how similar are sample replicates to each other?

Response: We completely agree your points. We performed all the differentiation, including NSCs, VSMCs, VECs and MSCs, and profiling using two independent clones (**Figure 7,** below and **Extended Data Fig. 5a-b**). Moreover, the similarity of our datasets evaluated between the replicates was greater than 0.90 with Pearson correlation coefficient in all cases (**Figure 2, below**). We have included these results in the revised manuscript (**Revised manuscript, Extended Data Fig. 18**).

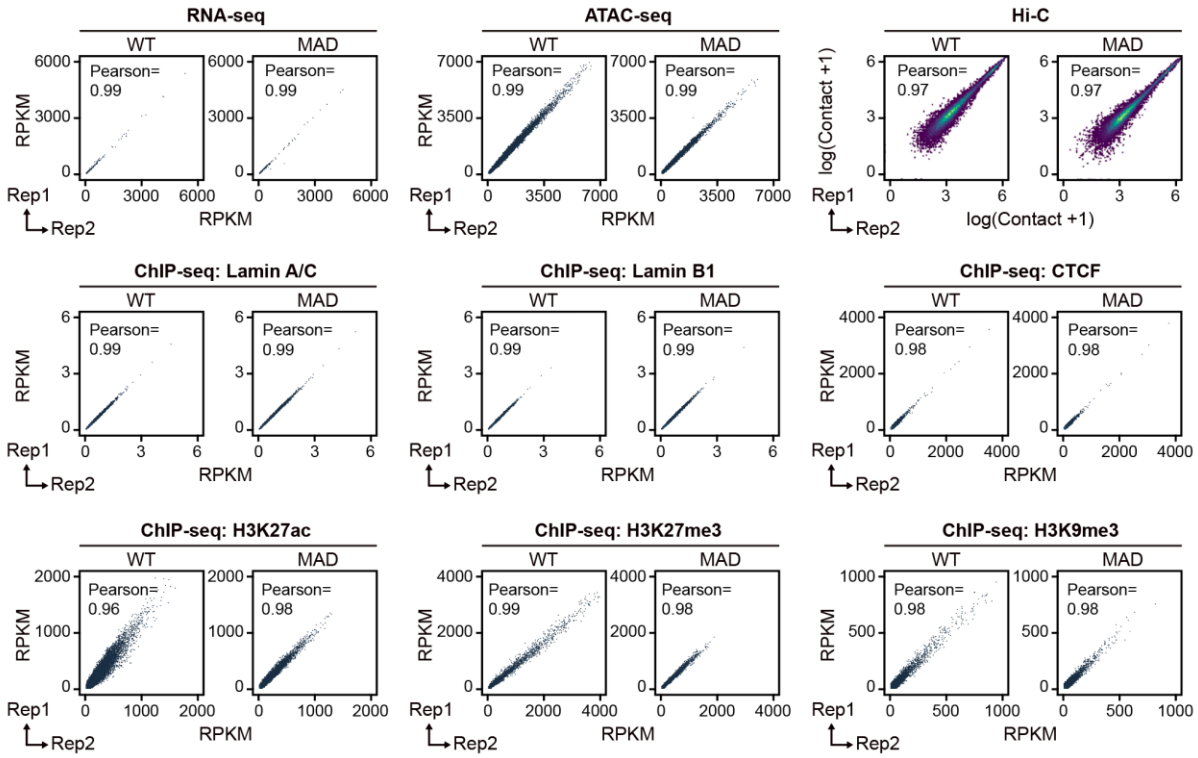

Figure 2: Similarity between replicates. Scatter plots showing overall signal changes of indicated datasets. For RNA-seq, ATAC-seq and ChIP-seq, x- and y-axis values represented RPKM (reads per kilobase million mapped reads). For ATAC-seq and ChIP-seq of CTCF, H3K27ac, H3K27me3 and H3K9me3, quantification was performed on the consensus peaks of the two replicates. For ChIP-seq of LMNA and LMNB1, quantification was performed on 100kb genomic bins.

4. The extent of alteration to lamina-association observed in Figure 3, 4, and 6 seems limited and overstated to me. By eye, both the EDD and peak callers seems to be overly sensitive and calling differences in LADs that reflect minor (if any) differences in ChIP-seq signal (e.g. Fig3a and 4a). In the case of Figure 6J, I do not see any LAD changes at all. Such subtle differences could easily be due to technical variation due to the variable nature of profiling Lamin-chromatin interactions. This extent of this variance is impossible to judge without a comparison of replicate variability. How similar are the replicates? Was peak calling performed on a merged replicate profile or separately on each replicate after which only consensus peaks were called? Regardless, even if these minimal differences were not technical noise, I would argue the claim of “profound” LAD re-organization is overstated. Indeed far more extensive LAD reorganization have been observed previously during, for example, differentiation (Peric Hupkes et al, 2010) and senescence (Chandra et al, 2015). What’s more, this seems to directly contradict the claim made by the EM from a single nucleus that there is massive loss of peripheral heterochromatin. As such, I find myself

very skeptical of the reported altered lamin-association and its extent.

Response: Thank you for the comments. Regarding the observation of LAD reorganization, our conclusions are not merely derived from visual assessments but are backed by rigorous statistical analyses. The high Pearson correlation coefficient (>0.90 **Figure 3**, below) between biological replicates underscores the reproducibility and robustness of our experimental results. The calling of LADs or peaks were performed on each replicate before calling the consensus LADs or peaks, affirming that the LADs/peaks change are not due to random variation but are consistent and reproducible findings (**Methods**).

Thank you for your feedback regarding the language used to describe the LAD reorganizations observed in our study. We appreciate your point on the potential implications of the term "profound." To ensure clarity and precision, we have revised the manuscript to describe these changes as "statistically significant alterations" in LAD configurations (**Revised manuscript**).

Lastly, we want to point out that EM images of peripheral heterochromatin cannot represent all the LADs conformation. H3K9me3 and LMNB1 were decreased at early passage (P9) (**Fig. 2g, h**), and more severe at late passage (P13), as well as HP1a. The increased coverage of LMNA LADs (**Main Fig. 3b**) aligns well with increased nuclei size and lower LMNA binding strength (**Fig.2g, h** and **3b**). In addition, the EM images of peripheral heterochromatin were taken at late stage of MSCs (P13) when LMNB1 and H3K9me3 decreased more severe.

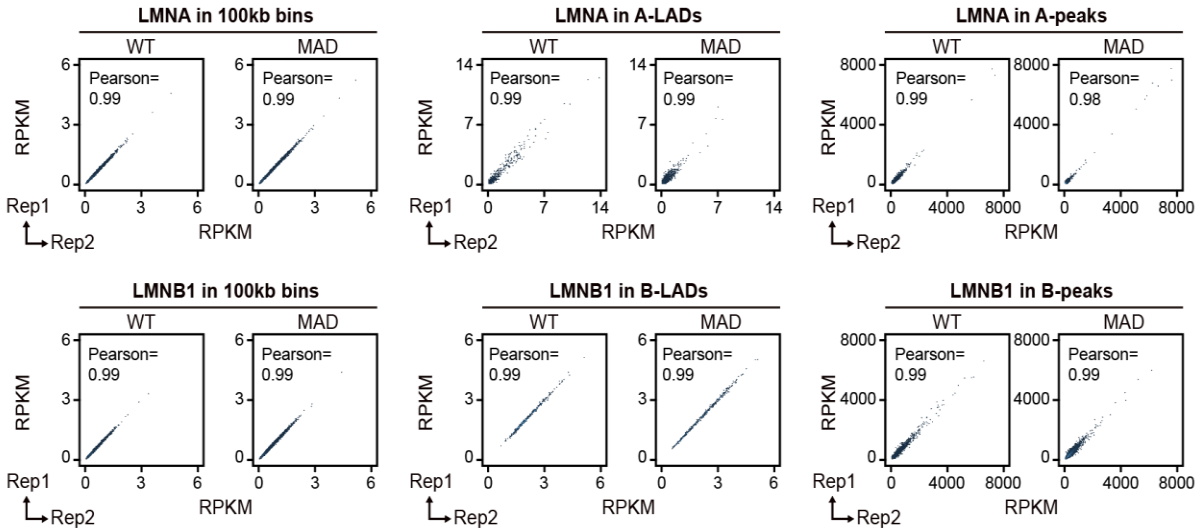

Figure 3: Similarity between replicates. Scatter plots showing overall signal changes of indicated datasets. x- and y-axis values represented RPKM. For LADs and peaks, quantification was performed on the consensus A-/B-LADs or A-/B-peaks of two replicates.

5. Figure 6J and 7d. The authors claim the SETDB2 TAD is split in the MAD MSCs. However, again, this feels like an excessive overreach. It is hard to say with the dotted line added, but it seems like TAD structure is only weakly affected (if at all). Combined with the very minimal change to lamina-association, this makes me question if this cherry-picked example represents the extent of changes reported in panel k, for example. Similarly, for fig 7d, while the altered enhancer-promoter contact for Tgfb2 seems convincing, I cannot say the same for Cbx7 and Kdm6a.

Response: Thank you for the important question. Following your valuable suggestions in the minor concerns #4, we removed the original dotted line annotations from the HiC map and add a TAD caller track below in the revised **Fig.6j** (also shown **Figure 4A** below). Besides, we added a zoom in of the altered. Contacts in the revised **Fig.7d** (also shown **Figure 4B** below). We hope these revisions will help showing the difference between WT and MAD.

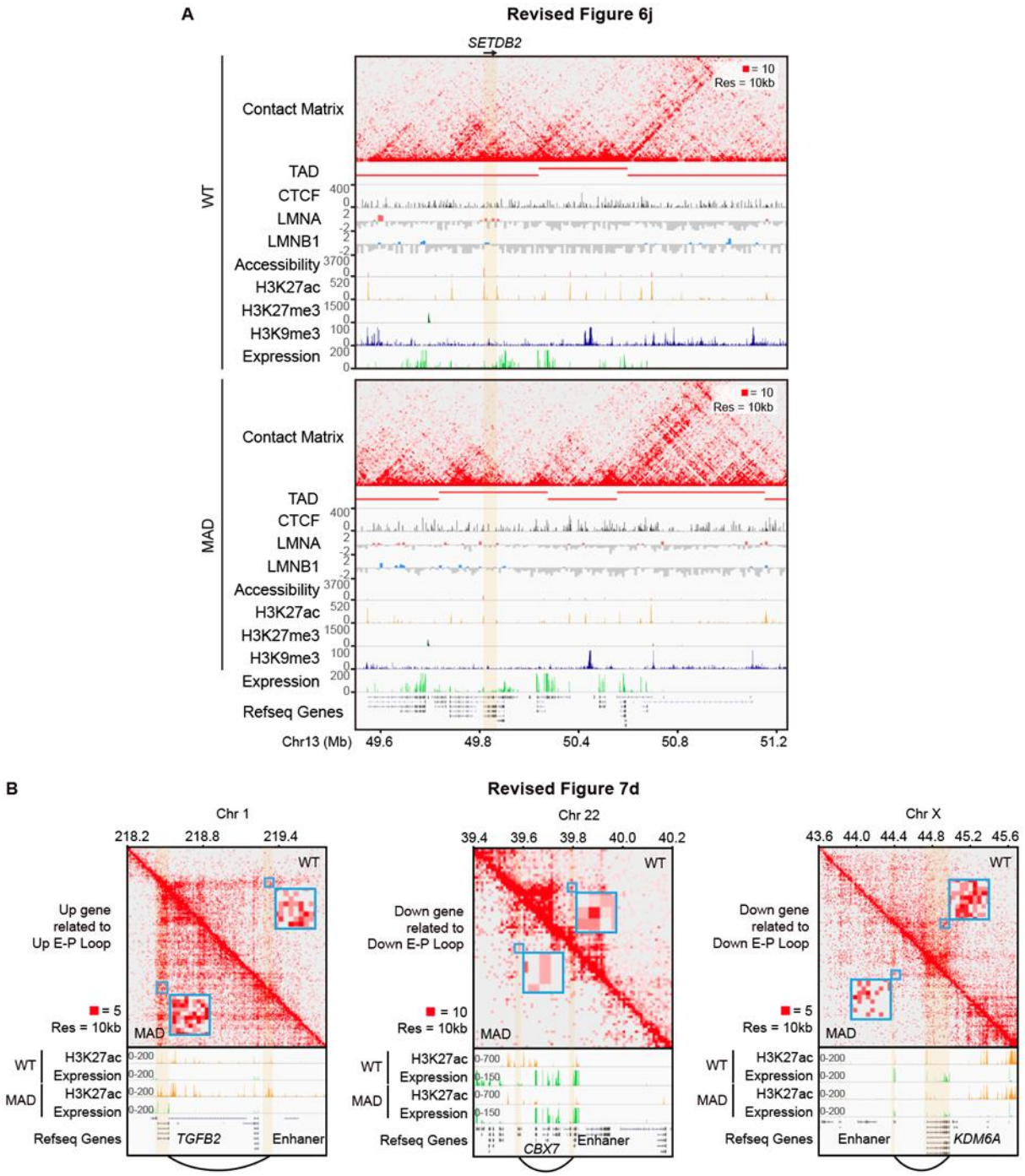

Figure 4: Revised genomic tracks. Representative genomic browser views of reorganized TADs or E-P loops in MAD. The y-axis values of LMNA or LMNB1 represented log_2RPKM (Signal vs Input). The y-axis values of accessibility, histone modifications and expression represented RPKM.

6. In general, the manuscript while well written is very dense and has complex figures. I feel the main messages would be more accessible if some panels were removed and the text shortened. As an example, Figure 5 and its associated main text was very dense and hard to get through.

Response: In the revised manuscript, we shortened the main text associated with **Fig.5** accordingly.

Minor points

1. Figure 1D. I may have misunderstood but I could not see HP1 in the western to show it is down regulated in MAD fibroblasts.

Response: Sorry for the mistakes. We added both IF and Western blotting of HP1a in the revised manuscript (revised manuscript (**Figure 1D, Extended Data Fig 1**; also shown below as **Figure 5**).

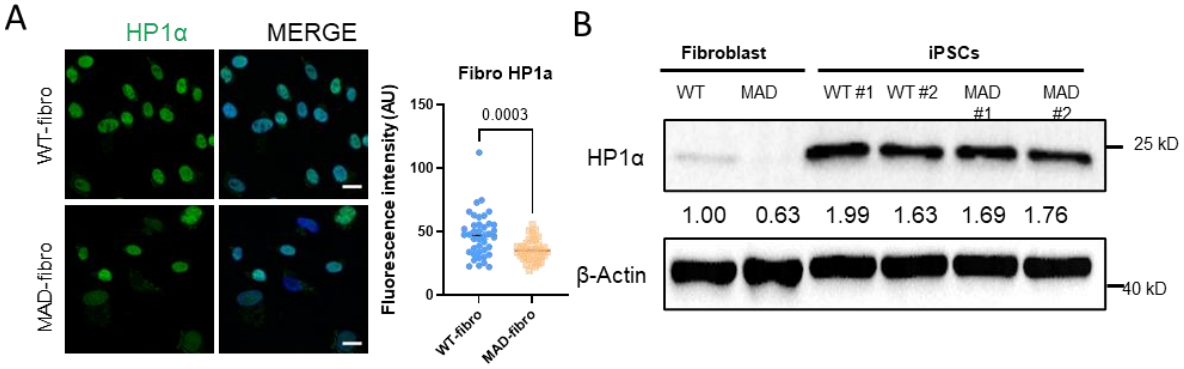

Figure 5: The expression of HP1a in MAD fibroblasts. A, Immunostaining of HP1a and quantification of fluorescence intensity in WT and MAD fibroblasts. Data represent the mean ± s.e.m.; n> 40 (unpaired t-test). B, WB of HP1a in WT, MAD-fibroblasts and iPSCs.

2. Figure 1e-g, S4 and S5. The authors claim that no differences in differentiation efficiency were observed for iPSCs and MSCs but it is hard to estimate this from images alone as there is no quantification. I believe adding this quantification would better highlight the authors beautiful and careful characterization of the cells.

Response: We added the quantification of two independent iPSCs clones' differentiation efficiency to NSCs, VSMCs, VECs and MSCs using flow cytometry (**Extended Data Fig. 4j, 7a;** also shown below as **Figure 6**).

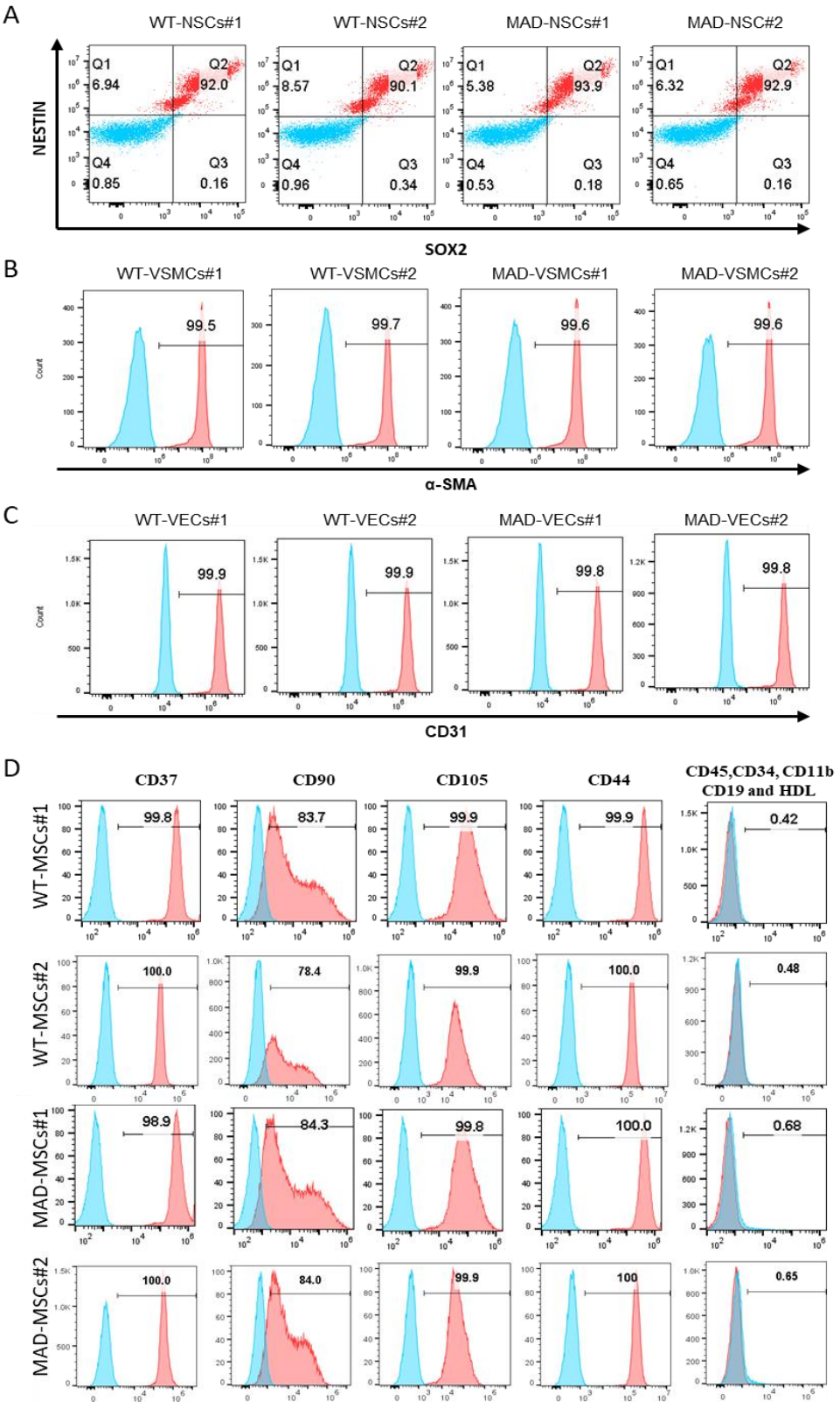

Figure 6: The quantification of iPSCs differentiation efficiency to NSCs, VMSCs, VECs and MSCs.

3. Lines 165-170 – The authors state chromatin is “budding off from nuclei”. However, are these not nuclear envelope ruptures and micronuclei? I believe it would be worth stating as such as this is an interesting finding that matches similar effects seen in HGPS.

Response: We observed both nuclear envelope ruptures and micronuclei in MAD cells. We added this statement in the revised manuscript.

4. Figures 6j and 7d. Could the authors remove the annotations from the HiC map and add a TAD caller track below (Fig 6j)? For figure 7d could they also add a zoom in of the contact they highlight as altered? It very hard currently to see the differences that the authors claim.

Response: Thank you for the suggestions. We have revised these two figures according to your advice (**revised manuscript Fig.6 and 7**, also see **Figure 4** above).

Reviewer #2 (Remarks to the Author):

This manuscript investigates the role of chromatin and gene regulation alterations in an understudied disease system: the atypical laminopathy Mandibuloacral dysplasia type A (MAD). The study uses iPSCs generated from a MAD patient with a homozygous lamin mutation and examines the phenotypes of mutant MSCs derived from these iPSCs. The study proposes that the mutant lamin leads to alterations in lamin associated domains, chromatin structure, and therefore gene misregulation of key genes, leading to mesenchymal stem cell senescence and therefore the phenotypes observed. While the scope explored in this work is admirable, throughout the paper there are numerous problems that make it difficult to draw conclusions from the presented results. These problems overall include 1) a lack of clarity about exactly how experiments were done (for example, what passage of MSCs were used?) 2) poor resolution figures and lack of labeling which makes the results hard to interpret, 3) discrepancies between figures (for example, one showing no progerin expressed in MAD and another showing high progerin levels in MAD), 4) problems in data analysis that make conclusions difficult, and 5) lack of sufficient replicates or sequencing depth which makes interpreting the genomic data very difficult as presented. Overall, while the goals of this study, the system they set up, and the scope of experiments are all very interesting, the data as presented are not sufficient to enable clear conclusions and move the field forward.

