Journal of Cell Science

Nup93 and CTCF modulate spatiotemporal dynamics and function of the *HOXA* gene locus during differentiation

Ajay S. Labade, Adwait Salvi, Saswati Kar, Krishanpal Karmodiya and Kundan Sengupta
DOI: 10.1242/jcs.259307

Editor: Maria Carmo-Fonseca

Review timeline
Original submission: 24 August 2021
Editorial decision: 27 September 2021
First revision received: 8 October 2021
Accepted: 30 October 2021

Original submission

First decision letter

MS ID#: JOCES/2021/259307

MS TITLE: Nup93 and CTCF modulate spatiotemporal dynamics and function of the HOXA gene locus during differentiation

AUTHORS: Ajay Labade, Adwait Salvi, Saswati Kar, Krishanpal Karmodiya, and Kundan Sengupta
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

I have now received comments on your manuscript from one expert referee. I have invited approximately 8 additional reviewers (including those that you suggested) but unfortunately they all declined. I have carefully read the paper and in principle, I agree with reviewer #1 that your work is a good fit to JCS. However, I also agree with concerns raised by this reviewer. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewer.

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

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

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, Labade et al unravel an interesting mechanism by which Nup93 regulates the expression of some HOXA genes during differentiation. To examine the genome wide distribution of Nup93 in differentiated colorectal cancer cells (DLD-1), the authors performed ChiP-seq and found that it is widely associated with the genome, particularly at promoter regions. Many of these areas were connected with developmental pathways. These regions were enriched for transcription factor binding sites, especially overlapping with CTCF sites. In a previous study the authors found that Nup93 associated with the HOXA genes and so decided to focus on HOXA gene clusters and their regulation during differentiation using RA-induced differentiation in embryonic carcinoma N2T/D1 cells. siRNA KD of Nup93 either increased or decreased the expression of some of the HOXA genes, whereas KD of CTCF showed the opposite action for the different genes. The co-depletion of CTCF & Nup93 had a balancing effect such that expression levels remained basically the same. RA-induced differentiation increased expression even more when Nup93 was KD (CTCF KD had the opposite effect). This induction initiates quite early (several hours) after RA addition. The occupancy of Nup93 and CTCF were followed during days of differentiation with both showing a reduction in occupancy in the cognate binding sites by day 8, and then re-occupancy by day 21.

Comments for the author

Altogether, the study is well presented, clear and convincing. In my opinion this study is suitable for J of Cell Science. My main remarks are: 1) The authors should make an effort to integrate and explain both the changes in Nup93 and CTCF occupancy, so that we get a clear integrative idea of what is happening. I felt I knew what was occurring with each of these molecules alone, but was not sure how they connected. 2) See last remark regarding the model of the association with the nuclear envelope. I am not convinced that the data point in this specific direction.
Additional remarks:
Line 137: “the G2 subpopulation of cells decreased upon RA-induced differentiation (Fig. S2B-C).” – hard to see, and is this change significant? Also, why is N=2 only? (also in E). Same also to understand from the plots if there is a significant change or not in Fig. S3B.
Fig.2 – I suppose most of the first part of the figure could be supplemental as it is only confirming that the cells are undergoing differentiation, as expected from them.
Also, I am not sure what is the difference between N= to n= ?
Fig. 3A – qRT-PCR is shown in Fig. 3B and a Western blot for 3C, so why not show the protein levels also for 3A?
Fig. 3D (compared to 3A) the Y axis's are different, so are the authors trying to say that the expression levels when RA was added are even 50 fold higher, compared to 2 fold without RA? If so, then this should be stated clearly. Also, is this change seen also on the protein levels?
Fig. 4 – the said change in location of the genes is not very convincing not in the images, nor in the plots.
Fig. 6 – Once again, this reviewer doesn’t see the close association with the nuclear periphery for the HOXA1 loci. In fact, at day 0 there is no association with the periphery, and definitely no association with the nuclear envelope as stated in the title. The territories are peripheral in day 0, but actually are mostly peripheral in all days. Therefore, I disagree with the model showing the locus at the nuclear lamina, when clearly in all images it is not. In microscopy units, these small distances from the envelope are probably more than a micron away if not more. Also, I am not convinced that the Nup93 the authors are measuring is Nup93 associated with the NPC but in my opinion it is nucleoplasmic Nup93 (the data from Fig. 5A don’t prove this either). But this is not a problem, as described in the Discussion, since there are several studies showing that nucleoplasmic Nups interact with chromatin and affect gene expression