Specific concerns, issues and suggestions:

1) More carefully defining the issue with the lamin protein in these cells:

a. The authors show the R527C mutation, but did they sequence the entire Lamin A gene to ensure that this was the only mutation?

Response: Thanks for this question. The identification of this MAD mutation is by whole-exon sequencing reported before (PMID: 25286833, https://pubmed.ncbi.nlm.nih.gov/25286833/). After collected the patient sample, we further confirmed this LMNA R527C mutation.

b. To demonstrate that this mutation is purely an amino acid change rather than any splicing issue (like HGPS), it would be helpful if the authors could use their RNA-seq data to show the full transcript across the LMNA gene.

Response: We checked the RNA transcripts cross the whole *LMNA* gene locus and found no abnormal RNA splicing events (**Figure 1**, below). The same LMNA p.R527C was also reported with no LMNA splicing change in MAD. Padhiar et al., BioRxiv 2022

https://www.biorxiv.org/content/10.1101/2022.08.31.504639v1.abstract;

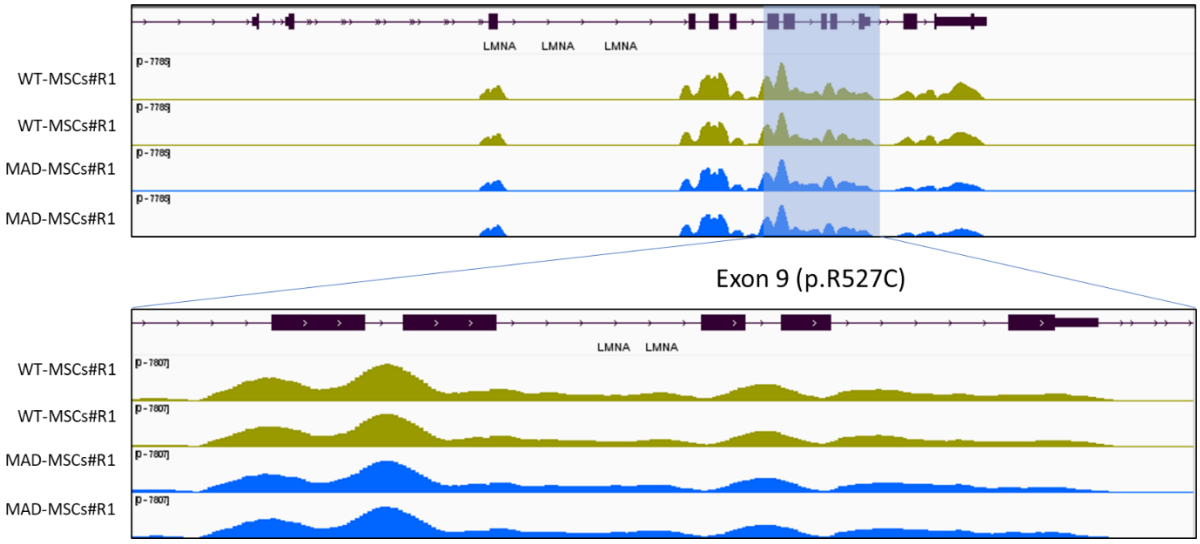

Figure 1: RNA transcripts cross the whole *LMNA* gene locus.

c. It is very confusing that Fig. S3b shows detectable progerin levels in MAD fibroblasts, when the authors earlier claim that progerin is not expressed in this condition, shown in the Western blot in Fig. S1. Finally, What does the Lamin A/C western blot look like in MAD-MSCs? Is the progerin form expressed?

Response: Apologies for the confusing presentation. Progerin is expressed in neither fibroblasts nor iPSCs derived MSCs from MAD patient as Western blotting showed no expression of progerin in MAD fibroblast or iPSCs-derived MSCs (**Fig.2B, C**). The qPCR signal is likely unspecific amplification of LMNA transcript as the two primers only differ in 2 nucleotides. The expression of progerin likely reflected the *LMNA* gene expression in fibroblasts. As LMNA gene expression is lost in iPSCs, such unspecific amplification also went down. To avoid confusing, we have deleted the data in the revised manuscript.

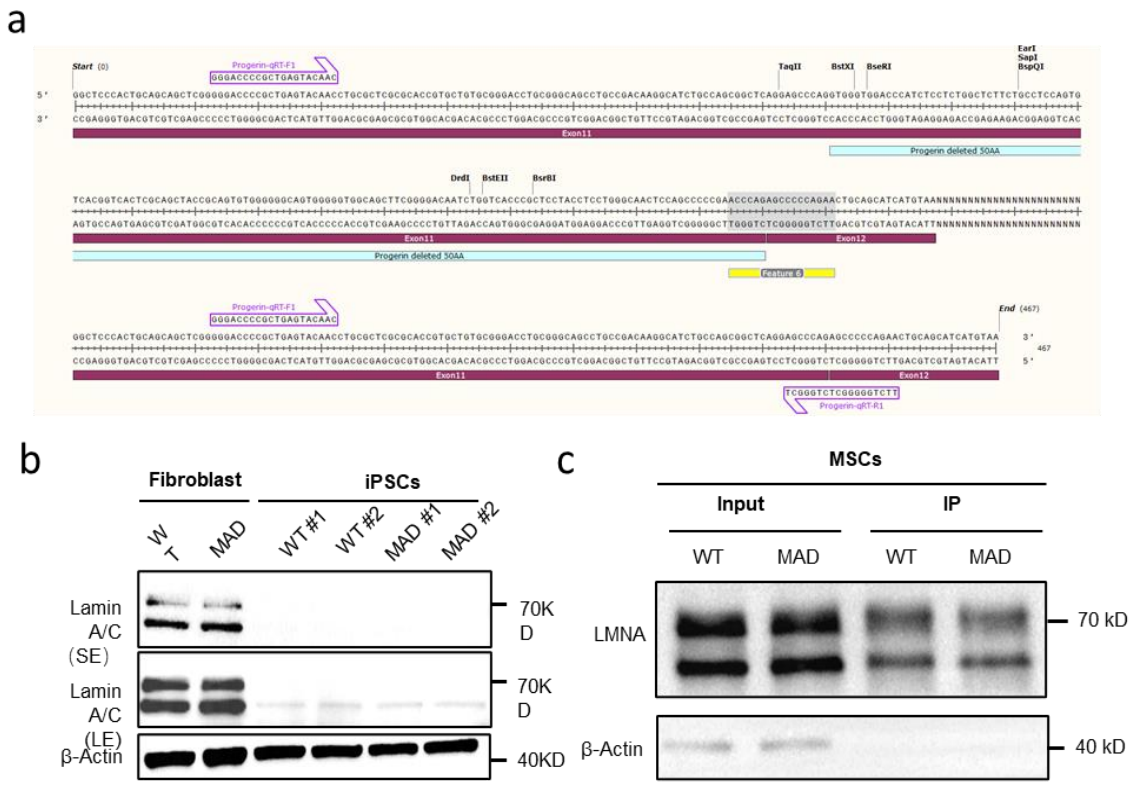

Figure 2: No progerin expression in MAD cells. A, The progerin qPCR reverse primer has potential to pair with LMNA Exon 12. When compared LMNA isoforms expression between iPSCs and fibroblast, it is possible to detect this product after normalized to iPSCs clones. B-C, Western blotting of LMNA in MAD fibroblast and iPSCs derived MSCs shows no progerin expression.

d. What is the localization of the mutant Lamin A/C in the nucleus? To examine this, it will be necessary to show single central slice through the nucleus from confocal imaging. Currently, it is not clear at all what mode of imaging is used (that must be clarified in figure legends and methods), but it looks like WT Lamin B and Lamin A/C are more localized to the periphery (as in Figure 1c, 2g) while mutant LaminA is not peripheral, but instead all throughout the nucleus (Figure 2g). But if these images are only epifluorescent, that is not sufficient to quantify localization. If indeed the MAD-MSCs do not properly localize their lamin A/C to the periphery, this might contribute to the extreme blebbing of their nuclei. This Lamin A/C localization should be further investigated and carefully examined with confocal microscopy Z-stacks.

Response: Lamin A/C mainly exists in the nuclear periphery though it can also be detected in the nucleoplasm. We have updated the Z-stacks confocal images with new antibody which shows high specificity.

2) Concerns about Western blot presentation

a. Original gels should be provided in the supplement for all Western blots (Fig1d, S2b, etc.) b. The blots use a single loading control (B-actin) for all proteins, but these must have come from separate gels. A corresponding loading control should be shown for each separate blot. c. The Lamin A/C panel for Figure 1d looks very underexposed compared to other blots, making it difficult to judge whether any Lamin A/C is actually expressed in the iPSCs.

Response: All the original gels were provided in **Supplementary Fig.2** and also shown **Figure 3** below. We have performed the WB of different targets using separate loading control referred in **Supplementary Fig.2**, but only shown a single loading in the main figure due to the limited space for figure organization. When reprogrammed back to iPSCs, LMNA expression is silenced, therefore little or no expression LMNA was found in iPSCs

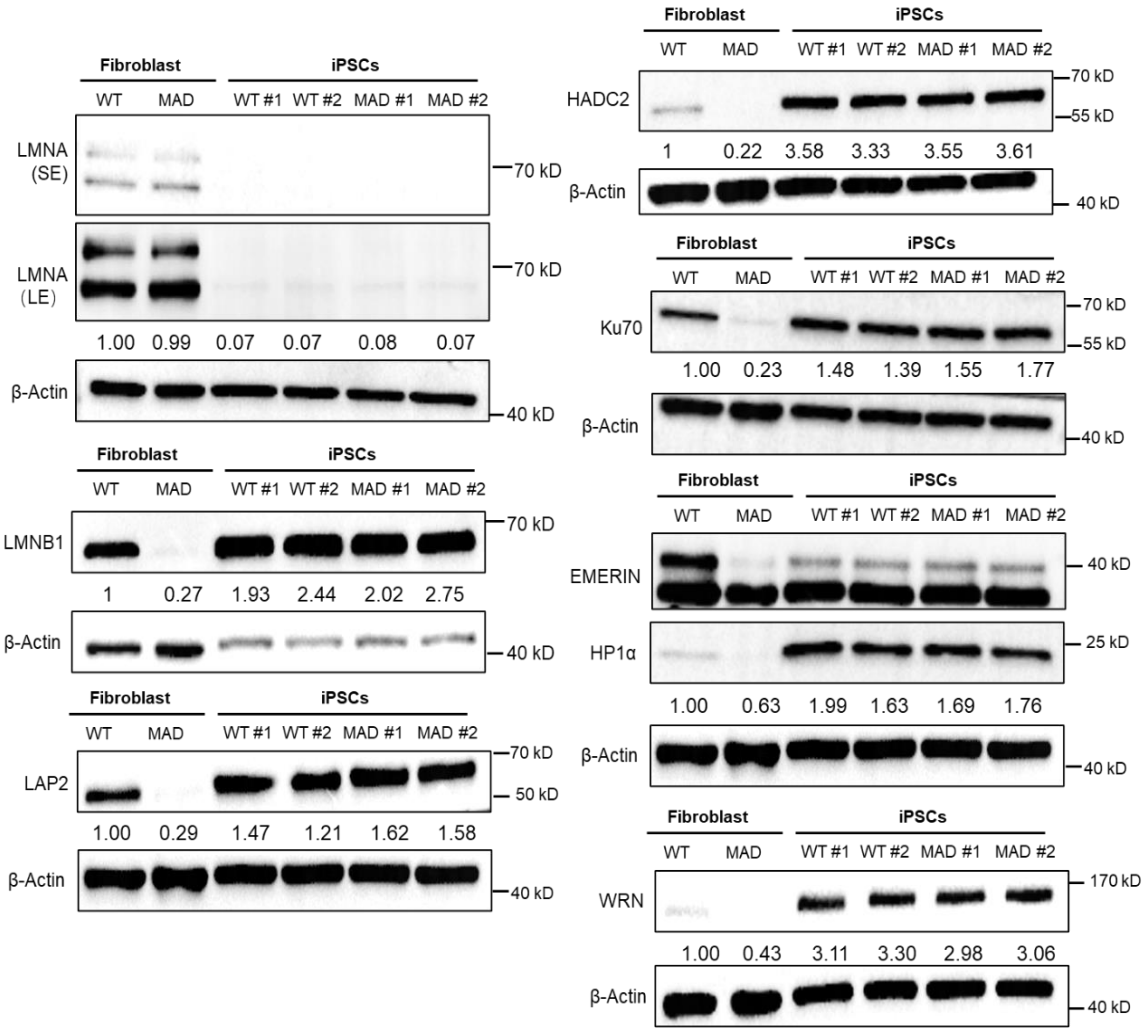

Figure 3. Western blotting with corresponding internal β-actin controls.

3) Lack of proper data to enable comparisons between MAD fibroblasts and iPSCs

a. In lines 115-121, many protein levels are discussed as being “restored” in iPSCs, but not all show appropriate comparisons to MAD-fibroblasts. (For example, there is no indication of what HP1a or Ki67 look like in fibroblasts before reprogramming, so there is no way to judge whether the levels are altered in iPSCs).

Response: We have added more data of comparison between MAD fibroblasts and iPSCs in the revised manuscript. The comparison of HP1a in IF and WB were shown in **Figure 4 a, b** (below) and in **Extended Data Figure 3a,b** in the revised manuscript, and the restored proliferative capability indicated by Ki67 was shown in **Figure 4c-d**, below.

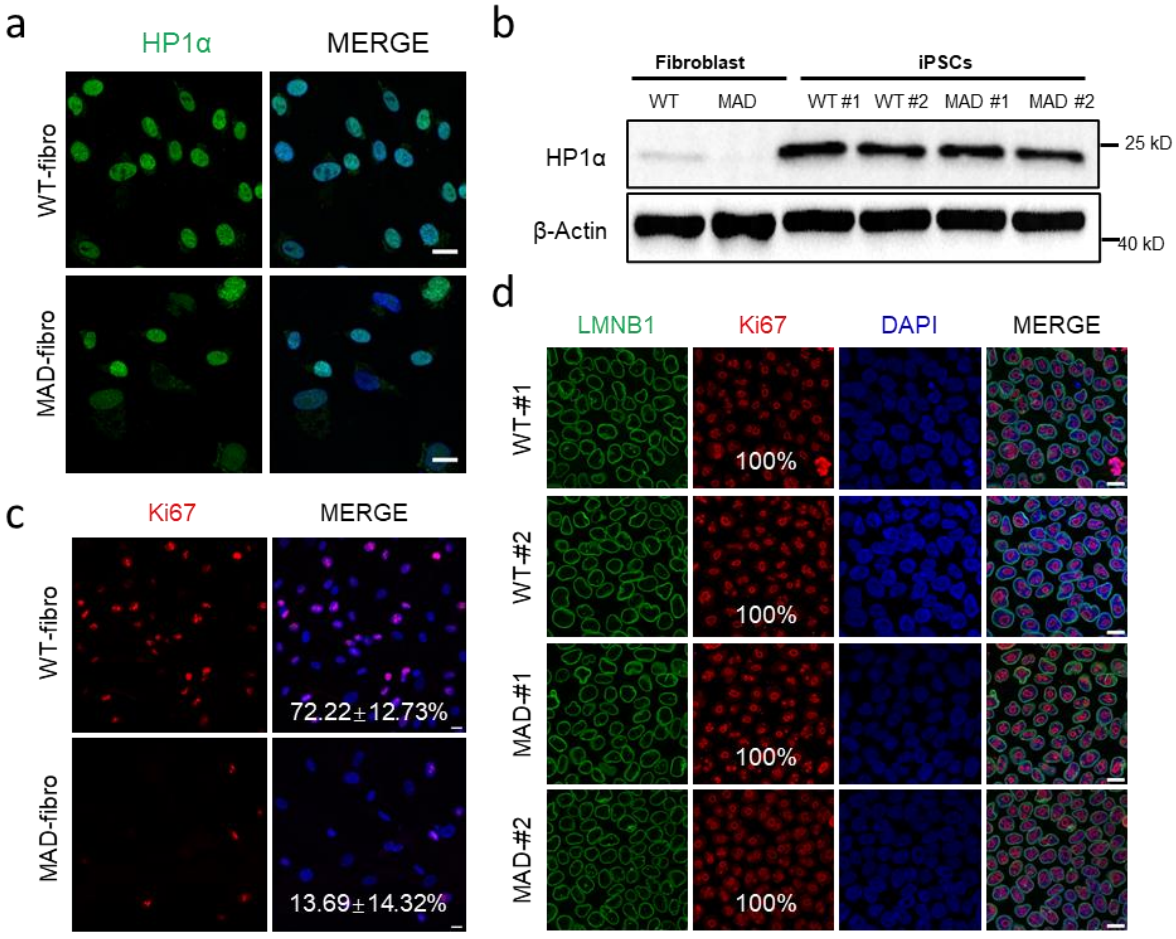

Figure 4. Comparison of HP1a or Ki67 between MAD fibroblast and iPSCs. A, IF of HP1α in WT and MAD fibroblasts. B, WB of HP1α in WT and MAD fibroblasts and iPSCs clones. C, Ki67 staining in WT and MAD fibroblasts, the number indicates percentage of Ki67 positive cells (n=3). D, Ki67 and LMNB1 co-staining in WT and MAD iPSCs clones, the number indicates percentage of Ki67 positive cells (n=3).

b. The claim in line 120 that the premature senescence in MAD fibroblasts is rejuvenated in the pluripotent state only makes sense if you have first shown that MAD fibroblasts are prematurely senescent. This is not explicitly shown. Only wrinkled nuclear morphology and the downregulation of some proteins are shown as defects in MAD fibroblasts.

Response: We have added more data of senescence including β-GAL staining, p16, p21, etc. in the revised manuscript (**Extended Data Figure 1**, also shown **Figure 5** below) in MAD and WT fibroblasts to show the premature aging defects of MAD fibroblasts.

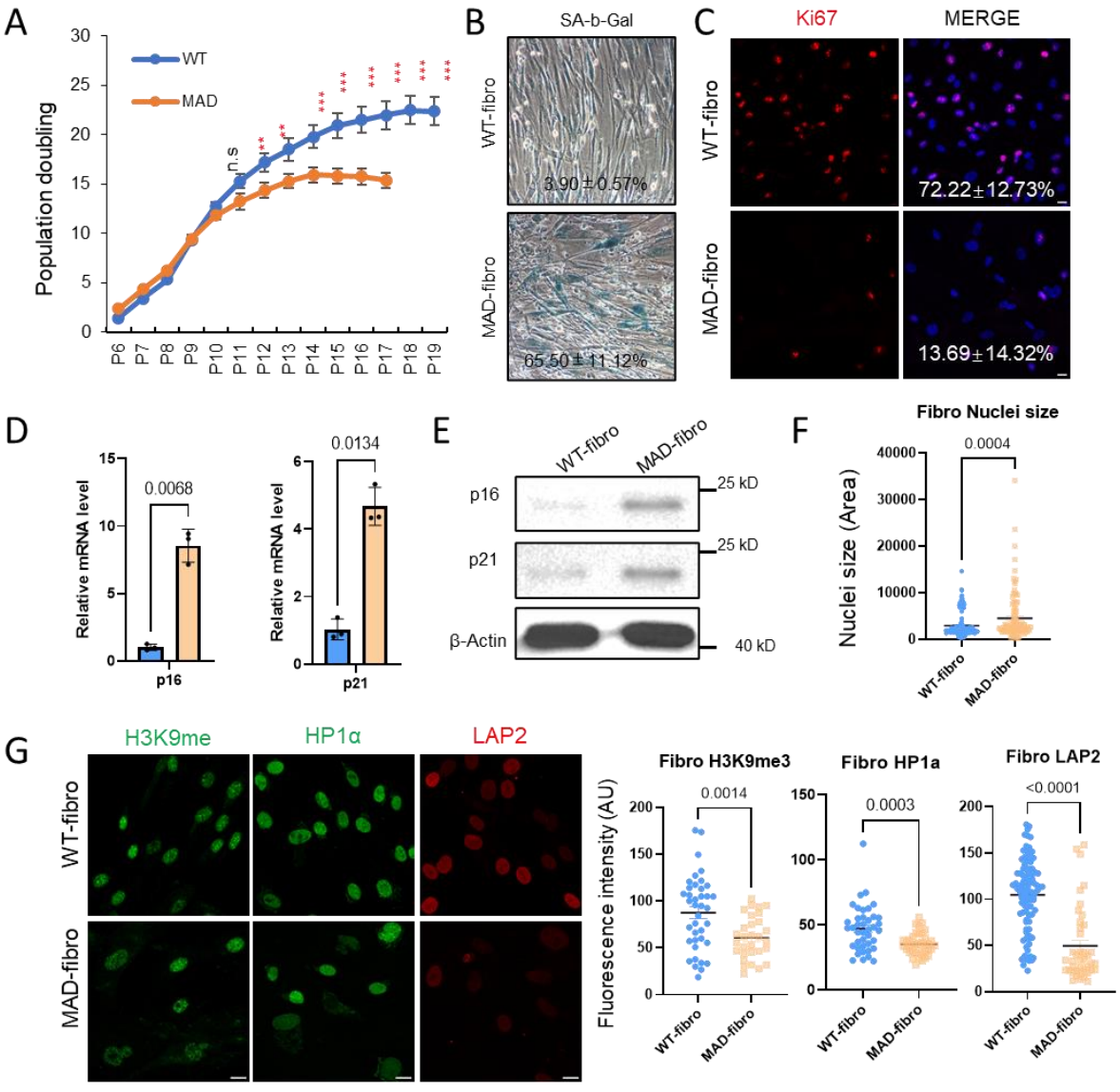

Figure 5. MAD fibroblasts manifest premature aging defects. A, Growth curve of WT and MAD fibroblasts. Data represent the mean ± s.e.m.; n=3 (unpaired t test). B, SA-β-Gal staining of WT and MAD fibroblasts, the labled number indicates SA-β-Gal positive cells. Data represent the mean ± s.e.m.; n=3. C, Ki67 staining in WT and MAD fibroblasts, the number indicates percentage of Ki67 positive cells (n=3). D, qPCR of senescence marker p16 and p21 in WT

and MAD fibroblasts. Data represent mean ± s.d., n=3; one-way ANOVA coupled with Tukey's post hoc test. E, WB of senescence marker p16 and p21 in WT and MAD fibroblasts. F, Nuclei size measurement in WT and MAD fibroblasts. n>150 (unpaired t test). G, IF and fluorescence intensity quantification of H3K9me3, HP1a and LAP2 in WT and MAD fibroblasts. n>50 (unpaired t test).

4) Discrepancies between images and quantification or claims

a. It is odd that Figure 1e shows 90% nuclear deformation in the quantification of VSMCs, but the image shown looks like only half the nuclei have deformations and only few of the nuclei have deformations in Fig S4f.

Response: Apologies for the unclear labeling. Nuclear deformation increases and becomes more severe with passages. **Extended Data Figure 4f** imaging were captured at early passage of iPSCs-derived VSMCs (P5) to demonstrate the cellular fate, while the quantification of 90% nuclear abnormality were taken at late passage (P8). We have now labelled the passage number in the figures legends to avoid confusion (**Revised manuscript, Extended Data Figure 4f legend**).

b. Figure S6b left panels are very saturated in the green channel making the patterns hard to observe.

Response: We have updated the **Extended Data Figure 6B** images in revised manuscript (also shown **Figure 6** below)

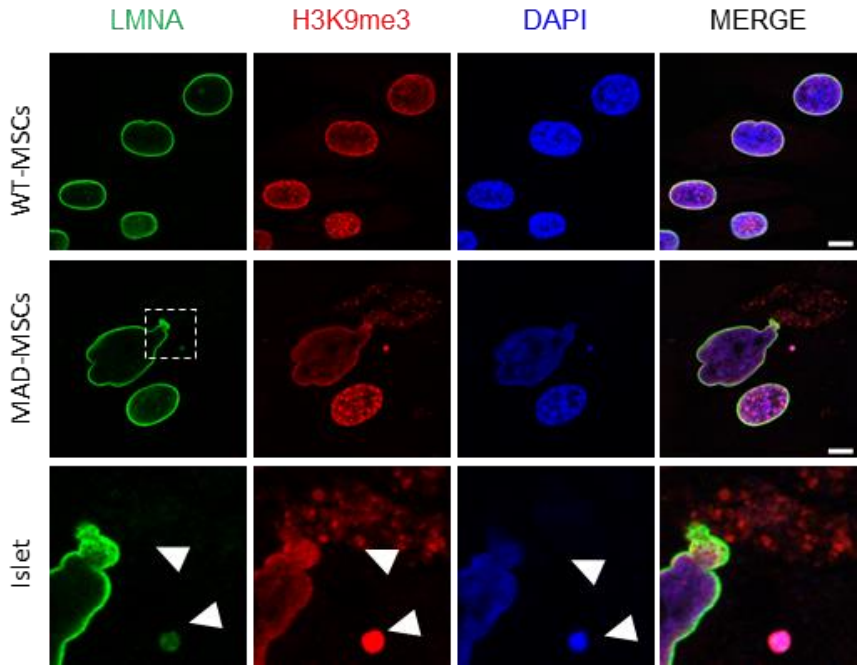

Figure 6. Co-staining of LMNA (green) and H3K9me3 (red) in passage 9 MSCs.

c. It is really hard to judge from this one image in Figure S6a what would count as a “more diffused centromere”.

Response: We initially want to emphasize the bigger nuclei of MAD-MSCs and have removed this confusing description in the revised manuscript.

d. The authors claim that there is no change in LAP2 in MAD-MSCs, which may be true from simple average intensity, but from the images in Fig 2g, it is striking that LAP2 is focused in a few very bright small foci in MAD-MSCs rather than spread out as in WT.

Response: Yes, we did observe the phenomenon that nuclear envelop associated proteins formed very bright foci at the nuclear budding region before nuclear envelop ruptures. We think that MAD mutation leads to nuclear lamina assembly problems where nuclear nicks easily appear, nuclear envelop associated proteins attends to aggregate at these regions to protect the nuclear integrity.

e. The one EM example in figure 2f is not sufficient to show whether this loss of heterochromatin at the periphery is consistent across many nuclei or just seen for the most abnormal ones.

Response: Thank you for your feedback regarding Figure 2f. To address concerns about the consistency of heterochromatin loss observed at the nuclear periphery, we have included additional EM images (**Figure 7**, below). These images were selected through a systematic random sampling process to minimize selection bias and to representatively illustrate the typical nuclear states observed. Furthermore, the loss of nuclear peripheral heterochromatin is corroborated by immunofluorescence (IF) staining and statistical analysis of proteins typically enriched in these regions, such as LAP2, HP1a, H3K9me3, and LMNB1 (**Extended Data Figure 6**). These additional data provide strong support for the phenomena observed in the EM images. We believe these additional examples and supplementary analyses convincingly demonstrate the consistency of the phenomenon across multiple nuclei.

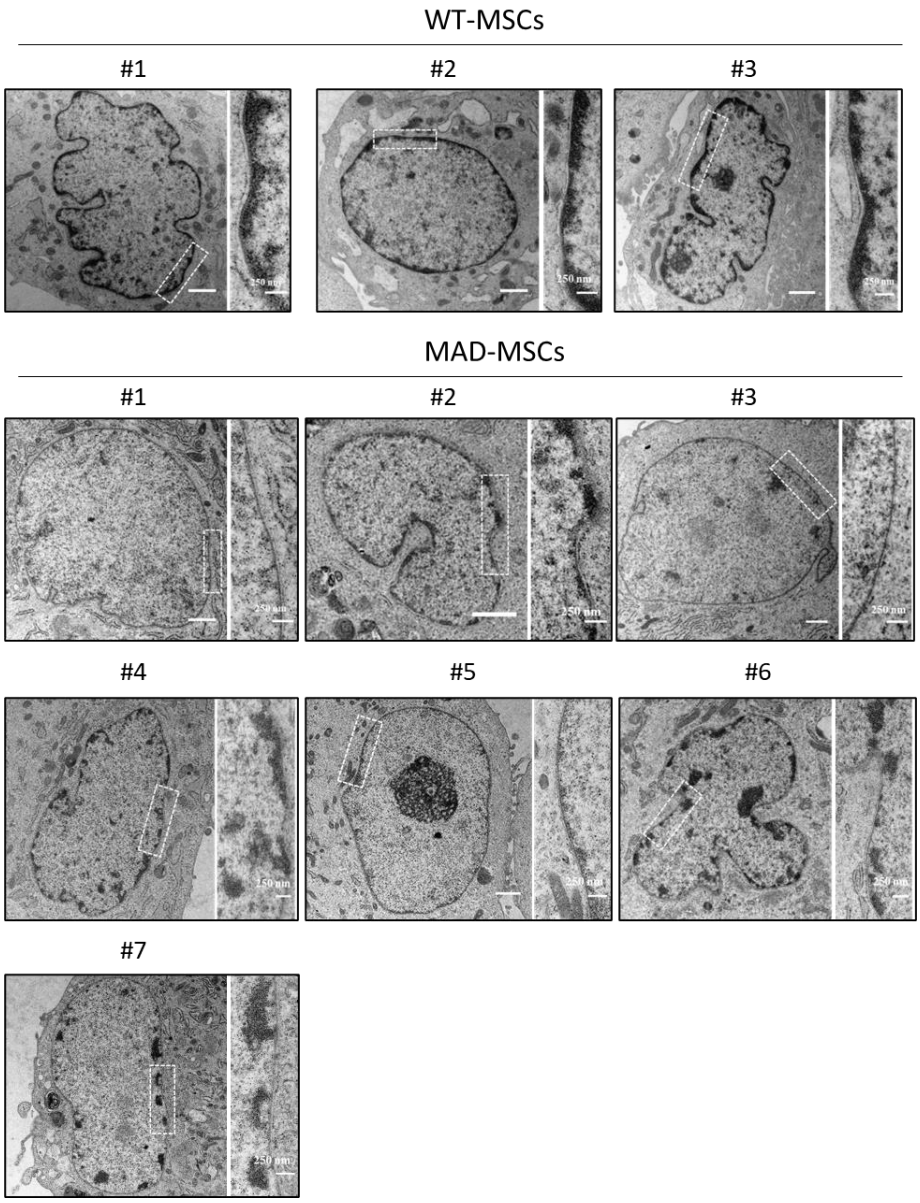

Figure 7. Electron microscopy (EM) imaging of nuclear peripheral heterochromatin. Late passage of MSCs (P13) were used.

f. Figure S6C: p16 marker is significant value and is marker for senescence, so how would this be corroborated to P9 not showing a senescence phenotype?

Response: The qPCR assay was performed using later passage of MSCs (Passage 13) indidcated in the figure legend. P9 cells were used for driving force investigation according to the MSCs growth curve, and only 6.37% cells were positive for SA- β -GAL staining (**Figure 8** below).

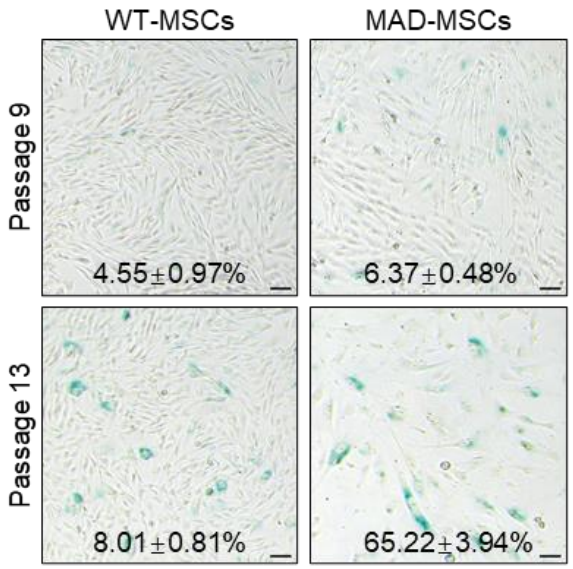

Figure 8. SA-β-Gal staining of WT and MAD MSCs at different passages, the labled number indicates SA-β-Gal positive cells. Data represent the mean ± s.e.m.; n=3.

5) Lack of details about MSC passages in the experiments shown:

a. In all the figures showing phenotypic quantifiers of the MAD-MSCs, a passage number needs to be specified. Senescence staining and DNA damage would be expected to be higher at later passage, for example.

Response: We apologize for the unclear statement and confusing caused. We have now included passage number in all figures or figure legends.

b. Also, with the results presented in the paper, it seems as if only a single passage number is used to do the study, how are the targets mentioned changing or not changing over passages?

Response: The RNA-Seq was performed at passage 9. It is expected that the geroprotection-associated genes are downregulated and senescence-associated genes are upregulated over passages.

6) Description of and data regarding MSC differentiation into osteo/adipo/chondro lineages is lacking.

a. Details need to be included in the Methods section about how the adipogenesis, osteogenesis, or chondrogenesis was induced, what passage of MSCs were used, and at what day after induction the stains were performed.

Response: We have added this information in the **revised manuscript, Methods**.

b. Figure S5b needs a paired negative control (these same stains on uninduced populations of MSCs).

Response: Though uninduced control is not necessary in MSCs differentiation assay, we nevertheless added the negative control in the revised manuscript (**Extended Data Fig. 5b**; also shown **Figure 9** below).

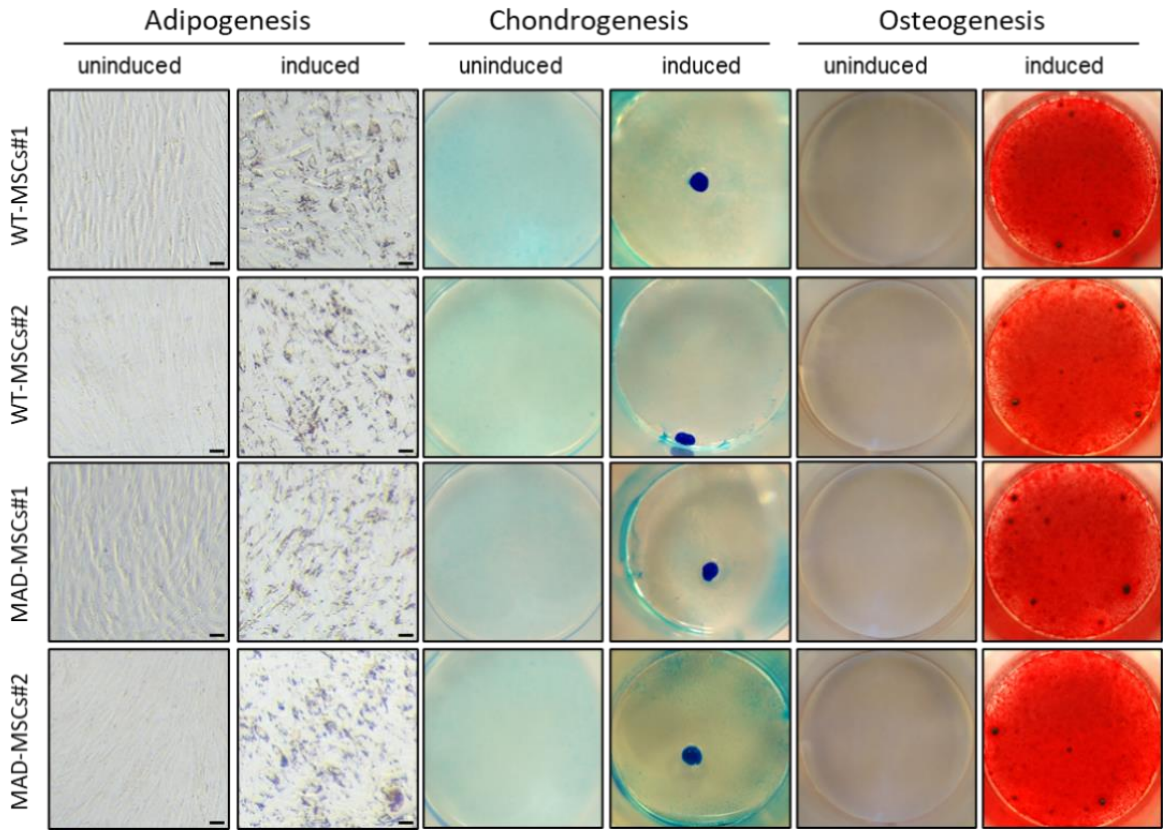

Figure 9. iPSCs derived MSCS for trilineage differentiation. Adipogenesis, osteogenesis and chondrogenesis in WT- and MAD-MSCs were examined by Oil red (Scale bars 100 μm), Alizarin red and Alcian blue staining. Uninduced cells for negative control.

c. Conceptually, it is overall very surprising that with the degree of DNA damage, senescence, and nuclear deformation that the MAD-MSCs show, they apparently differentiate equally efficiently as WT into these different lineages? HGPS MSCs are impaired in their differentiation into these lineages. The very limited amount of basic staining shown is not enough to prove that the differentiation is “fine” in MAD-MSCs. The expression of key marker genes during the time-course of differentiation should be tracked. If the authors do not want to emphasize this angle, that is fine, but they should then remove this off-handed but not well supported comment about how these MSCs differentiate.

Response: The downstream differentiation of WT and MAD-MSCs were performed at passage

3-5 to demonstrate the cellular identity, with flow cytometry data. That may explain why we do not observe differential efficiency of tri-lineage differentiation. We also believe the late passage (senescent) MAD-MSCs may exhibit impaired differentiation, but this kind of phenotypes should be the effect of cellular senescence but not the *LMNA* R527C mutation. In addition, using the early passage of HGPS-MSCs as stem cell aging model, other groups also did not observe obvious impaired differentiation (PMID: 29476423, https://pubmed.ncbi.nlm.nih.gov/29476423/;

PMID: 35292115, https://pubmed.ncbi.nlm.nih.gov/35292115/). To be more careful about this conclusion, we would like to accept your suggestion to remove the comments in the revise manuscript.

7) Figure 2c-e labeling and representation problems:

a. There is no scalebar for WT-MSCs in c and d and the length represented by the scalebar is not specified in the legend. Because of this scalebar issue, it is not clear whether MAD-MSC nuclei are truly larger than WT as they appear, or if that is just a zoom issue. From Figure S5, it appears that it really is true that the MAD nuclei are often 2 times larger than WT. Is this true? This should be commented on.

Response: We have added the scale bar to all the represented figures and we did observe bigger nuclei in MAD-MSCs (**Figure 10** below). Cellular senescence is usually accompanied by increased nuclei.

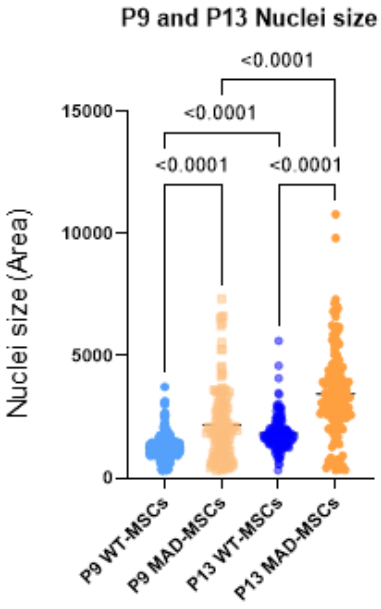

Figure 10. Nuclei size measurement of p9 and p13 WT and MAD-MSCs. n>100 (unpaired t test)

b. What kind of replicates are the quantifications representing? Different biological experiments or different fields from the same experiment?

Response: We refer biological replicates. We have clarified in the revised manuscript.

c. The senescence representative images are taken at such different focal planes (one with very rounded appearance, the other more in the middle of the cells) that it is hard to judge the staining. I see some blue in the WT but it is hard to tell the gal staining from the already bluish tinge of the image.

Response: We have replaced the images with new set of data in the revised manuscript (revised **Fig.2B;** also shown above **Figure 8**).

8) Other figure labeling and description problems:

a. Figure S6c: what do error bars represent? What internal control gene was used to normalize the qPCR? (this is particularly important since almost all the genes are increased, which could also just be more RNA in the reaction).

Response: The error bar represents expression variation of three repeats. All the genes were normalized to 18S rRNA. Additionally, these SASP genes are inflammation activation associated, and high expression levels in passage 13 MAD-MSCs align well with accelerated senescence.

b. Figure S7b, what does the color bar represent? Z score of RPKM?

Response: The color bar represented the Z score of RPKM. We have included this information in the revied manuscript (**Extended Data Figure 7b**; also see **Figure 11** below) as well as in the figure legend.

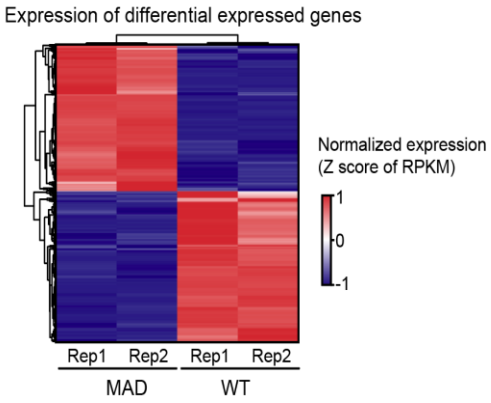

Figure 11. Expression of differential expressed genes. Heatmap showing the Z score of RPKM of differential expressed genes between MAD and WT.

c. Plots in Figure S8b and c are not described at all in terms of what they are showing (what are the connected vertical lines? What is on the x axis?)

Response: These two plots were used to show the intersection between the indicated gene sets, generated by UpSetR v1.4.0 (PMID: 28645171, https://cran.r-project.org/web/packages/UpSetR/index.html). This type of plot was a scalable alternative to Venn diagram. The connected vertical lines indicate what gene-sets are part of the intersection. The x-axis represents different gene-sets and the y-axis represents the size of the corresponding intersections (number of overlapped genes in the connected gene-sets). We have included this information into the revised manuscript (**Revised manuscript, Methods**) and figure legend of Figure S8.

d. Figure 3f claims to show "all expressed genes" but no genes are plotted between the -1 to 1 log2FC range. These "unchanged' genes should be plotted as well for context of how many are differentially regulated.