First revision

Author response to reviewers' comments

Advance Summary and Potential Significance to Field:
In this study, Labade et al unravel an interesting mechanism by which Nup93 regulates the expression of some HOXA genes during differentiation. To examine the genome wide distribution of Nup93 in differentiated colorectal cancer cells (DLD-1), the authors performed ChiP-seq and found that it is widely associated with the genome, particularly at promoter regions. Many of these areas were connected with developmental pathways. These regions were enriched for transcription factor binding sites, especially overlapping with CTCF sites. In a previous study the authors found that Nup93 associated with the HOXA genes and so decided to focus on HOXA gene clusters and their regulation during differentiation using RA-induced differentiation in embryonic carcinoma N2T/D1 cells. siRNA KD of Nup93 either increased or decreased the expression of some of the HOXA genes, whereas KD of CTCF showed the opposite action for the different genes. The co-depletion of CTCF & Nup93 had a balancing effect such that expression levels remained basically the same. RA-induced differentiation increased expression even more whenNup93 was KD (CTCF KD had the opposite effect). This induction initiates quite early (several hours) after RA addition. The occupancy of Nup93 and CTCF were followed during days of differentiation with both showing a reduction in occupancy in the cognate binding sites by day 8, and then re-occupancy by day 21.

Response: We thank the reviewer for the summary of our work, where we have investigated a mechanism involving nucleoporin Nup93 and the chromatin organizer CTCF in regulating HOXA gene expression during differentiation.

1. Altogether, the study is well presented, clear and convincing. In my opinion this study is suitable for J of Cell Science. My main remarks are: 1) The authors should make an effort to integrate and explain both the changes in Nup93 and CTCF occupancy, so that we get a clear integrative idea of what is happening. I felt I knew what was occurring with each of these molecules alone, but was not sure how they connected. 2) See last remark regarding the model of the association with the nuclear envelope. I am not convinced that the data point in this specific direction.

Response: We thank the reviewer for the comments on our manuscript. We also thank the reviewer for bringing this critical aspect to our notice. Our responses are as below and reiterated in response #7.

(i) We have modified the explanation about the dynamic association of Nup93 and CTCF during differentiation in the discussion section *"Mechanistic role of Nup93 and CTCF mediated HOXA regulation during differentiation"* of the manuscript.

(ii) We elaborate on a fascinating finding that despite being one of the most stable nuclear architectural proteins at the nuclear periphery, Nup93 shows considerable overlap in its occupancy with the chromatin organizer CTCF (Fig. 1). Since Nup93 and CTCF do not interact with one another, as revealed by co-immunoprecipitation experiments (Fig.5 & Labade et al., 2016), we hypothesize that Nup93 and CTCF have temporally resolved and mutually exclusive roles in regulating the HOXA locus during differentiation. Although Nup93 and CTCF binding shows considerable overlaps genome-wide, Nup93 and CTCF have unique and non-overlapping binding sites on HOXA locus (Fig. 6D) and therefore exert mutually exclusive regulatory functions to further fine-tune the expression of genes required for development and differentiation, such as the HOXA gene locus. It is well established that HOXA genes are expressed in a temporal manner during differentiation. Therefore, differentiation serves as an important and useful paradigm to study the role of a novel axis of chromatin organizers such as Nup93 and CTCF in the regulation of HOXA gene expression.

(iii) Furthermore, ChIP-qPCR analysis revealed interesting binding dynamics of CTCF and Nup93 within the HOXA locus during differentiation. In undifferentiated cells (Day0), the HOXA gene locus was repressed, and both Nup93 and CTCF were enriched on their respective binding sites. Previous findings have shown that RA-mediated induction of HOXA locus disrupts looping of the HOXA gene

locus and reduced CTCF occupancy suggesting increased chromatin accessibility for RNA polymerase II (Oh et al., 2018; Wang et al., 2015; Xu et al., 2014). Therefore, RA-mediated induction of HOXA expression is associated with local chromatin opening of the HOXA gene locus resulting in a pattern of temporal expression. It is likely that RA-induced activation of HOXA considerably "opens up" HOXA gene loci, thereby destabilizing Nup93 and CTCF occupancy on HOXA. Consistent with previous findings, our ChIP-qPCR results showed decreased occupancy of both Nup93 and CTCF on their respective binding sites after the induction of HOXA expression during RA treatment (Day2-8) (Fig. 5B-H).

(iv) Interestingly, the HOXA gene locus undergoes re-repression upon differentiation, where both Nup93 and CTCF re-occupy their conserved binding sites by Day21 (Fig. 5B-H). In addition to ChIP-qPCR, we corroborated this finding by 3D-FISH analyses of the HOXA gene locus during differentiation. These assays give different readouts - while ChIP-qPCR provides protein-DNA interaction dynamics, 3D-FISH analyses reveal gene loci positioning during differentiation in the interphase nucleus. This has been detailed in response#7. Taken together, these results suggest that Nup93 and CTCF drive the re-looping and re-tethering and hence repression of the HOXA gene locus.

(v) We also synthesized a number of different FISH probes to label the individual HOX genes within the HOXA gene locus. However, these smaller probes (~1kb probes) were undetectable by FISH, potentially due to the reduced incorporation efficiency of fluorescently coupled dUTP in smaller DNA segments. After optimizing the labeling for these smaller FISH probes, using oligo-FISH, we aim to perform 3D-FISH followed by super-resolution imaging in order to resolve the differential dynamics of the 3' and 5' HOXA genes at the sub-micron level within the HOXA locus.

(vi) Collectively, these experiments unravel the potential mechanisms by which Nup93 and CTCF regulate HOXA gene expression. The observed overlap in binding sites combined with the localization of NPCs results in the differential dynamics of the HOXA gene locus. Competition for these binding sites in the context of RA treatment and altered loci positioning could explain the differential binding observed which could further result in CTCF-mediated looping. The mechanisms by which Nup93 and CTCF are connected needs detailed investigation, considering the absence of a direct interaction between them. However, our study proposes a fascinating mechanism that relies on the structural/positional aspect of Nup93 and the looping function of CTCF (Detailed explanation in Discussion section of the manuscript).

(vii) Of note, another recent finding suggests crosstalk between nucleoporin Nup153 and CTCF in regulating gene expression during mouse ES cell differentiation (Kadota et al., 2020). These results highlight the crucial structure-function relationship between nucleoporins and chromatin organizers such as CTCF.

2. Additional remarks: Line 137: "the G2 subpopulation of cells decreased upon RA-induced differentiation (Fig. S2B-C)." – hard to see, and is this change significant? Also, why is N=2 only? (also in E). Same also to understand from the plots if there is a significant change or not in Fig. S3B.

Response: We thank the reviewer for bringing this to our notice. We agree that the decrease in the G2 subpopulation upon RA treatment is not significant. Upon re-evaluation, we find that this data does not contribute to our current findings, as we have presented additional results which demonstrate RA-induced differentiation (Figure S2). We have therefore deleted the cell sorting data from the supplemental data of the manuscript.

3. Fig.2 – I suppose most of the first part of the figure could be supplemental as it is only confirming that the cells are undergoing differentiation, as expected from them. Also, I am not sure what is the difference between N= to n=?

Response:
(i) Yes, we agree that most of this data is supplemental as this confirms that the NT2/D1 cells are undergoing differentiation (New Fig. S2).

(ii) N: refers to the number of independent biological replicates, while n: represents technical replicates or number of nuclei as mentioned in each figure legend.

4. Fig. 3A – qRT-PCR is shown in Fig. 3B and a Western blot for 3C, so why not show the protein levels also for 3A?

Response: We thank the reviewer for this important suggestion. Our response is as below:
(i) We determined protein levels for HOXA1 and HOXA5. However, we were unable to detect HOXA protein levels in DLD1 colorectal cells, potentially due to the poor quality of these antibodies **(Fig. R1)**. We did not detect a specific band for either HOXA1 (sc-17146) or HOXA5 (ab55239) at the expected molecular weight (37-49kDa) as suggested by the antibody data sheets.