Response: We apologize for the confusion and thank you very much for the advice.

Please allow us to provide details about Figure 3f. The "expressed genes" in this figure referred to genes with average RPKM ≥ 1 in WT or RPKM ≥ 1 in MAD. The Figure 3f analyzed all the "expressed genes" in the re-organized LADs. We have revised the figure legend for further clarification.

We also summarized the number of genes in gained and lost LADs to show a global view between alterations in LADs and gene expression. As shown in the figure below, the gained LADs contained more down-regulated genes while the lost LADs contained more up-regulated genes (**Figure 12** below). We have included these results into the revised manuscript (**Revised manuscript, Extended Data Figure 9**).

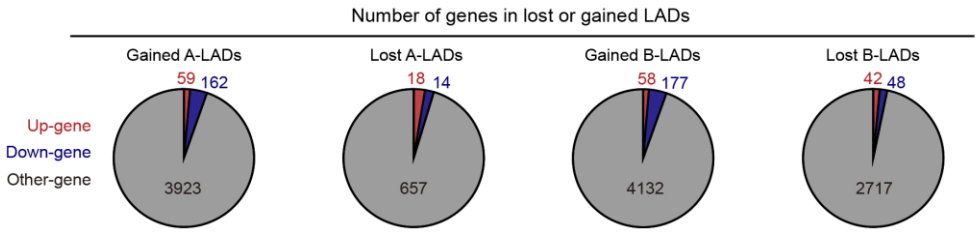

Figure 12. Link between alteration in LADs and gene expression. Pie charts showing the summary of the number of genes in the gained and lost LADs.

e. Figure S17 shows CTCF and ChIP-seq histone modification data that is so blurry as to be completely unreadable.

Response: We apologize for the low resolution of the figure. We have replaced the figures with higher resolution in the revised manuscript (see **Figure 13** below, **Revised MS, Extended Data Fig. 17d**).

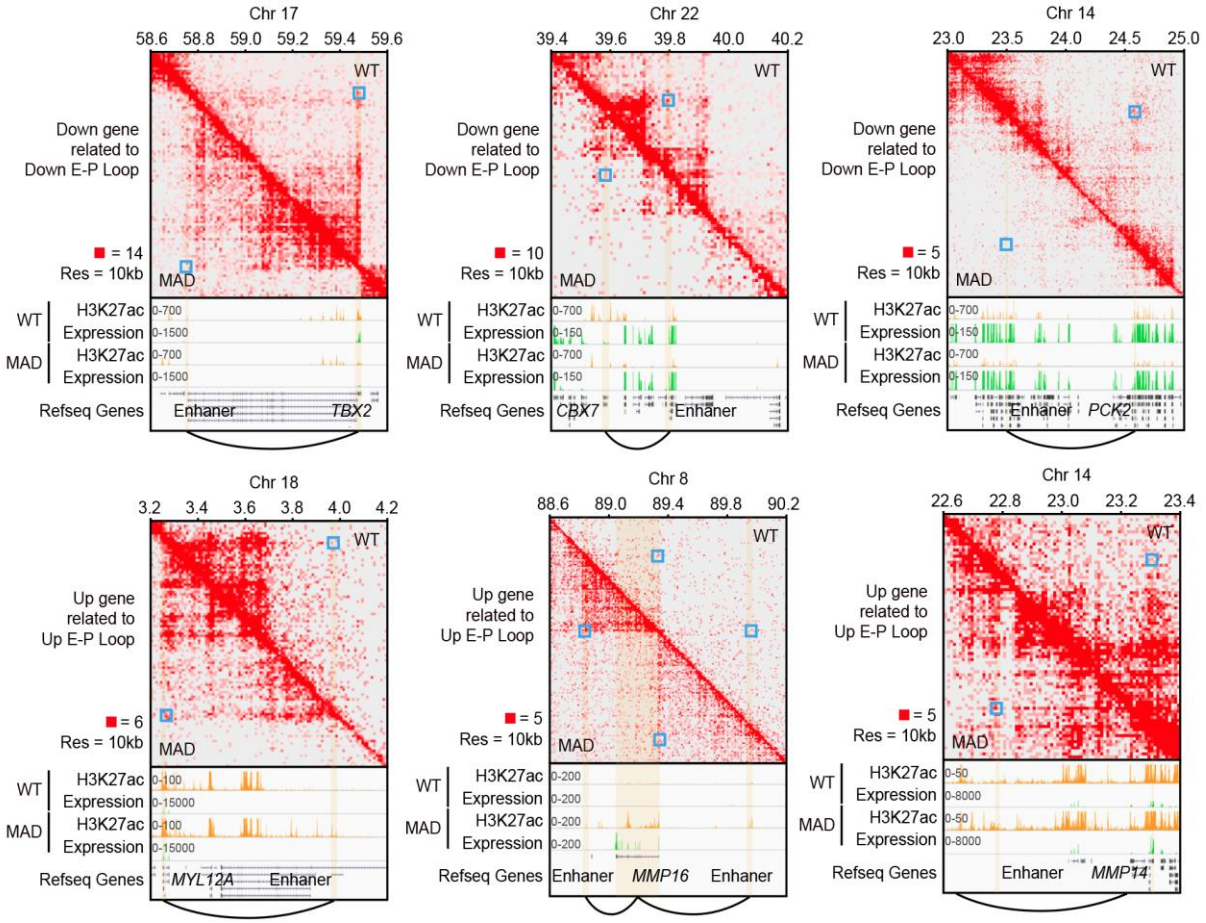

Figure 13. Revised genomic tracks. Representative genomic browser views of reorganized E-P loops in MAD. The y-axis values of accessibility, histone modifications and expression represented RPKM.

f. Figure S5a is not high resolution enough for readers to be able to read the percentages on the gates shown.

Response: We have revised the figure with the gating shown in **Figure 14** below (also **revised manuscript Extended Data Fig. 5a**).

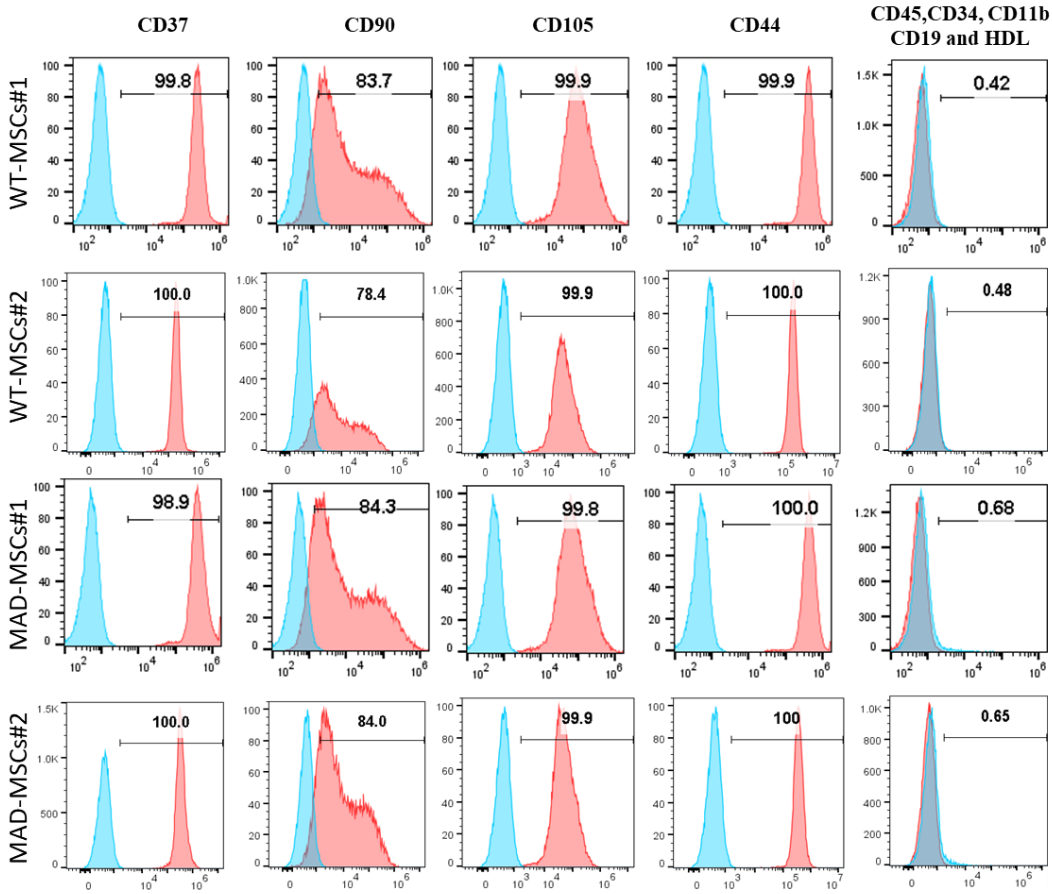

Figure 14: The quantification of iPSCs differentiation efficiency to MSCs.

9) Vastly inconsistent gene expression analyses in comparison datasets make interpretation impossible

a. Comparing MAD-MSC gene expression to other disease models is a very good idea, but the datasets are processed in all kinds of different ways, which makes the comparison hard to evaluate. Some differentially expressed genes are calculated by p-value, others by q-value, others with log2 fold change >2, others >0.58. Most confusingly, DMSO_HGPS reports the top 100 average RPKM genes. This does not indicate differential expression, just highly expressed genes. It is also not clear what "GAtreat" means. Likely owing to these very different processing methods, WS_DMSO does not correlate well with WRN_KO and HGPS does not correlate well with HGPS_DMSO. Given these discrepancies, it is not clear what we can learn from the MAD profile being similar to some and not others. In fact, MAD is similar both to accelerated senescence conditions (HGPS) and alleviated (TertOE). It would be much clearer if all these datasets could be processed in the same way and analyzed together with batch correction.

Response: Thank you very much for these important comments. We sincerely apologize for

the lack of clarity in our original manuscript. Please allow us to provide more details of the methods behind comparing MAD to the published human MSCs (hMSCs) aging models.

First, we compared MAD with the published hMSCs aging models by comparing the up-regulated and down-regulated genes in MAD with the gene lists considered as "senescence-associated genes" and "geroprotection-associated genes" in different models. Please allow us to emphasize that this comparison was between gene lists, all of which were backgrounded by all genes. The similarity between the two lists was demonstrated using hypergeometric tests, without considering the expression levels of genes in different datasets, thus minimizing the influence of batch effects.

Second, the core of the above analysis is to determine the lists of "senescence-associated genes" and "geroprotection-associated genes" in different published models. The reason for using different criteria is that those were the criteria used in the respective articles. Therefore, we directly used their analysis results and listed their criteria. For articles that did not identify relevant genes, we performed a unified analysis using the same criteria and provided detailed information in the Methods section. This is the best we can do in this analysis.

Third, "GA treat" means that the cells were treated by gallic acid (GA), which is a natural phenolic compound with antioxidant, anti-inflammatory, and antineoplastic properties. GA was reported to show beneficial effects in alleviating human mesenchymal stem cell (hMSC) senescence (PMID: 34542813).

Lastly, the low similarity in gene lists from different articles may reflect cells being treated by different treatments, especially DMSO was reported to have cellular toxicity (PMID: 29125561). Besides, it can be observed that the same phenotype of accelerated or alleviated aging can have very different expression profiles. We have toned down the statement in the revised manuscript. In all, we thank you very much for your insightful comments.

b. As a control for co-enrichment in Fig 3g, the opposite from expected comparison should be done. That is, the Upregulated MAD genes should be checked for enrichment among “geroprotection” genes. And vice versa.

Response: Thank you very much for the constructive suggestion. Following your advice, we evaluated the similarities between up-regulated MAD genes and "geroprotection-associated genes", as well as down-regulated MAD genes with “senescence-associated genes”. From the results, it can be observed that the similarities between up-regulated MAD genes and "geroprotection-associated genes" were relatively lower than those with “senescence-associated genes”, and down-regulated MAD genes were more similar to geroprotection-associated genes" (see **Figure 15** below). We have included these results into the revised

manuscript (**Revised manuscript, Extended Data Fig.8d**). Thank you again for the helpful advice.

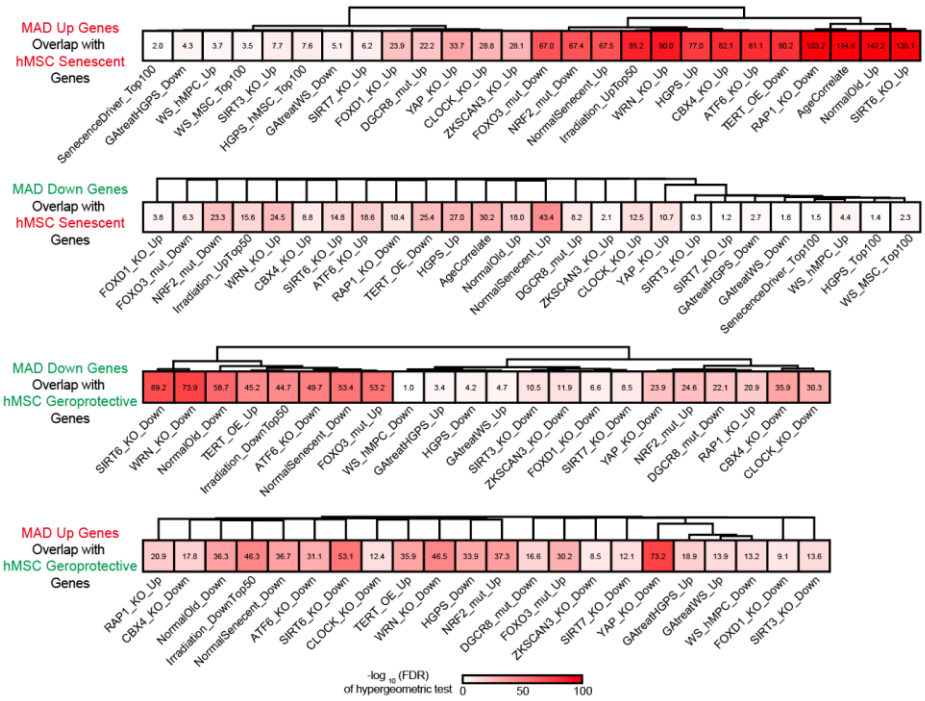

Figure 15. Cross-analysis of hMSCs aging models with MAD-MSCs. Similarities between the indicated gene lists were demonstrated using hypergeometric tests.

It also seems an overstatement to call these “LAD mediated gene expression changes” when many go in the opposite direction that you would expect (Gained LAD upregulated genes). Similarly, line 229 is overstating things to say that HDAC4 was downregulated “as a consequence of gained A-LADs”.

Response: We have toned down the statements in the revised manuscript.

c. With the transcriptome data collected in this study, are there any micro RNAs/long non-coding RNAs enriched in the intronic regions along with the LADs?

Response: Thank you for the important question. We did find that there are some microRNA and long non-coding RNAs in the LADs and some of them were differentially expressed in MAD compared to WT (see **Figure 16** below).

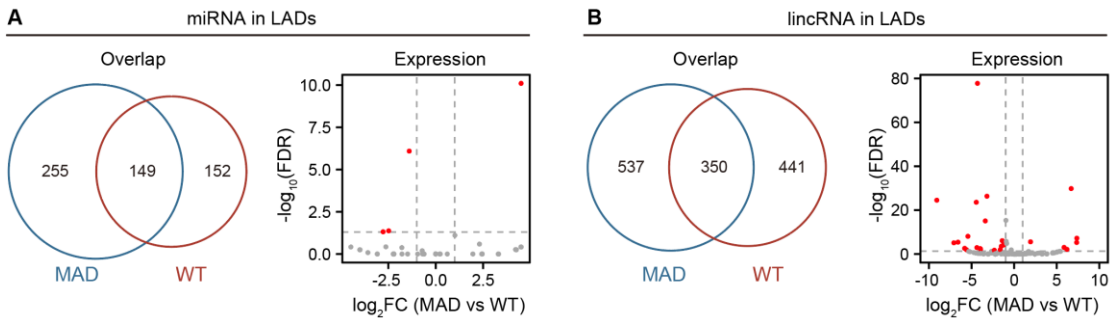

Figure 16. Expression of miRNA and lincRNA in LADs. (**A-B**) Left: Venn plots showing overlap of miRNA or lincRNA in LADs in MAD and WT. Right: Volcano plots showing the differential expression of overlapped miRNA or lincRNA in the Left.

10) Lamin association data needs replicates to be believable

a. Lamin ChIP can often have low/noisy signal, so the data appearance in Fig 3a is somewhat expected, but overall, the differences are hard to evaluate. Some regions classified as “gained or lost” LADs look to be very minor differences in signal. A biological replicate would help clarify this.

Response: Thank you for the comments and constructive suggestions.

We did have biological replicates for identifying LADs and the differential LADs. We evaluated the similarity between the two biological replicates in LADs and found that the Pearson correlation coefficient between replicates was larger than 0.99 (see **Figure 17 A** below). The calling of LADs was performed on each of the two replicates before calling the consensus LADs, and the consensus LADs were used for identifying the differential LADs. We have included this information into the Methods in the revised manuscript (Revised manuscript, Methods).

We also evaluated the differences in Lamins signals in the differential LAD regions, and observed that the signals changed as expected. The “gained LADs” showed higher Lamins signals in MAD than WT while the “lost LADs” showed lower Lamins signals in MAD than WT in both replicates (See **Figure 17B** below). These results indicated that the differential LADs indeed exhibit different Lamin signals. We have included these results into the revised Manuscript (**Revised MS, Extended Data Fig.18**)

Thank you again for the helpful suggestions.

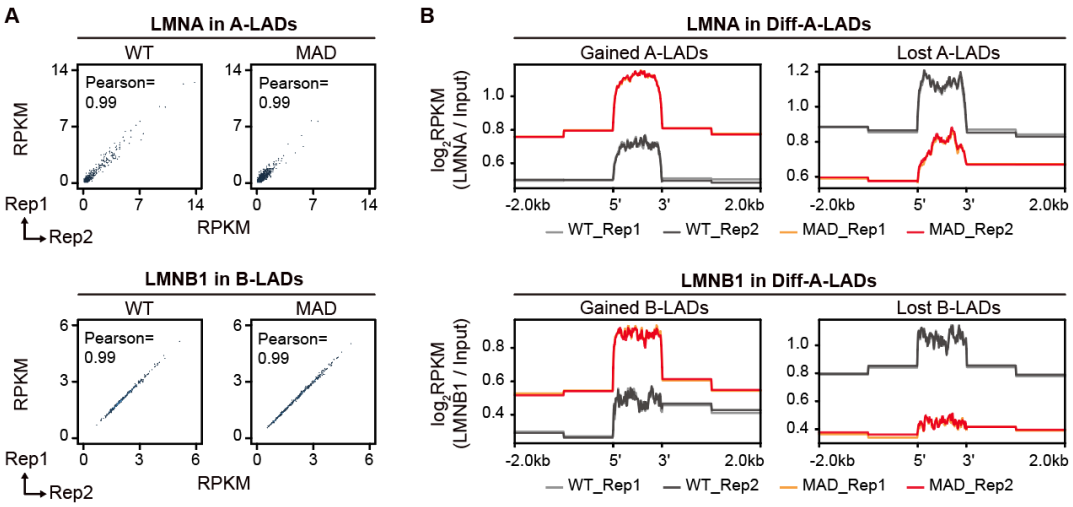

Figure 17. Similarity of Lamins signals between replicates in LADs or Diff-LADs. (**A**) Scatter plots showing overall signal changes of indicated datasets. x- and y-axis values represented RPKM. Quantification was performed on the consensus A-/B-LADs of two replicates. (**B**) Average plots showing the Lamins signals in the differential LADs. Log_2RPKM was calculated in 10kb-resolution.

b. The Gained A peaks and lost A peaks also are very hard to trust without clear replicates. In some cases, it is hard to see how the raw Lamin data even leads to these peaks. In Fig S13, the Myo6 figure, there is a gained A peak and a lost A peak under the same bin of Lamin A signal, which shows little change between MAD and WT, so that doesn't make sense.

Response: Thank you for the comments. We evaluated the **similarities** of Lamin signals between the two replicates in A/B Lamin-peaks out of LADs and found that the Pearson correlation coefficient between replicates were above 0.90 (see **Figure 18A** below). The differential Lamin peaks were determined by DiffBind from the two replicates. Peaks were considered different when FDR < 0.05. We have included this information into the Methods in the revised manuscript (**Revised manuscript, Methods**).

In addition, we evaluated the **differences** in Lamins signals in the differential Lamin-peaks, and observed that the signals changed as expected. The "gained peaks" showed higher Lamins signals in MAD than WT while the "lost peaks" showed lower Lamins signals in MAD than WT in both replicates (see **Figure 18B** below). Theres results indicated that the differential Lamin-peaks indeed exhibit different Lamin signals. We have included these results into the revised Manuscript (**Revised manuscript, Extended Data Fig.**18)

Lastly, we have replaced the original genomic tracks of Lamins in SFigure13 to those of 10-bp resolution to show the difference of Lamin peaks (see **Figure 18C** below and **revised**

manuscript, Extended Data Fig.13).

Thank you again for your insightful comments.