(ii) We used transcript levels of HOXA genes as a readout of its expression due to the following reasons:

- We focused on determining the co-regulatory role of Nup93 and CTCF in regulating chromatin organization, spatial positioning, and expression levels of the HOXA genes during differentiation. We have not studied the downstream effects of HOXA proteins in the current paradigm.
- Previous studies on HOXA chromatin organization and function during differentiation have also examined transcript level changes as a readout of HOXA gene function using RTPCR or transcriptomics-based approach (Ferraiuolo et al., 2010; Rousseau et al., 2014; Xu et al., 2014). It would certainly be interesting to study HOXA at the protein level as well.

(iii) We acknowledge the profound importance of HOXA transcription factors in controlling the expression levels of downstream targets and in turn, cell fate decisions.

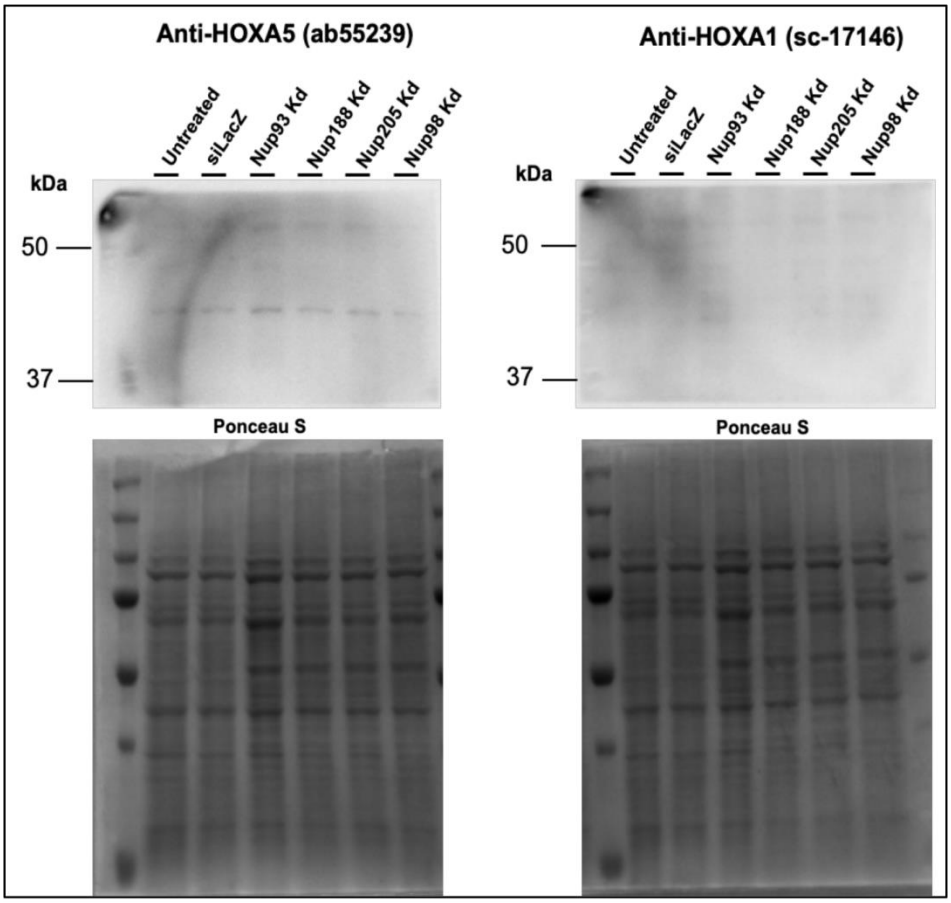

Figure R1: Immunoblotting performed to detect HOXA5 and HOXA1 proteins. However, we did not detect any specific bands at the expected molecular weight, using the antibodies as mentioned above, despite detectable and uniform protein loads and transfer, as shown by Ponceau staining in the lower panels.

5. Fig. 3D (compared to 3A) the Y-axis are different, so are the authors trying to say that the expression levels when RA was added are even 50 fold higher, compared to 2 fold without RA? If so, then this should be stated clearly. Also, is this change seen also on the protein levels?

Response: We thank the reviewer for bringing this to our notice. Yes, an upregulation of HOXA gene expression upon RA treatment is considerably higher (~50-fold) as compared to Nup93 knockdown alone. Retinoic acid is a potent inducer of HOXA gene expression whereas Nup93 depletion is only responsible for activation of the HOXA gene locus. RA activates the retinoic acid receptor (RAR) which in turn, associates with the retinoic acid response elements (RAREs) located within the HOXA gene locus. This association leads to eviction of CTCF and opening of HOXA gene locus which undergoes a wave of temporal activation. Our results suggest that Nup93 depletion untethers the HOXA locus from the nuclear periphery which initiates its activation but not overexpression.

6. Fig. 4 – the said change in location of the genes is not very convincing, not in the images, nor in the plots.
Response: (i) The movement of the HOXA gene locus was calculated from the DAPI border (edge of the nucleus). Of note, the movement is significant as computed from the comparison between median 3D distances (Fig. 4B). FISH images presented in our manuscript are only 2D representations derived from maximum intensity projections of 3D stacks of nuclei and therefore do not represent actual 3D distances from the nuclear periphery that were obtained upon quantifying 3D reconstructions of each nucleus. However, these measurements are performed from the DAPI edge to the center of mass of the HOXA FISH signal in a 3D reconstructed nucleus.

(ii) As mentioned in response #2, local remodeling/reopening of the HOXA locus may not necessarily translate into a detectable change of more than ~200 nm in the positioning of the FISH loci signal. All our FISH measurements were performed across independent biological replicates, and the data were pooled from >200 independent nuclei. Statistical tests (non-parametric tests, Mann-Whitney Wilcoxon Test) showed a significant change in the 3D locations of the HOXA gene loci as compared to the controls (Fig.4 and Fig.6).

7. Fig. 6 – Once again, this reviewer doesn't see the close association with the nuclear periphery for the HOXA1 loci. In fact, at day 0 there is no association with the periphery, and definitely no association with the nuclear envelope as stated in the title. The territories are peripheral on day 0 but actually are mostly peripheral on all days. Therefore, I disagree with the model showing the locus at the nuclear lamina, when clearly in all images it is not. In microscopy units, these small distances from the envelope are probably more than a micron away if not more. Also, I am not convinced that the Nup93 the authors are measuring is Nup93 associated with the NPC but in my opinion, it is nucleoplasmic Nup93 (the data from Fig. 5A don't prove this either). But this is not a problem, as described in the Discussion, since there are several studies showing that nucleoplasmic Nups interact with chromatin and affect gene expression.

Response: We thank the reviewer for critically evaluating our speculative model. Based on the emerging literature, the reviewer's comments, and our own experimental data, we have incorporated this evidence as follows:
(i) Previous studies show that Nup93 is a stable nucleoporin localized at the nuclear periphery (Rabut et al., 2004; Toyama et al., 2013).
Furthermore, there are no detectable levels of Nup93 in the nucleoplasm. We overexpressed Nup93-GFP in differentiated colorectal cancer cells (DLD-1), followed by high-resolution imaging of Nup93-GFP by Structured Illumination Microscopy (SIM) to determine its nuclear localization. Again, we did not detect any nucleoplasmic localization of Nup93 even upon its overexpression in DLD-1 cells **(Fig.R2A)**. We also performed immunofluorescence assays followed by co-labeling of Nup93 and Mab414 (anti- nuclear pore complex antibody) and found that Nup93 colocalizes with Mab414 at the nuclear periphery **(Fig. R2B)**.