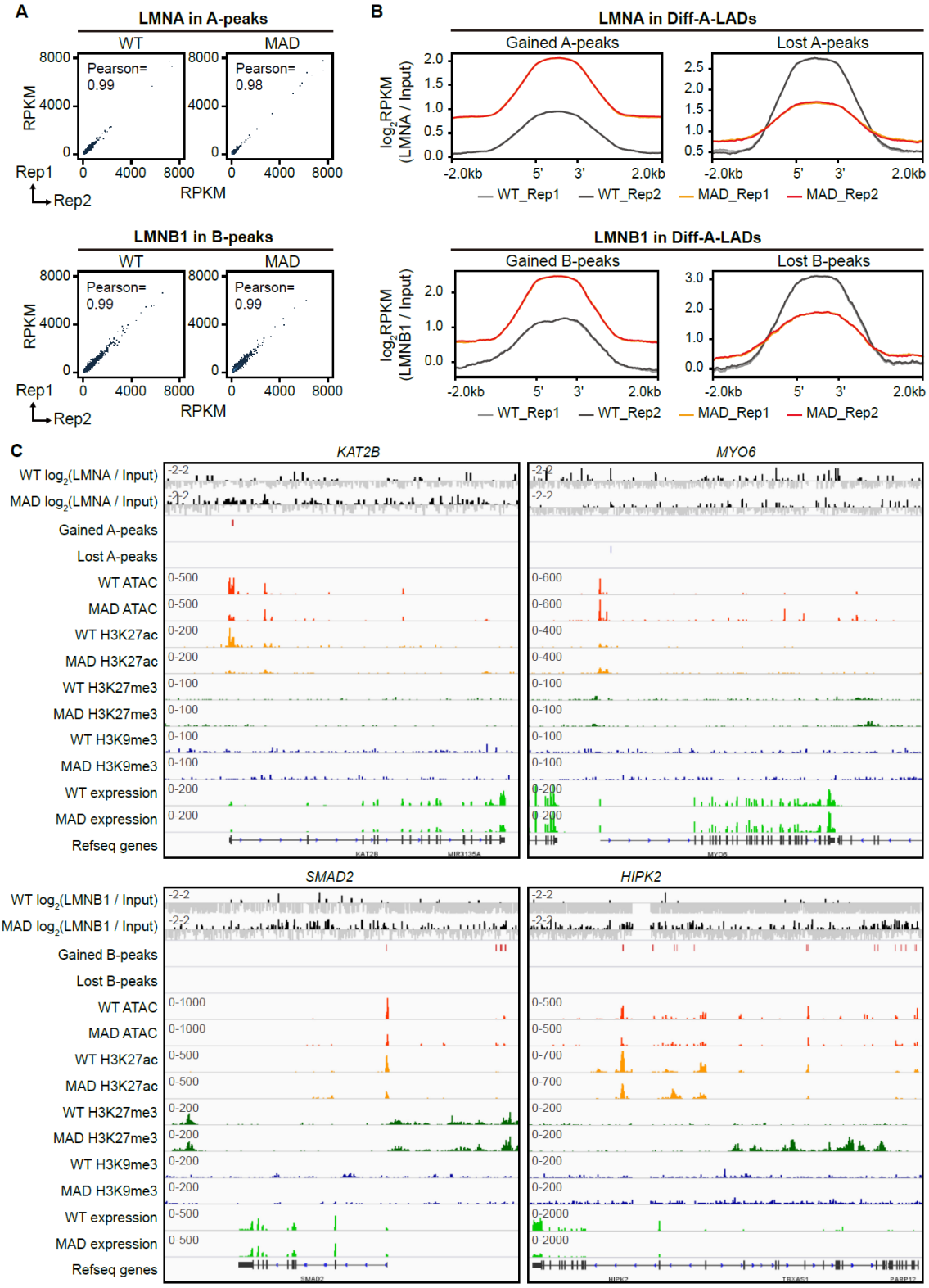

Figure 18. Similarity of Lamins signals between replicates in Lamin peaks or Diff-Lamin-peaks and example tracks. (**A**) Scatter plots showing overall signal changes of indicated datasets. x- and y-

axis values represented RPKM. Quantification was performed on the consensus A-/B-peaks of two replicates. (**B**) Average plots showing the Lamins signals in the differential Lamin-peaks. Log_2RPKM was calculated in 1kb-resolution. (**C**) Representative genomic browser views showing Lamin-peaks reorganization and chromatin features. The y-axis values of Lamins represented log2 RPKM vs input. The y-axis values of ATAC, histone modifications and expression represented RPKM.

11) Insufficient Hi-C data sequencing to draw conclusions at the TAD or loop level

a. The sequencing depth for the Hi-C is very low, and certainly not enough valid pairs to accurately represent the data at 10 kb resolution. 14-16 million unique valid pairs per replicate is not enough to detect TADs and loops reliably at 10 kb resolution. To reliably gain information at this resolution, there should be at least 100 million unique valid pairs, and likely much more than that.

Response: Thank you for the important comments. We have followed your suggestion and enhanced the Hi-C data to a resolution of 10 kb. Both the WT and MAD samples now have a total of more than 100 million valid pairs. Using this enhanced Hi-C data, we re-analyzed and validated the previous identified differential TAD boundaries and loops. Similarly, we found the difference between WT and MAD with enhanced data (see **Figure 19** below). In detail, the "gained TAD boundaries" showed lower insulation scores in MAD compared to WT, while the "lost TAD boundaries" exhibited higher insulation scores in MAD than in WT (see **Figure 19 A** below). Besides, the "up loops" demonstrated higher average contacts in MAD than in WT, while the "down loops" displayed lower average contacts in MAD compared to WT (see **Figure 19B** below). These results further validated our observations regarding TADs and loops. We have included these results in the revised manuscript (**Revised manuscript, Extended Data Fig.19**). Thank you again for the constructive advice.

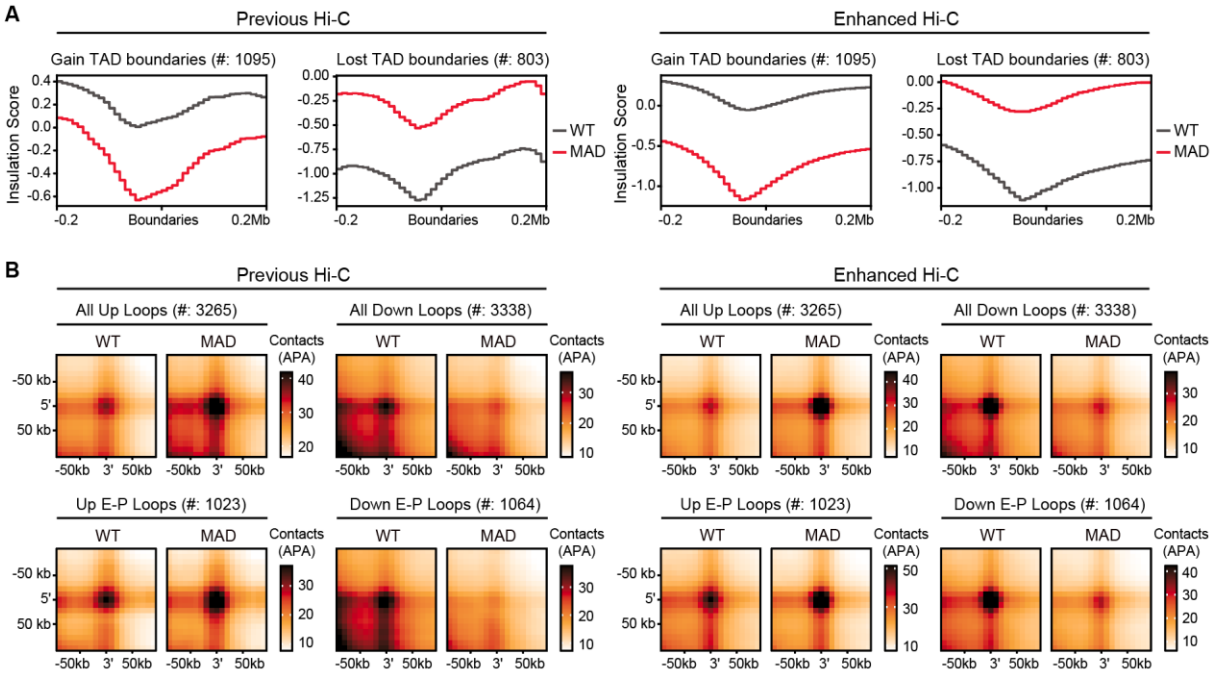

Figure 19. Validation of differential TADs and loops with enhanced Hi-C data. (**A**) Average plots of insulation scores around differential TAD boundaries. The insulation scores were calculated by GENOVA at 10kb resolution. Lower insulation score represented stronger TAD insulation. (**B**) APA analysis at 10 kb resolution showing the contacts of the indicated interactions in the indicated groups.

b. Additionally, TAD callers can be very sensitive to minor variations in the data, making metrics like TAD size and shifts in TAD boundaries hard to interpret. Before TAD and loop data can be interpreted reliably, more reads are needed and then the data should be analyzed by a continuous quantitative method such as insulation score that will allow the TAD boundary strength at given locations to be compared rather than a “yes/no” answer about whether a TAD boundary exists.

Response: Thank you for the constructive suggestions. In our analysis, the TADs were called by TopDom (PMID: 26704975; https://pubmed.ncbi.nlm.nih.gov/26704975/), which is a continuous quantitative method similar to insulation score and has been proven to be robust to resolution and sequence depth (PMID: 28334773; https://pubmed.ncbi.nlm.nih.gov/28334773/). The differential TAD boundaries were then identified using diffHiC (PMID: 26283514; https://pubmed.ncbi.nlm.nih.gov/26283514/) with two biological replicates. Only the TAD boundaries with a false discovery rate (FDR) < 0.05 were considered as reorganized. We have included these details into the revised manuscript (**Revised manuscript, Methods**).

As we mentioned in our response to your comment **11)-a**, we followed your valuable advice and enhanced the Hi-C data. We found that the “gained TAD boundaries” showed lower insulation scores in MAD compared to WT, while the “lost TAD boundaries” exhibited higher

insulation scores in MAD than in WT (see **Figure 19A** above). These results further supported the reliability of our conclusion regarding TAD reorganization in MAD. We have included these results in the revised manuscript (**Revised manuscript, Extended Data Fig.19**).

Thank you again for the important suggestions.

c. Finally, claims about "shorter TADs" (if validated by more data) would need to be discussed further in light of what is known about gene regulatory mechanisms and TADs.

Response: Thank you for your valuable comment. We tried to discuss this issue. We observed relatively higher expression levels within the shortened TADs (**Revised manuscript, Fig.7i**). In principle, the enlarged nuclei of MAD-MSCs due to abnormal nuclear lamina (**Revised manuscript, Extended Data Fig.5c**) were associated with genome-wide chromatin relaxing and TAD shortening. The relatively higher gene expression within the shortened TADs may be attributable to their relatively higher level of accessibility (**Revised manuscript, Fig. 7i**). This observation is consistent with previous studies showing that ESCs have shorter TADs (PMID: 29053968; https://pubmed.ncbi.nlm.nih.gov/29053968/), accompanied by a more open genome (PMID: 25768910; https://pubmed.ncbi.nlm.nih.gov/25768910/) and higher gene expression. Moreover, it has been reported that the same gene exhibits higher expression within smaller TADs. Specifically, the expression of *Tsix* was found to be higher in the allele with the smaller TAD (PMID: 24813616; https://pubmed.ncbi.nlm.nih.gov/24813616/). We have included these discussions in the revised manuscript.

12) Comparison to other literature needed:

a. Padhiar et al., BioRxiv 2022 deals with similar work and the field would benefit from a cross comparison of results from these studies which do or don't match (in particular, this preprint suggests impaired MSC differentiation, unlike the work currently under consideration by Jin et al.)
https://www.biorxiv.org/content/10.1101/2022.08.31.504639v1.abstract

Response: Thanks for the suggestion. The differentiation of WT and MAD-MSCs in our study were performed at passage 3-5 to demonstrate the cellular identity, accompanied by flow cytometry data. Late passage (senescent) MAD-MSCs should exhibit impaired differentiation. Therefore, this kind of phenotypes should be carefully interpreted as the defects may be related with senescence itself. Indeed, patients with HGPS or MAD develop normally to term and only exhibit developmental defects 12 months after birth. As no detailed information available in their manuscript, we guess the differences may resulted from cell passages used between our experiments and their experiments. In addition, using the early passage of HGPS-MSCs as stem cell aging model, other groups did not observe obvious impaired differentiation (PMID:

29476423, https://pubmed.ncbi.nlm.nih.gov/29476423/;

PMID: 35292115, https://pubmed.ncbi.nlm.nih.gov/35292115/). To be more careful about this conclusion, we have removed all the discussion part of MAD-MSCs differentiation in the revised manuscript.

b. Perepelina et al. Cells, 2019 also investigates MSCs with this same mutation and their osteogenic potential https://www.mdpi.com/2073-4409/8/3/266#B26-cells-08-00266. This work should be cited and discussed.

Response: Thanks for this comment. We must point it out that MAD is caused by the recessive mutation and forced expression of mutant lamin A/C isoform in WT cells to manifest cellular defects is debatable. Considering the various phenotypes may be observed using different passages of MAD-MSCs, we have removed all the discussion part of MAD-MSCs differentiation in the revised manuscript.

c. The sentence in abstract: “revealed an essential role for Lamin A/C in the maintenance of chromatin architecture”. Is an overstatement that doesn’t acknowledge that this was already well known before…

Response: Thanks for this comment. We add references following this sentence.

d. In the discussion section, a study is cited which identifies PSG4 as a key locus-- how is that locus changing with respect to this disease model?
Response: We did not observe a significant change in chromatin structure in PSG4 locus.

13) Other Minor issues:

a. While the gene name LMNA should be written in all caps, LAMIN is not usually capitalized (line 76) when just referring to the type of intermediate filament.

Response: Thanks for the comment. We have changed this accordingly in the revised manuscript.

b. Line 73: “A different mechanism as TPS” should be “A different mechanism than TPS”

Response: Thanks for the correction. We have corrected this sentence accordingly.

c. Supp Fig 1c y axis should either be labeled “percentage” and listed as whole numbers (20, 40 60 etc.) or “fraction” and listed as shown (0.2, 0.4). Otherwise, it looks like 0.7% of cells showed abnormalities.

Response: Thanks for the comments. We have changed the labeling accordingly.

d. Supplementary Table Legends are needed.

Response: we have added the table legends accordingly.

Reviewer #3 (Remarks to the Author):

In this study authors put together an impressive amount of epigenomic datasets to describe nuclear and genome alterations in Mandibuloacral dysplasia type A (MAD).

They generated iPSCs from a MAD patient with LMNA p.R527C mutation. They performed different levels of epigenome analysis on mesenchymal stem cells (MSCs) generated from iPSCs. They started with RNA-seq and integrated with ChIP of lamins, histone marks and HiC. They found a subset of genes involved in geroprotection and cell fate determination affected by chromatin remodeling.

Chromatin remodeling is one of the hallmarks of the pathological premature aging and the identification/characterization of molecular mechanisms driving this aberrant process is of interest for the scientific community. I did not understand if all analysis were done on MSCs generated by a unique iPSCs clone, but this is an important point. Considering that genome reprogramming is accompanied by chromatin remodeling the authors should have performed the study starting from at least two different iPSCs clones, to be independent on putative aberrancies generated during the cell reprogramming.

I recommend a major revision prior the publication in Nature Communication.

Criticisms:

1. Introduction: the authors stated: “The difference between TPS and APS stems primarily from the production and accumulation of progerin or prelamin A which competes with LAMIN A/C for the interaction with lamina associated proteins, including DNA damage repair-associated proteins (DNAPKcs [7], PARP1[8], TRF2[9], SIRT1[10] and SIRT6[11]) and epigenetic modifiers (RBBP4/7[12], SUV39H1[13] and HDAC2[14]), while specifically impairing the mitochondrial fitness associated PGC1α[15] and anti-oxidation related NRF2[16].” However, among the epigenetic regulators the authors should have included Polycomb as described in several works: Lin YR et al., Biochim Biophys Acta Mol Cell Res. 2021; Lionetti MC et al., Biophys J. 2020 and Sebestyén E et al., Nat Comm 2020.

Response: Thanks for this suggestion, these references are now included in the revised manuscript.

2. Figure 1d: Lamin B1 and Lap2 are overloaded in the western blot and, although it is clear that their levels are restored in MAD derived iPSCs, I cannot appreciate differences between WT and MAD derived iPSCs.

Response: The declining of Lamin B1 and LAP2 is aging-associated and we found

decreased lamin B1 and LAP2 in senescent MAD-fibroblast. The expression of Lamin B1 and LAP2 restored upon reprogramming regardless of LMNA mutation as LMNA is not expressed in iPSCs. Quantification of LMNB1 and LAP2 by IF and WB did not show obvious difference in iPSCs state (**Figure 1** below).

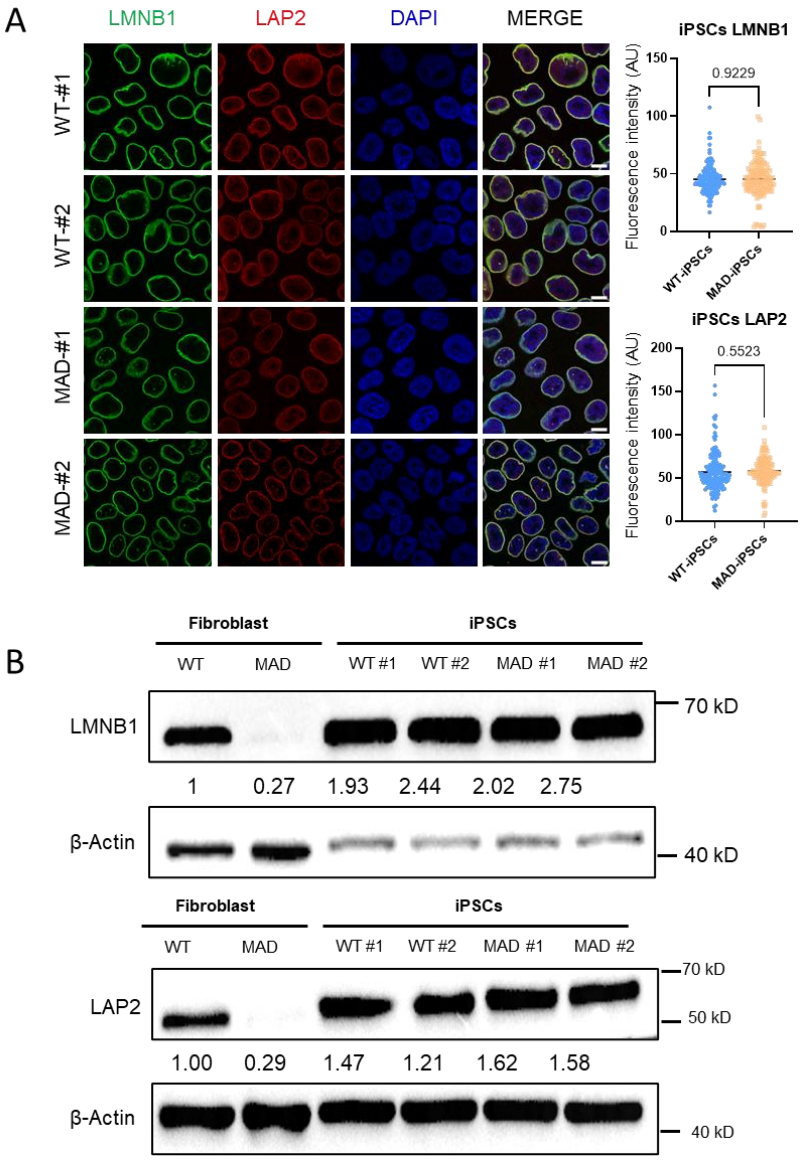

Figure 1. Quantification of LMNB1 and LAP2 in WT and MAD-iPSCs. A, IF of LMNB1 and LAP2 in two WT and MAD-iPSCs clones, quantification of fluorescence intensity of LMNB1 and LAP2 in WT and MAD-iPSCs (cells from two clones were combined, n>100); B, WB of LMNB1 and LAP2 in two WT and MAD-iPSCs clones.

3. Figure 2g-h: this part of the work should be improved, also considering what the authors found by ChIP-seq experiments. ChIP-seq of histone marks (H3K27ac, H3K27me3 and H3K9me3) precipitated more genomic regions in MAD compared with WT. However, the histone levels are dropped. This is possible when there is a redistribution of the histone

marks along the chromatin fiber or unspecific binding that render the IF signal more diffused. For this reason, the figure 2g-h need a more accurate quantification, by segmentation of intranuclear H3K9me3 and H3K27me3 bodies. Moreover, H3K27ac should be added in the IF analysis because the authors performed H3K27ac ChIP-seq assay.

Response: Thanks for suggestion. Following your suggestion, we have performed a variety of histone marks including H3K27ac, H3K27me3 and H3K9me3, H3K4me3, etc. (see **Figure 2** below, revised **Fig.2g-h**). In addition, we quantified the fluorescence intensity plotted by single nuclei instead of the whole image presented before.