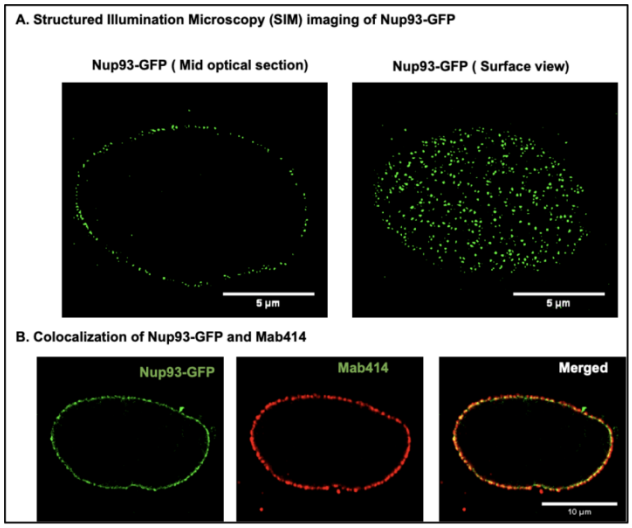

Fig. R2: (A) We performed structured illumination microscopy (SIM) of cells overexpressing Nup93-GFP, which reveals distinctive foci of Nup93-GFP at the nuclear border (mid-optical section), with countable foci (surface view). (B) Furthermore, Nup93-GFP largely colocalizes with an independent nucleoporin marker (Mab414) at the nuclear border, which was undetectable within the nucleoplasm.

(ii) Since Nup93 is localized at the nuclear envelope, we reasoned that Nup93 associates with the HOXA gene locus at the nuclear periphery. To further strengthen our findings, we performed Nup93 ChIP-qPCR which largely captures direct or indirect protein-chromatin interactions. We found that Nup93 indeed associates with and represses the HOXA gene locus. However, this association decreases during differentiation, consistent with an active HOXA gene locus. Since ChIP-qPCR is a population-level assay, we also examined the localization of the HOXA gene locus using 3D FISH/imaging at the single-cell level, which reveals the status of the HOXA gene locus when these cells are subjected to various conditions of RA treatment, Nup93 or CTCF knockdown.

(iii) Indeed, our 3D-FISH measurements showed a significant shift in the nuclear localization of HOXA when its distance was measured in 3D from the nuclear edge (border demarcated by DAPI). Since 3D-FISH was performed using a BAC clone covering the entire HOXA locus (~107kb) we were unable to determine sub-micron level changes in HOXA chromatin organization. We attempted high-resolution 3D-FISH imaging using STED microscopy. While we were able to detect Chromosome 7 Territory (CT7) territory (green), we were unable to detect the labeled HOXA gene locus due to a significant loss in its fluorescence, because of high-power laser depletion filters during STED microscopy.

(iv) In this study, we have not calculated the 3D distances of CT7 from each nucleus during differentiation/Nup93/CTCF depletions, as we focused on determining the 3D distances of the HOXA gene locus (which maps to CT7). Also, gene loci (average ~1-10 kb) which are orders of magnitude smaller than chromosome territories (average ~100 Mbp), show independent dynamics compared to that of chromosome territories. We hybridized CT7 (green) as a landmark to ascertain that we indeed visualized the HOXA gene locus, which is on CT7 territory (iv) Based on these observations and previous findings (Xu et al., 2014) (Ferraiuolo et al., 2010) (Rousseau et al., 2014) (Kadota et al., 2020), we speculate a model visualizing the HOXA gene locus which re-organizes during differentiation with respect to Nup93 and CTCF.