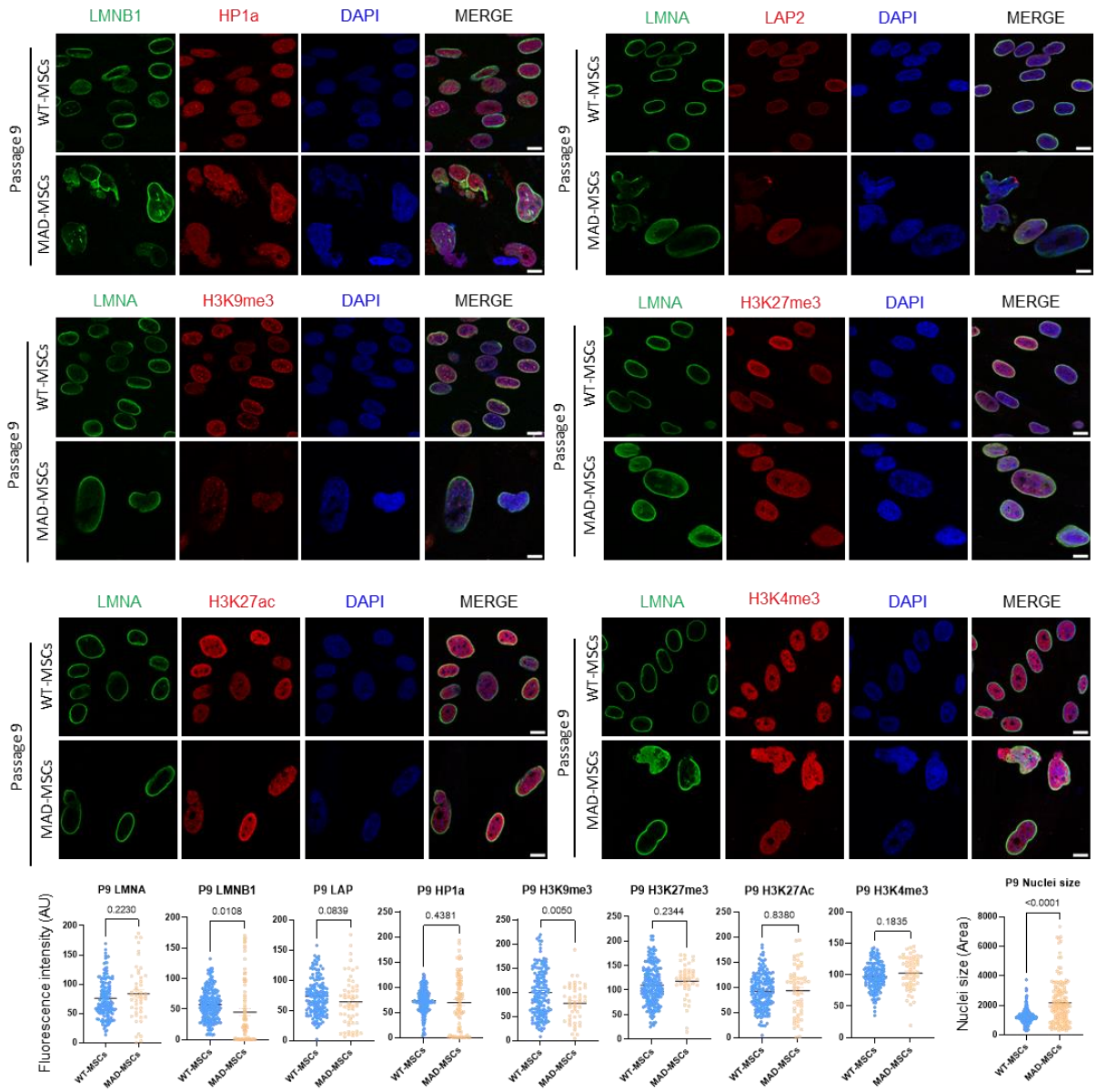

Figure 2. IF and quantification of fluorescence intensity of histone modifications using WT and MAD-MSCs at passage 9.

4. Figure 2g-h: it is strongly suggested to perform the IF analysis in both clones of iPSCs to see when the histone remodeling take place. A western blot done in parallel on iPSCs and MAD-MSCs at distinct point of differentiation will clarify if there is a global decrease in histone amounts along differentiation or an intranuclear redistribution.

Response: We thank you for the suggestion. The aging-associated changes in histone modifications do not occur synchronously, making it very challenging to track all of these modifications and their dynamics. In this study, we found the declining of H3K9me3 and LMNB1 at P9, while the loss of HP1 α and LAP2 were observed at P13 (see **Figure 2** above and **Figure 3** blow). Indeed, this is another important question which should be addressed in the future. However, it goes beyond the scope of the current study. We will perform a more comprehensive analysis in the future.

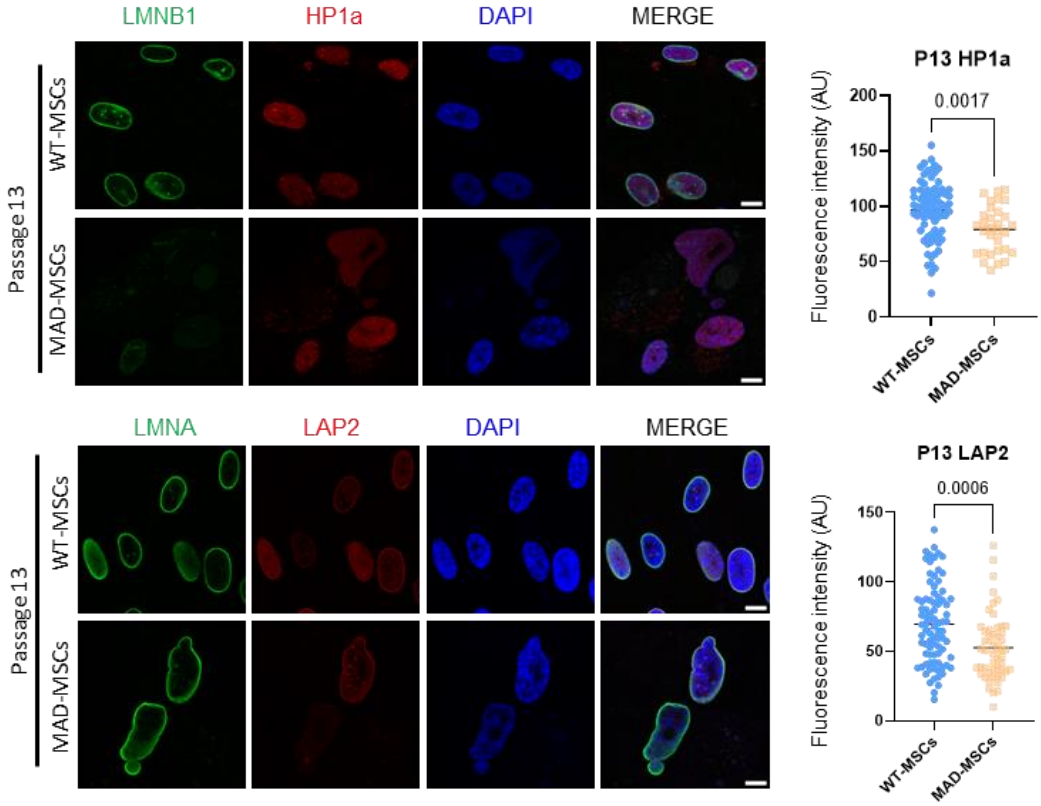

Figure 3. IF and quantification of fluorescence intensity HP1 α and LAP2 using MSCs at passage 13.

5. Figure 2g-h: the authors stated: “Although loss of nuclear structure protein LAP2a and heterochromatin associated HP1a were observed in multiple senescence conditions and MAD fibroblasts (Figure 1d), change in neither protein were observed in MAD-MSCs at p9 (Figure 2g-h)”. However, in the figure LAP2a and HP1alpha seems to be less in MAD-

MSCs cells. Thus, images chosen by the authors are not representative.

Response: The declining of LAP2a and HP1alpha are aging-associated as demonstrated in senescent MAD fibroblasts (**Revised manuscript, Extended Data Fig.1**). The quantification of fluorescence intensity of LAP2a and HP1alpha at were averaged. IF analysis did not reveal a significant change (p value<0.05) in the total intensity of two proteins between WT and MAD at passage 9. However, LAP2a and HP1alpha did exhibit uneven distribution. In the regard, less LAP2a and HP1alpha were observed in some regions in the nuclei in MAD cells whereas high density of LAP2a and HP1alpha at nuclear blebbing regions can also be found (see **Figure 2** above). Along with cell passaging, we did observe declining LAP2a and HP1alpha at passage 13 (see **Figure 2** above).

6. Figure S6b: the H3K9me3 immunofluorescence is overexposed.

Response: Thanks for the comment. We replaced the figure with new double-staining of LMNA and histone modifications images in the revised manuscript Figure S6b (also see figure below), showing co-staining of LMNA and H3K9me3.

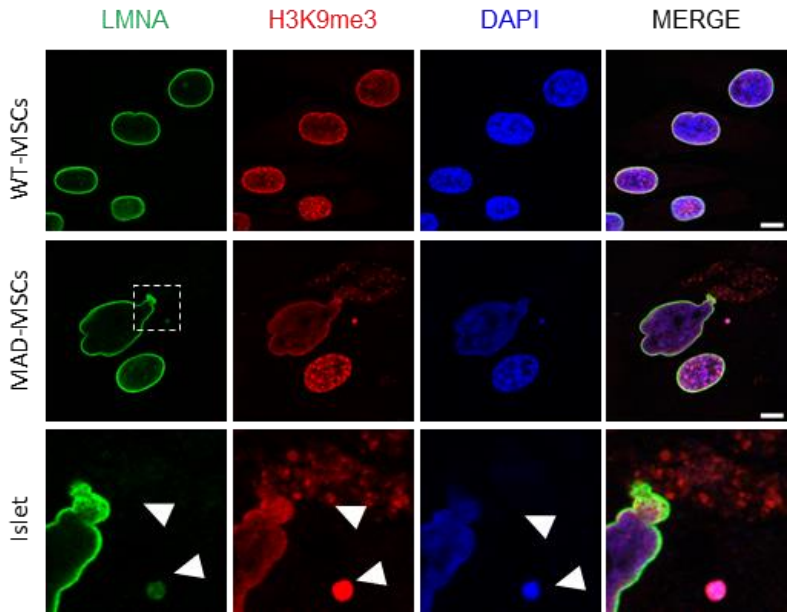

Figure 4. Co-staining of LMNA (green) and H3K9me3 (red). The islet is zoom-in view of nuclear blebbing region.

7. Figure 3a-f and Figure 4a-f: ChIP-seq of Lamin A is very informative, but the ChIP-seq assay is dependent on Ab-antigen binding that could be affected by the MAD mutation on Lamin A. The gained LADs, that also correspond to 10% of downregulated genes in Lamin A ChIP, could be dependent on the efficiency of immunoprecipitation. The authors should show the ab binding efficiency in WT and MAD-MSCs by IP followed by western blot.

Response: Thanks for the comment. The lamin A/C antibody (Santa Cruz Biotechnology, sc-376248) is specific for an epitope mapping between amino acids 2-29 at the N-terminus of Lamin A/C of human origin (*https://www.scbt.com/p/lamin-a-c-antibody-e-1*) and has been used for ChIP-seq assay in publications [PMID: 31316208 https://pubmed.ncbi.nlm.nih.gov/31316208/ and 32208162 https://pubmed.ncbi.nlm.nih.gov/32208162/]. Theoretically, the Ab-antigen binding is not affected by the MAD (LMNA R527C) mutation and the lysate IP (**Figure 5** below) indeed shows no obvious difference of Ab-antigen binding between WT and MAD cells.

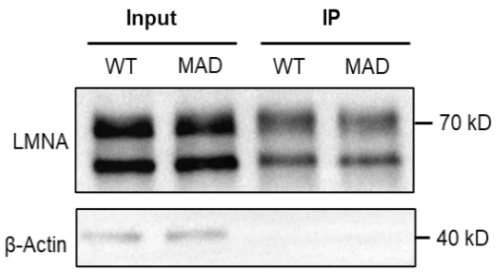

Figure 5. Validation the binding efficiency of lamin A/C antibody for MAD-MSCs. Both WT and MAD-MSCs were lysed with RIPA buffer, incubated with 5ug lamin A/C antibody at 4°C overnight, followed by protein A/G-beads binding. The binding complex was boiled for WB after three times washing.

8. The number of genes in gained and lost LADs should be indicated. I can understand that not all the genes belonging to a specific gained or lost LADs will behave the same, but this should be clearly commented in the text. For example in the region od HDAC4 what about the flanking genes in that gained LAD? The authors could show the transcription reads of the entire gained or lost domains to support the link of lamin binding/transcription. I would expect an higher correlation between gained LAD/transcriptional repression rather than lost LAD/transcriptional activation, because the activation requires additional steps of activator recruitments or DNA/DNA interactions.

Response: Thank you very much for these suggestions.

First, we summarized the number of genes in the gained and lost LADs. We found that the gained LADs contained more down-regulated genes while the lost LADs contained more up-regulated genes (**Figure 6A** below).

Following your suggestion, we revised the example tracks to show all the transcription reads of the entire gained or lost domains, including *HDAC4*. It can be observed that many genes exhibited down-regulated expression in the gained LADs while many genes showed up-regulated expression in the lost LADs (**Figure 6B** below).

In addition, we analyzed the average expression across all the gained and lost LADs and fount that gene expression was relatively lower in MAD than WT in the gained LADs and higher in MAD than WT in the lost LADs (**Figure 6C** below).

Overall, it suggested that the gained LADs were linked to transcriptional repression. Besides, from the differential gene numbers shown in **Figure 6A** below, indeed there is a higher correlation between gained LAD/transcriptional repression rather than lost LAD/transcriptional activation, exactly as you expected.

We have included these results in the revised manuscript (**Revised manuscript, Extended Data Fig.9**), thank you again for the helpful advice.

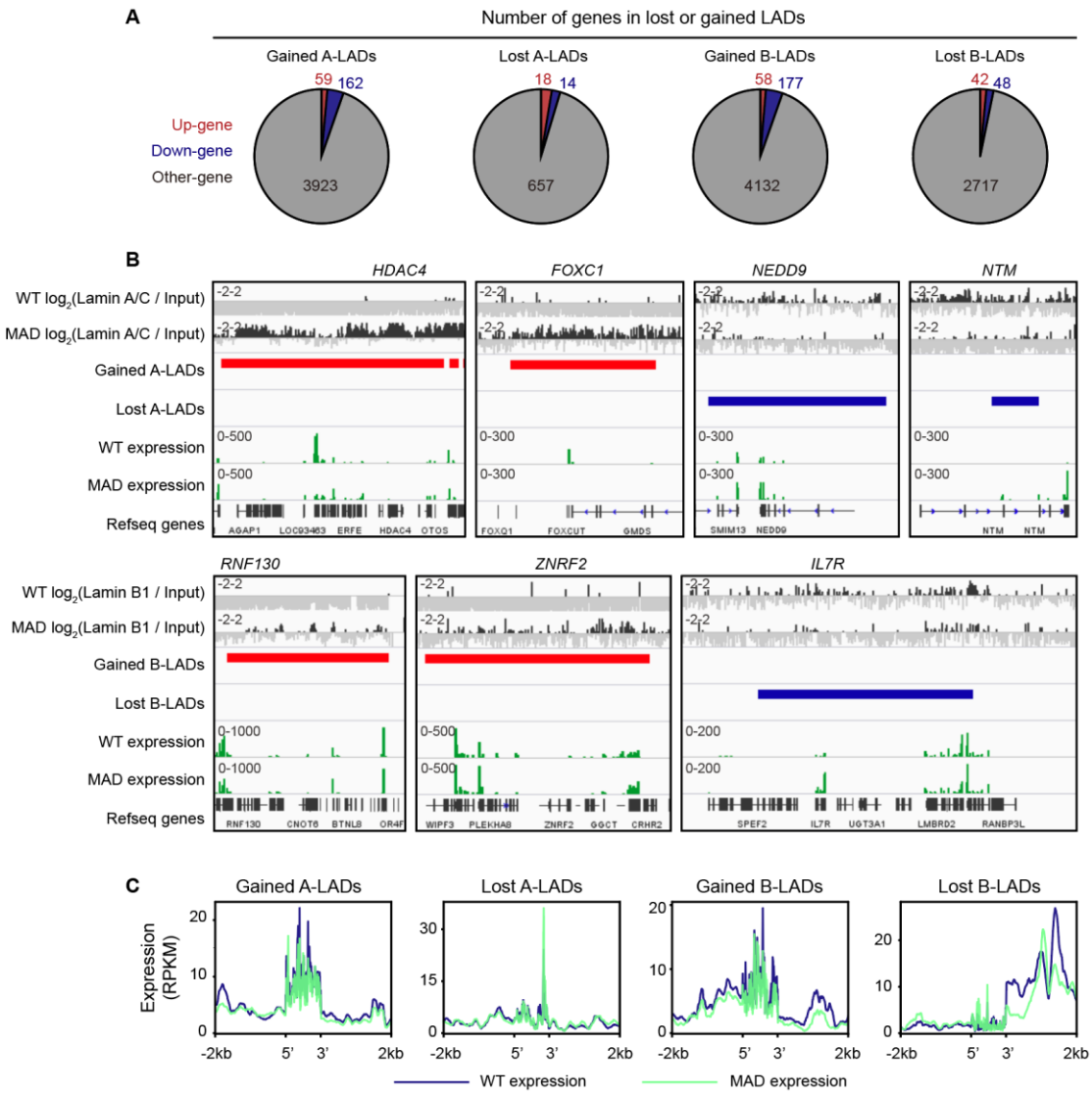

Figure 6. Link between alteration in LADs and gene expression. (**A**) Pie charts showing the summary of the number of genes in the gained and lost LADs. (**B**) Representative genomic browser views showing LAD reorganization and changes in gene expression. The y-axis values of Lamins represented log2 RPKM vs input. The y-axis values of expression represented RPKM. (**C**) Average plots

showing average gene expression in the indicated domains. The y-axis values represented RPKM.

9. One of the controls required when working with high coverage ChIP (as lamins) is to randomize the domains and to see how many DEGs fall inside random domains. This will unequivocally demonstrate that the selected genes were specific.

Response: Following your advice, we combined all the differential LADs and non-LADs lamina-chromatin binding peaks and randomized these domains for 1000 times to see how many DEGs fall inside random domains. According to the results, significantly more DEGs overlapped with the differential domains compared to the random domains (**Figure 7** below). These results further supported that the selected genes were specific.

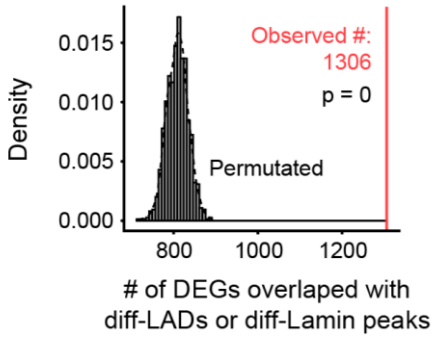

Figure 7. DEGs significantly overlapped with the differential Lamin binding domains. Genome-wide co-occurrence of differential LADs and non-LAD lamina-chromatin binding peaks with DEGs using permutation test. The y-axis density represented the frequency of co-occurrence of differential regions with DEGs while x-axis represented predicted co-occurrence number. The observed co-occurrence number was indicated.

10. Figure 4a-f: the tool used to call out-of-LADs peaks is not indicated.

Response: Thank you for pointing this out and sorry for the omission. Please allow us to provide more details of calling out-of-LADs peaks. First, we called Lamin broad peaks by MACS2 (PMID: 18798982, version 2.1.2, parameters: '-g mm -q 0.05 -m 5 50 --broad' for Lamin A/C and Lamin B1). Next, we called LADs using Enriched Domain Detector (PMID: 24782521, EDD, version 1.1.19, http://github.com/CollasLab/edd, parameters: 'max_CI_value = 0.25, required_fraction_of_informative_bins = 0.80, p_hat_CI_method = agresti_coull, log_ratio_bin_size = 10'). Finally, The non-LAD lamin-chromatin binding sites were Lamin peaks out of LADs, calculated by bedtools (PMID: 20110278, version 2.29.1, https://bedtools.readthedocs.io/en/latest/, intersectBed -v). We have included these details in the revised manuscript, thank you again for the important suggestion (**Revised manuscript, Methods**).

11. Figure 6a: although the lack of interactions is very clear in MAD-MSCs the eingen vector that generate compartment analysis is very similar between WT and MAD-MSCs. In fact, they found only 20% of compartment switches. Moreover, despite the higher amounts of genomic regions bound by lamins they found more B to A than A to B switches. I would not expect that all different levels of epigenome analysis are in line, but the authors should comment in the text more about not expected data.

Response: Thank you for the insightful comments.

First, let us provide additional explanations for the significant decrease in the interactions in MAD-MSCs, but only 20% of genomic regions underwent compartment switches. In our datasets, the decrease in interactions was consistent with a significant decrease in the absolute values of PC1 (**Figure 8A** below). However, to determine a switch, PC1 needs to change from negative to positive or from positive to negative. In our data, 20% of the genomic intervals underwent a switch, indicating that 20% of the intervals had a change in PC1 from <0 to >0 or from >0 to <0. Meanwhile, more genomic regions exhibited a decrease in interactions without changing their A/B status, and these two observations are not contradictory.

Second, let us explain why there are more Lamins-bound genomic regions, yet more B-to-A than A-to-B compartment switches. In normal cells, Lamin-associated domains (LADs) were often part of the B compartment, where Lamins interacted with condensed chromatin (PMID: 28525751). In MAD-MSCs, the nucleus is enlarged due to abnormal nuclear lamina ((**Revised manuscript, Extended Data Fig.5c**), accompanied by an increased surface area within the nucleus and more genomic regions interacting with Lamins ((**Revised manuscript, Extended Data Fig.**3b). However, concurrently, with the increase in nuclear volume, the overall chromatin became more relaxed (**Figure 8B** below). Importantly, we observed that in MAD-MSCs, the chromatin interactions within Lamin-associated genomic regions were weakened ((**Revised manuscript, Extended Data Fig. 6c**). These results indicated that Lamin-associated chromatin in MAD-MSCs was more relaxed, and LADs were not necessarily part of the B compartment (**Figure 8C** below). Therefore, the increased Lamins binding and the more B-to-A compartment switches in MAD-MSCs are not contradictory (**Figure 8D** below).

Thank you again for the valuable comments.

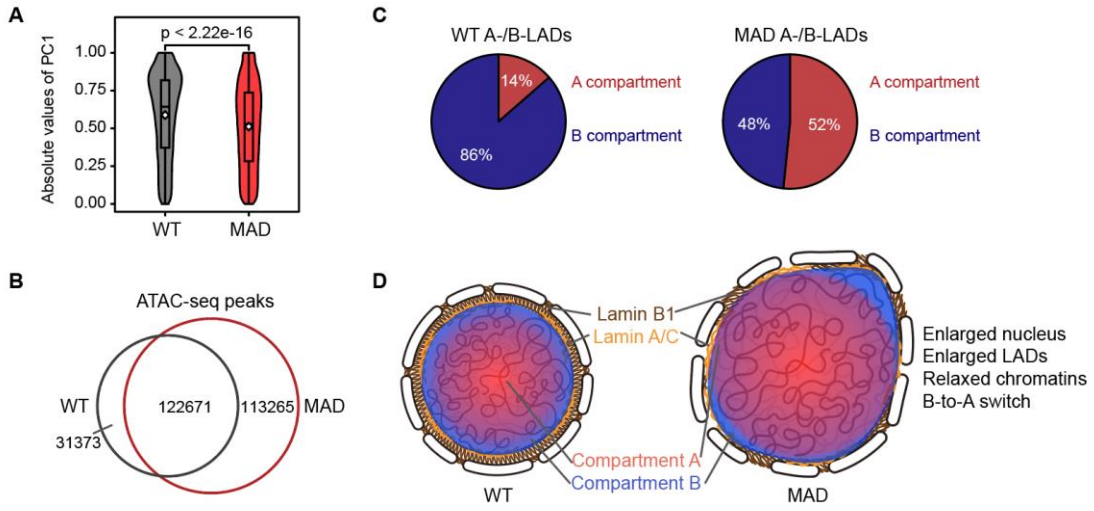

Figure 8. Models explaining the changes of compartment switches and LAD in MAD-MSCs. A Violin plot showing the decrease in the absolute values of PC1 in MAD, consistent with the decrease in interactions. The p value was determined by the Wilcoxon rank-sum test. **B**, Venn diagram showing more ATAC-seq peaks in MAD than WT. **C**, Pie charts showing more A compartments in LADs of MAD than WT. **D**, Models showing the enlarged nucleus, enlarged LADs, relaxed chromatins and B-to-A compartment switches in MAD-MSCs.

12. Figure 6g: ChIP of CTCF should be supported by quantification of CTCF protein amounts by western blot.

Response: Thanks for this suggestion. We didn't find significant change of CTCF expression at passage 9 MSCs by both IF and WB (**Figure 9**, below). The increased CTCF binding may be explained by the bigger nuclei size of MAD-MSCs and the ChIP-seq is performed at bulk level.

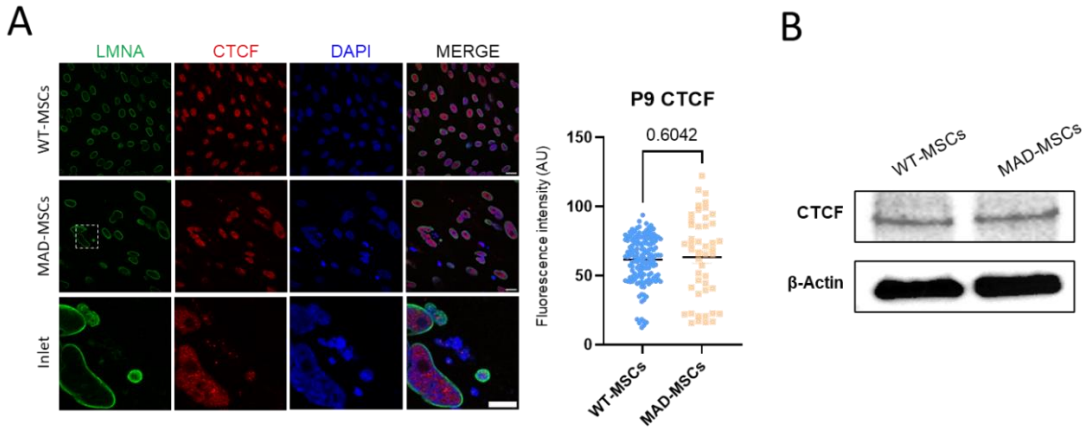

Figure 9. CTCF expression in P9 MAD-MSCs. A, IF staining of LMNA and CTCF in MAD-MSCs at passage 9, and the quantification of CTCF fluorescence intensity, n>50. B, WB of CTCF in MAD-MSCs at passage 9, β-Actin were used as loading control.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Jin et al have made impressive efforts to address the extensive comments of all three review and have significantly improved the manuscript. They should be commended for their hard work. In terms of addressing my concerns in particular, they explained the nature of their replicates, in particular clarifying how all genomics experiments represent replicates from two independent iPSC clones. They also significantly improved the quality of their analyses, Hi-C datasets, Figures and text.

I have only minor comments.

1. The explanation of Lamin A/B ChIP-seq replicates and additional examples shown (e.g. Extended Fig 9, 12 and 13) show convincing LAD reorganization in MAD-MSCs. Despite this, the example region shown in Fig. 3a makes these changes appear underwhelming. Could the authors double or triple the relative height the actual log2(LMNA/input) tracks? This will help highlight differences. This is also true for figure 4.

Finally, could the authors show the replicate tracks separately at some example loci, e.g. Extended Fig. 9a)? This will visually emphasize that consistency of the replicates at single loci and match the way LAD differences were called (i.e. separately on each replicate).

2. With regard to my previous point regarding number of replicates, it seems the primary fibroblast analysis (extended Figure 1) was still performed with a single control line. While it is understandable why there is only one MAD line, it is strange that there are not multiple controls to confirm the results are not inter-individual variability. It should at least be clearly stated in the main text that the comparisons are made only with a single control.

3. The authors should remove any dotted lines from the HiC maps to indicate TADs in extended figure 16. It is mis-leading, the reader should be allowed to interpret the differences themselves. Also, the Topdom calling in Fig. 6b is nice but I believe an additional track showing insulation score would also help (both in Fig. 6b and extended fig. 16.D). Insulation score is a continuous measurement and so does not use an arbitrary threshold to call TADs. It will give the reader an additional metric to determine the extent of the changes observed.

4. It is still impossible to say which of the many observed alterations are the direct primary effects of the MAD mutation or are indirect secondary consequences. This should be highlighted in the discussion as an important, but understandable, limitation.

Reviewer #2 (Remarks to the Author):

With this revision, the authors have clarified and improved several aspects of their work, though important concerns and issues remain. The revision has improved the following satisfactorily:

- The RNA splicing issue and progerin expression clarification is addressed

- Senescence is now more thoroughly shown.

- Changes in nuclear morphology with increased passage number is now better annotated. Likewise, MSC passage numbers are clarified.

- Previous literature is better discussed and incorporated

Remaining concerns and issues, particularly with methods presentation:

1) While clear explanations were given in the rebuttal, a bit more clarification is needed about the MSC differentiation in the manuscript itself:

a. The rebuttal contains useful clarification that initial differentiation of MAD-MSCs (ED Fig. 5b) is likely reflective of early passage competence at differentiation, while later passages show senescence. To make sure this is clear in the manuscript, the authors need to add (around line 155 and in the figure legend) a note about the passage at which the tri-lineage differentiation was carried out.

b. While it is reasonable for the authors to back off on any conclusions about MAD-MSC differentiation potential, it is still important to cite other work done on MAD-MSCs, such as Perepelina Cells, 2019. The rebuttal explains well that there is a concern that this paper exogenously expressed the mutation, which may cause different effects, but it is still important to cite what has previously been done in this system.

2) Several descriptions of methods are still severely lacking:

a. Imaging and immunofluorescence conditions are not explained at all in the paper, figures, or methods. The rebuttal letter indicates that this microscopy (figures 1 and 2) is confocal Z stacks with a "new antibody" (no details as to which one was used before or now). But, in the manuscript, there is no microscopy methods description (how was staining done? What were antibodies? What catalog number and company? Are the images shown maximum projections or single slices from a Zstack? What b-Gal staining protocol was used? The fact that the images are confocal and whether they are single slices or maximum projections should also be noted in the figure legends.

b. Antibody catalog numbers should also be provided for all antibodies used in imaging, westerns, and ChIP experiments (in particular, what lamin antibodies were used?)

c. Information given in response to qPCR questions (that 18s rRNA is the control) needs to be added in the figure legend, not just rebuttal letter. Also, qPCR primers and approach need to be included in the methods section. Essentially none of the recommended information from the MIQE qPCR reporting guidelines is present https://academic.oup.com/clinchem/article/55/4/611/5631762). Especially given that some of the rebuttal explains results as "The qPCR signal is likely unspecific amplification of LMNA transcript as the two primers differ in only 2 nucleotides" this raises concerns— such non-specific amplification should be detectable and eliminated by melting curve analysis and primers should (and can) be designed to have bigger differences than this.

3) Full Westerns and RT-PCR gels are provided, which is good, but reveals a few issues.

a. EMERIN, HP1a, and LAP2 are all on gels with no corresponding loading control. Therefore, when a band is missing (for LAP2) in one lane, there is no proof that enough protein was loaded in that lane. The Figure 3 shown in the rebuttal places the same B actin control underneath LAP2, Emerin, and HP1alpha, even though these do not come from the same gel. So, that is incorrect (looks to me like they are duplicating the B actin from the Fox3a gel for this purpose).

b. Also, the data in Extended Data 2b shows Actin as an RT-PCR control, but that data isn't shown in the full gel supplementary figure 2. Instead, GAPDH is shown in that supplementary figure—is ED Fig 2b labeled wrong?

4) Remaining concerns about Lamin ChIP data:

a. The correlation between LMNB1 signal in B-LADs between replicates is so similar as to be almost hard to believe. The LMNA scatterplots show at least some expected spread, but the LMNB1 scatterplots are nearly a straight line. Similarly, it seems remarkable that the lines of average traces (rebuttal figure 17) are exactly overlapping between MAD Rep1 and 2 for Gained A-LADs. Both the LaminA and input signals were essentially identical for the biological replicates? Showing the data for both replicates for a specific region (such as is shown in Fig3a) would be informative. I don't know that I have ever seen true biological replicates that follow the exact same pattern of minor fluctuations along the genome.

b. The increased details about LAD peak calling and comparison are appreciated, and the aggregated plots are informative. But, I question the appropriateness of the settings and parameters used in peak calling. It seems very strange that there is, for example, only one narrow "gained A peak" in the KAT2B region shown in the rebuttal, when many other areas in that same picture show the same or larger differences between WT and MAD. The selection of some narrow regions over others doesn't look justifyable. Is there any way that the peak calls in the WT data could be benchmarked against other published fibroblast lamin ChIP data?

5) It is inappropriate that the poorly sampled Hi-C data is still used for the final loop and TAD calls, even when more deeply sequenced data has been collected.

a. The "validation" of loops called on poorly sequenced Hi-C data with better sequenced Hi-C data does not address the concern that many different loops may be called if you start from scratch with the better sequenced Hi-C data. The more deeply sequenced data needs to be used to call loops and TADs, not the original low read data.

Finally, there are a few remaining typos:

1) ED Fig 2b- one label says "MDA" instead of "MAD"

2) Line 130 refers to the wrong figure panel for proliferative capacity: should be Extended Data Fig 1i instead of 1c.

3) Extended Data Fig 17d typo: "Enhaner" rather than "Enhancer"

4) Figure 3g: "Enrichement" should be "Enrichment"

Reviewer #3 (Remarks to the Author):

The authors have done a reasonable job to address my major concerns.

I recommend it for publication.

Point-by-point Responses

We appreciate the constructive suggestions and in-depth comments made by the reviewers. In this letter, we will provide detailed point-by-point responses to the reviewers' comments, along with the revised Figures of our manuscript.

Reviewer #1 (Remarks to the Author):

Jin et al have made impressive efforts to address the extensive comments of all three review and have significantly improved the manuscript. They should be commended for their hard work. In terms of addressing my concerns in particular, they explained the nature of their replicates, in particular clarifying how all genomics experiments represent replicates from two independent iPSC clones. They also significantly improved the quality of their analyses, Hi-C datasets, Figures and text.

I have only minor comments.

1. The explanation of Lamin A/B ChIP-seq replicates and additional examples shown (e.g. Extended Fig 9, 12 and 13) show convincing LAD reorganization in MAD-MSCs. Despite this, the example region shown in Fig. 3a makes these changes appear underwhelming. Could the authors double or triple the relative height the actual log2(LMNA/input) tracks? This will help highlight differences. This is also true for figure 4.

Response: Thank you for the valuable suggestion! We have double the height of the fold change tracks in **Fig. 3a** and **Fig. 4a** following your suggestion, shown as below:

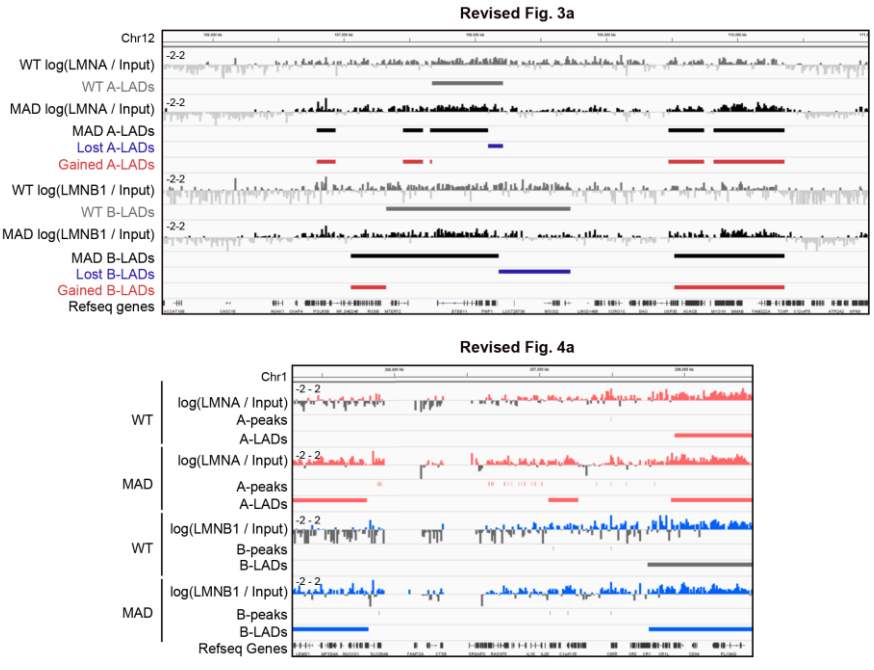

Figure 1: Revised Fig. 3a and Fig. 4a.

Finally, could the authors show the replicate tracks separately at some example loci, e.g. Extended Fig. 9a)? This will visually emphasize that consistency of the replicates at single loci and match the way LAD differences were called (i.e. separately on each replicate).

Response: Thank you for the important suggestion. We have added the replicate tracks separately at **Extended Fig. 9a**, shown as below:

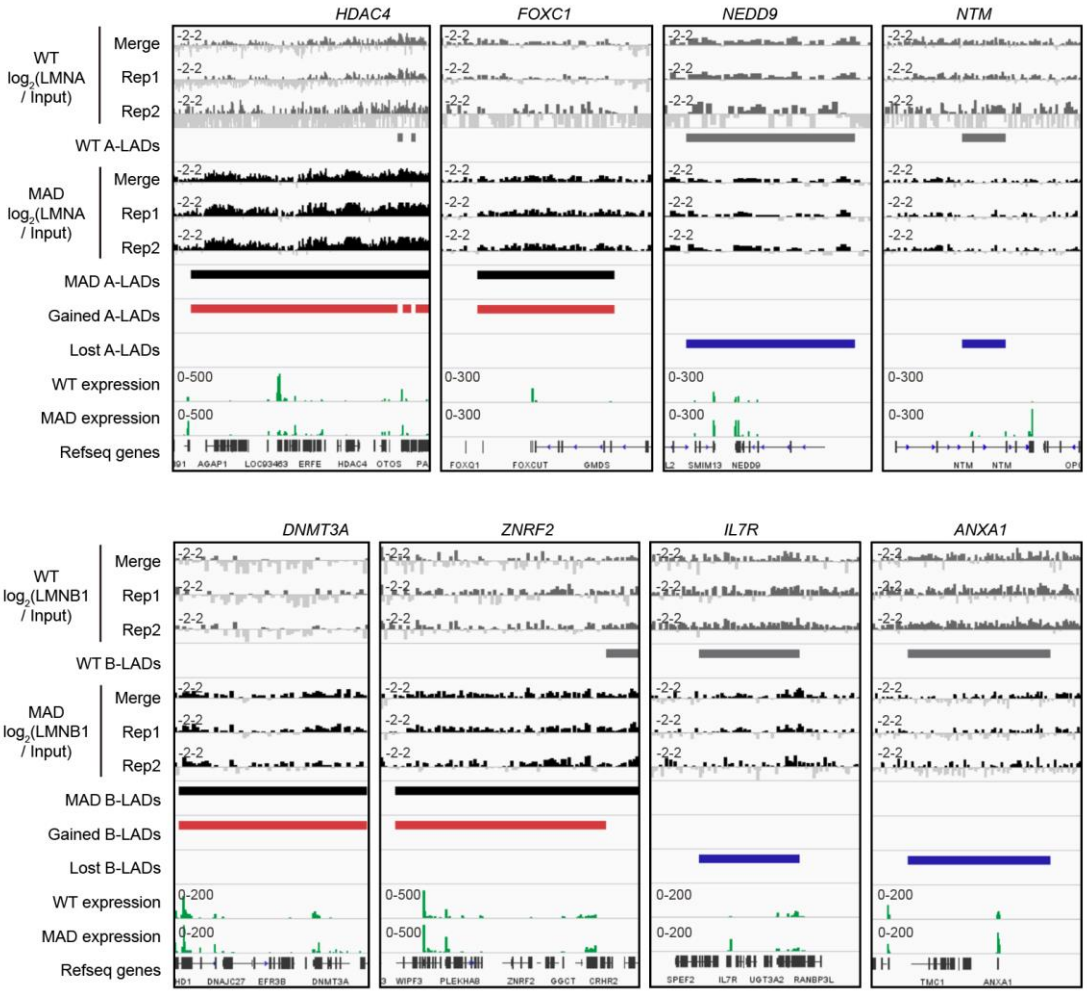

Figure 2: Revised Extended Fig. 9a.

2. With regard to my previous point regarding number of replicates, it seems the primary fibroblast analysis (Extended Figure 1) was still performed with a single control line. While it is understandable why there is only one MAD line, it is strange that there are not multiple controls to confirm the results are not inter-individual variability. It should at least be clearly stated in the main text that the comparisons are made only with a single control.

Response: We totally understand your concern and agree that inclusion of additional control samples at the initial biopsy stage would strengthen our study. Unfortunately, obtaining these samples was not feasible at that time. We have included the expanation in the methods as suggested.

Regarding the experiment, we ensured that primary cells from both the healthy donor and MAD patient were freshly isolated and maintained under identical conditions. The slightly faster proliferation rate of MAD fibroblasts compared to WT cells at passages 6-8 truthfully reflects the donor age difference (3-year-old male MAD patient and a 26-year-old female normal person) and accelerated senescence characteristic of MAD fibroblasts, thereby ruling out inter-individual variability. We appreciate your understanding of this matter.

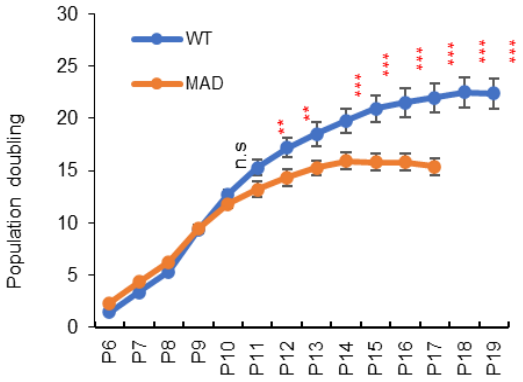

Figure 3. Growth curve of WT and MAD-fibroblasts.

3. The authors should remove any dotted lines from the HiC maps to indicate TADs in extended figure 16. It is mis-leading, the reader should be allowed to interpret the differences themselves. Also, the Topdom calling in Fig. 6b is nice but I believe an additional track showing insulation score would also help (both in Fig. 6b and extended fig. 16.D). Insulation score is a continuous measurement and so does not use an arbitrary threshold to call TADs. It will give the reader an additional metric to determine the extent of the changes observed.

Response: Thank you for this helpful advice. We have removed the dotted lines and added TAD tracks, Topdom bin signal tracks and insulation score tracks to **Fig. 6j** and **Extended Fig. 16d**, shown as below:

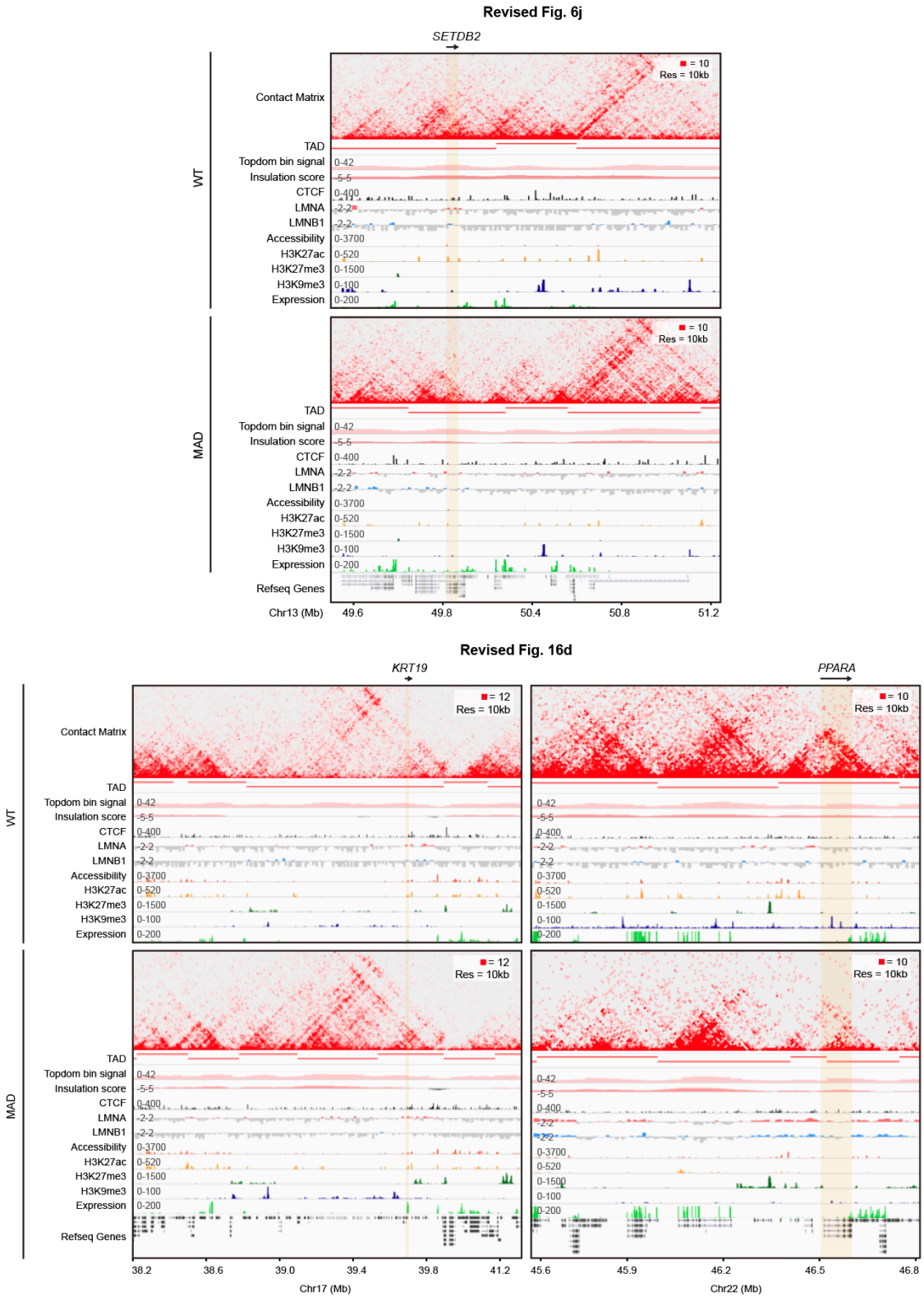

Figure 4: Revised Extended Fig. 6j and Fig. 16d.

4. It is still impossible to say which of the many observed alterations are the direct primary effects of the MAD mutation or are indirect secondary consequences. This should be highlighted in the discussion as an important, but understandable, limitation.

Response: Thanks for this suggestion. We added an additional discussion of the limitation in the discussion as suggested.

"*Our study suggests that a pathogenic LMNA mutation may lead to changes in the organization of chromatin at multi-levels. These changes could affect gene activity and contribute to the aging of stem cells in laminopathy-based atypical progeria. While it is difficult to definitively determine whether the multi-level chromatin alterations are a direct consequence of the MAD mutation or a result of secondary effects, further investigation is warranted to establish a clear causal relationship*"

Reviewer #2 (Remarks to the Author):

With this revision, the authors have clarified and improved several aspects of their work, though important concerns and issues remain. The revision has improved the following satisfactorily:

- The RNA splicing issue and progerin expression clarification is addressed

- Senescence is now more thoroughly shown.

- Changes in nuclear morphology with increased passage number is now better annotated. Likewise, MSC passage numbers are clarified.

- Previous literature is better discussed and incorporated

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a. The rebuttal contains useful clarification that initial differentiation of MAD-MSCs (ED Fig. 5b) is likely reflective of early passage competence at differentiation, while later passages show senescence. To make sure this is clear in the manuscript, the authors need to add (around line 155 and in the figure legend) a note about the passage at which the tri-lineage differentiation was carried out.

Response: Thanks for this suggestion. We have added this information in the revised manuscript in the methods and figure legends.

b. While it is reasonable for the authors to back off on any conclusions about MAD-MSC differentiation potential, it is still important to cite other work done on MAD-MSCs, such as Perepelina Cells, 2019. The rebuttal explains well that there is a concern that this paper exogenously expressed the mutation, which may cause different effects, but it is still important to cite what has previously been done in this system.

Response: Thanks for this suggestion. We have cited the paper Perepelina *Cells*, 2019 in the revised manuscript.

2) Several descriptions of methods are still severely lacking:

a. Imaging and immunofluorescence conditions are not explained at all in the paper, figures, or methods. The rebuttal letter indicates that this microscopy (figures 1 and 2) is confocal Z

stacks with a “new antibody” (no details as to which one was used before or now). But, in the manuscript, there is no microscopy methods description (how was staining done? What were antibodies? What catalog number and company? Are the images shown maximum projections or single slices from a Zstack? What b-Gal staining protocol was used? The fact that the images are confocal and whether they are single slices or maximum projections should also be noted in the figure legends.

Response: Thank you for this valuable suggestion. We have added this information in our revised manuscript in the Methods and figure legends.

b. Antibody catalogue numbers should also be provided for all antibodies used in imaging, westerns, and ChIP experiments (in particular, what lamin antibodies were used?)

Response: We have summarized all these information in the **Supplementary Table 2**, including the host species, Vendor, Catalogue Number and working concentration for WB, IF and ChIP-seq. Lamin A/C (Santa Cruz sc-376248) is recommended for both IF and ChIP.

c. Information given in response to qPCR questions (that 18s rRNA is the control) needs to be added in the figure legend, not just rebuttal letter. Also, qPCR primers and approach need to be included in the methods section. Essentially none of the recommended information from the MIQE qPCR reporting guidelines is present https://academic.oup.com/clinchem/article/55/4/611/5631762). Especially given that some of the rebuttal explains results as “The qPCR signal is likely unspecific amplification of LMNA transcript as the two primers differ in only 2 nucleotides” this raises concerns— such non-specific amplification should be detectable and eliminated by melting curve analysis and primers should (and can) be designed to have bigger differences than this.

Response: Thank you for your suggestion. We have added the information in our revised manuscript in the methods and figure legend accordingly. The sequences of qPCR primers are summarized in **Supplementary Table 1.** The qPCR primers and quantification methods are also added in **Methods** part.

For the progerin qPCR part, the primers can only be designed cross exons, particular in progerin splicing region, otherwise it is hard to distinguish progerin and lamin A. Please also refer to the *Nature* paper PMID: 21346760, Supplementary Figure 10 (**below**). BJ-iPSC is the control cell without LMNA mutation and higher progerin expression in EB (d14) than EB (d0)

could also be observed by qPCR, similar situation in our study, but it doesn't mean the expression of progerin in BJ-iPSC cells.

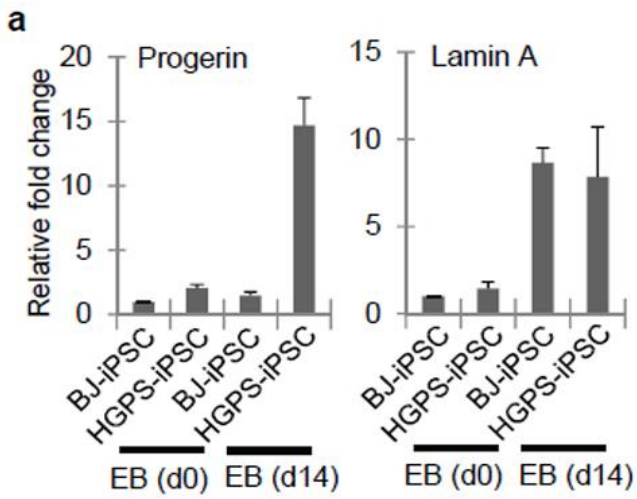

Figure derived from PMID: 21346760 Supplementary Figure 10.

3) Full Westerns and RT-PCR gels are provided, which is good, but reveals a few issues.

a. EMERIN, HP1a, and LAP2 are all on gels with no corresponding loading control. Therefore, when a band is missing (for LAP2) in one lane, there is no proof that enough protein was loaded in that lane. The Figure 3 shown in the rebuttal places the same B actin control underneath LAP2, Emerin, and HP1alpha, even though these do not come from the same gel. So, that is incorrect (looks to me like they are duplicating the B actin from the Fox3a gel for this purpose).

Response: LAP2, EMERIN, and HP1a were indeed run on separate gel with the same loading volume previously. We totally understand your high standard and concern of WB, so we have re-run the samples to ensure HP1a and LAP2 blotting in parallel with β-Actin. However, EMERIN share close size with β-Actin and we have tried several times but failed to blot these two proteins on the same gel.

To answer your question as much as possible, we run the gel with 15 lanes: half for LAP2/β-Actin/HP1a and half for LAP2/EMERIN/HP1a, with LAP2 and HP1a from the same membrane (**Below**). The results show the same tendency presented in **Figure 1d** and we appreciate your understanding of this matter.

On the other hand, WB results are not the only evidence, the declining and restoration of HP1a and LAP2 in MAD fibroblast and iPSCs were also accompanied by immunofluorescence (**Figure 1c** and **Extended Data Figure 1j and 3a**).

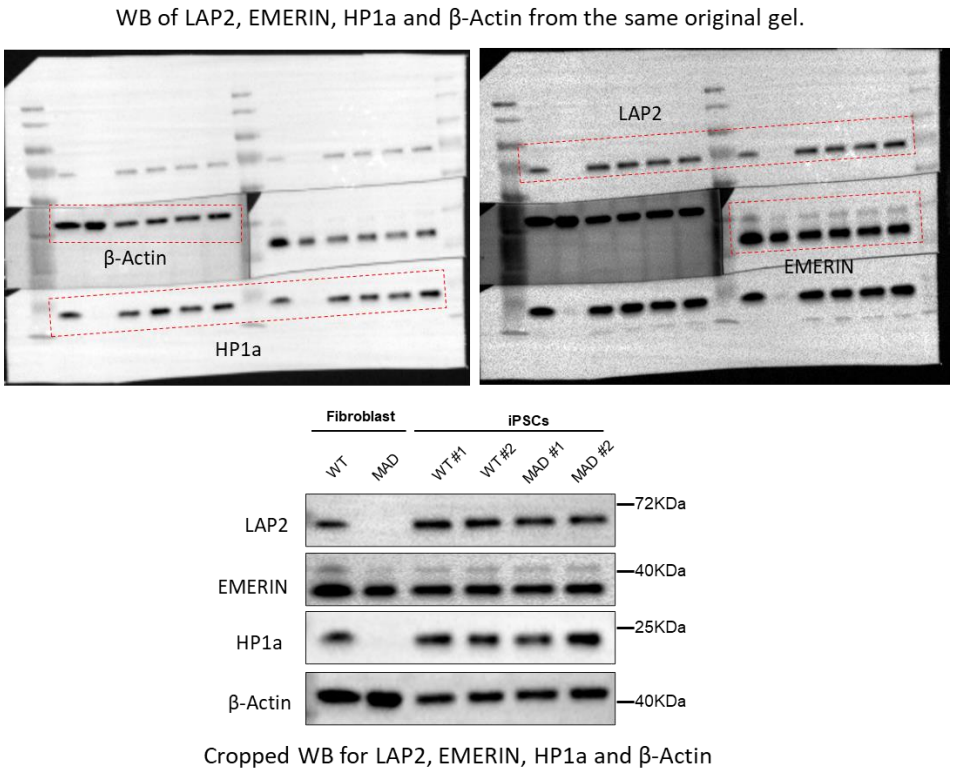

Figure 1: WB of LAP2, EMERIN, HP1a and β-Actin from the same gel.

b. Also, the data in Extended Data 2b shows Actin as an RT-PCR control, but that data isn't shown in the full gel supplementary figure 2. Instead, GAPDH is shown in that supplementary figure—is ED Fig 2b labeled wrong?

Response: Apologies for the mistakes. We used both GAPDH and Actin as RT-PCR control, while only presented Actin in **Extended Data 2b** while missed the Actin original gel in **Supplementary Figure 2** and have added in revised version.

4) Remaining concerns about Lamin ChIP data:

a. The correlation between LMNB1 signal in B-LADs between replicates is so similar as to be almost hard to believe. The LMNA scatterplots show at least some expected spread, but the LMNB1 scatterplots are nearly a straight line. Similarly, it seems remarkable that the lines of average traces (rebuttal figure 17) are exactly overlapping between MAD Rep1 and 2 for Gained A-LADs. Both the Lamin A and input signals were essentially identical for the biological replicates? Showing the data for both replicates for a specific region (such as is shown in Fig3a)

would be informative. I don't know that I have ever seen true biological replicates that follow the exact same pattern of minor fluctuations along the genome.

Response: Thank you for the comments. Following your suggestion, we show data for both replicates in **Fig3a** as the followings:

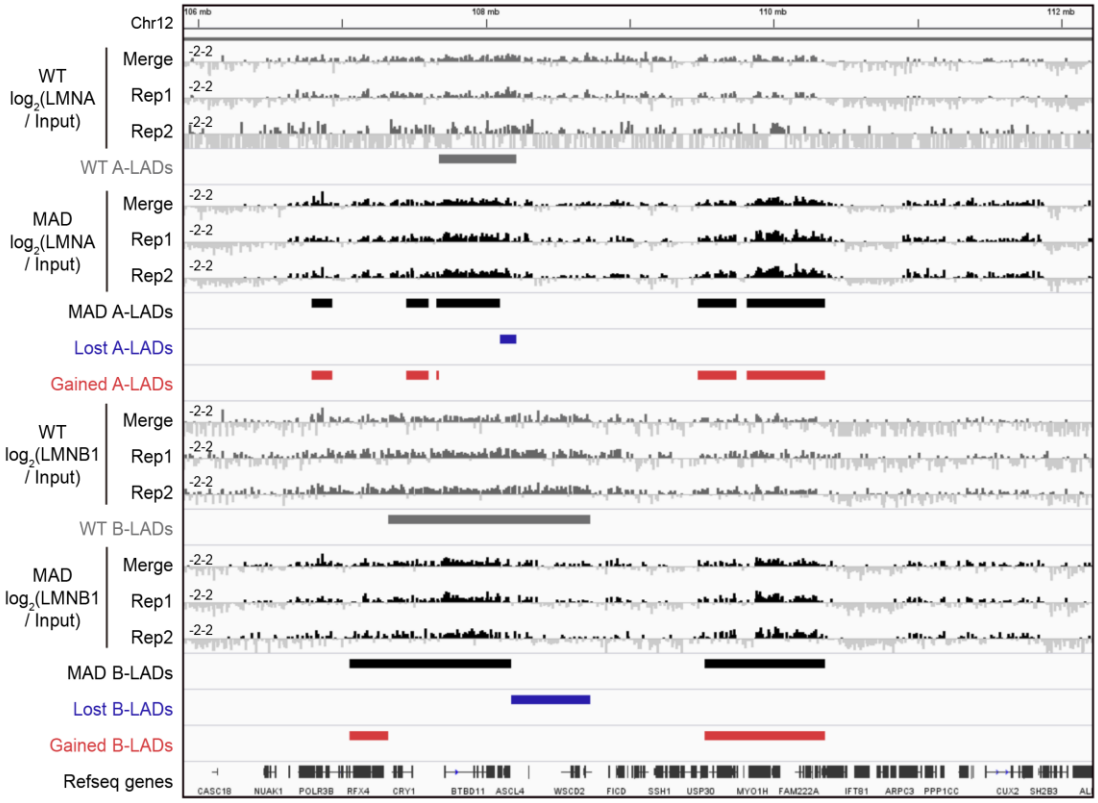

Figure 2: Genomic browser view with replicates in Fig. 3a

b. The increased details about LAD peak calling and comparison are appreciated, and the aggregated plots are informative. But, I question the appropriateness of the settings and parameters used in peak calling. It seems very strange that there is, for example, only one narrow "gained A peak" in the KAT2B region shown in the rebuttal, when many other areas in that same picture show the same or larger differences between WT and MAD. The selection of some narrow regions over others doesn't look justifyable. Is there any way that the peak calls in the WT data could be benchmarked against other published fibroblast lamin ChIP data?

Response: We attempted to benchmark our peak calling method with previous published datasets; however, there is no published Lamin ChIP data in MSCs. Therefore, we applied our peak calling method to published Lamin ChIP data in fibroblast to validate its reliability. Since Ser22-phosphorylated (pS22) LMNA has been reported to have binding sites outside of LADs (PMID: 32208162), we used the same parameters as in our manuscript to call peaks for

phosphorylated LMNA (MACS2, '-q 0.05 -m 5 50 –broad'). The resulting peaks exhibited similar patterns in signal enrichment and genomic distributions to those in our study (Response R2 Figure 3).

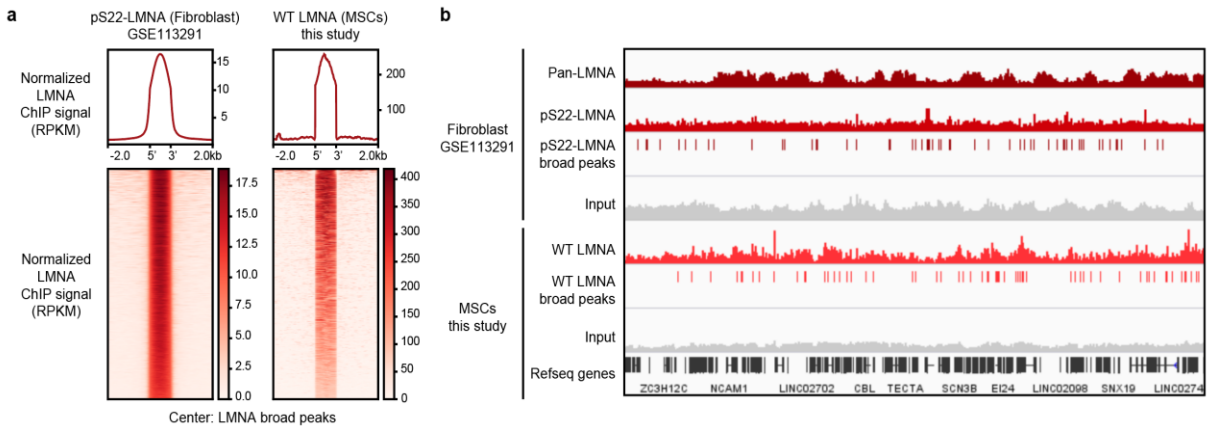

Figure 3: Benchmarking of Lamin peak calling.

5) It is inappropriate that the poorly sampled Hi-C data is still used for the final loop and TAD calls, even when more deeply sequenced data has been collected.

The "validation" of loops called on poorly sequenced Hi-C data with better sequenced Hi-C data does not address the concern that many different loops may be called if you start from scratch with the better sequenced Hi-C data. The more deeply sequenced data needs to be used to call loops and TADs, not the original low read data.

Response: Thank you for this comment. We appreciate your concern regarding the use of previous Hi-C data for calling TADs and loops. However, since the better data has confirmed the robustness of these TADs and loops, our current methodology does not affect the conclusions of our manuscript.

Finally, there are a few remaining typos:

1) ED Fig 2b- one label says "MDA" instead of "MAD"

2) Line 130 refers to the wrong figure panel for proliferative capacity: should be Extended Data Fig 1i instead of 1c.

3) Extended Data Fig 17d typo: "Enhaner" rather than "Enhancer"

4) Figure 3g: "Enrichement" should be "Enrichment"

Response: Apologies for our carelessness and we have corrected all the typos accordingly. Thank you for pointing out.

Reviewer #3 (Remarks to the Author):

The authors have done a reasonable job to address my major concerns.

I recommend it for publication.

Response: Thank you for your insightful comments.