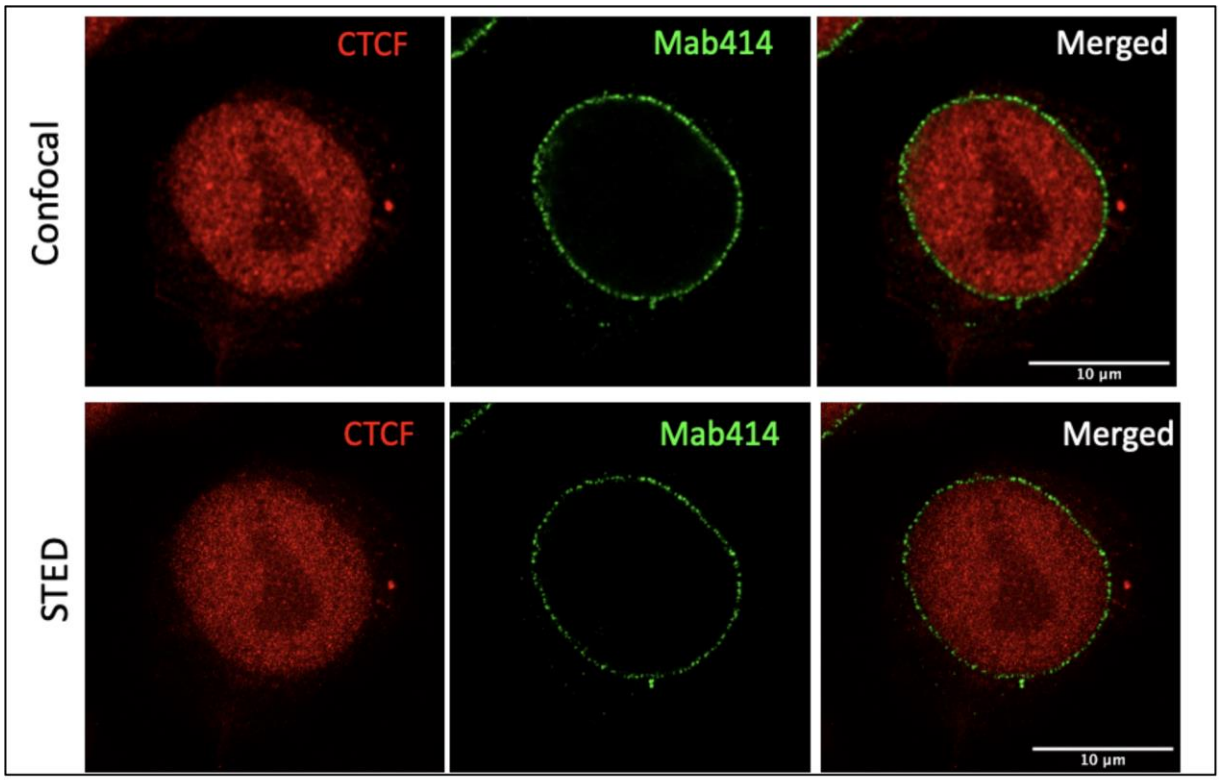

Fig. R3: We performed immunofluorescence assays, where we labelled CTCF and the nucleoporin using the antibody Mab414. Comparisons between confocal and STED imaging reveals the distinctively divergent localization of the nuclear pore complex at the nuclear border (Mab414), while CTCF shows a specific localization within the nucleoplasm. STED imaging resolves the nucleoporin and CTCF at a much higher resolution than confocal imaging.

Selected References

Ferraiuolo, M. A., Rousseau, M., Miyamoto, C., Shenker, S., Wang, X. Q. D., Nadler, M., Blanchette, M. and Dostie, J. (2010). The three-dimensional architecture of Hox cluster silencing. *Nucleic Acids Res.* **38**, 7472–7484.

Kadota, S., Ou, J., Shi, Y., Lee, J. T., Sun, J. and Yildirim, E. (2020). Nucleoporin 153 links nuclear pore complex to chromatin architecture by mediating CTCF and cohesin binding. *Nat. Commun.* **11**, 2606.

Labade, A. S., Karmodiya, K. and Sengupta, K. (2016). HOXA repression is mediated by nucleoporin Nup93 assisted by its interactors Nup188 and Nup205. *Epigenetics Chromatin* **9**, 54.

Oh, J. H., Kim, C. Y., Lee, J.-Y. and Kim, M. H. (2018). Retinoic acid and CTCF play key roles in inducing the collinear expression of the Hoxa cluster. *Acta Biochim Biophys Sin (Shanghai)* **50**, 555–559.

Rabut, G., Doye, V. and Ellenberg, J. (2004). Mapping the dynamic organization of the nuclear pore complex inside single living cells. *Nat. Cell Biol.* **6**, 1114–1121.

Rousseau, M., Crutchley, J. L., Miura, H., Suderman, M., Blanchette, M. and Dostie, J. (2014). Hox in motion: tracking HoxA cluster conformation during differentiation. *Nucleic Acids Res.* **42**, 1524–1540.

Toyama, B. H., Savas, J. N., Park, S. K., Harris, M. S., Ingolia, N. T., Yates, J. R. and Hetzer, M. W. (2013). Identification of long-lived proteins reveals exceptional stability of essential cellular structures. *Cell* **154**, 971–982.

Wang, X., Xu, M., Zhao, G., Liu, G., Hao, D., Lv, X. and Liu, D. (2015). Exploring CTCF and cohesin related chromatin architecture at HOXA gene cluster in primary human fibroblasts. *Sci. China Life Sci.* **58**, 860–866.

Xu, M., Zhao, G.-N., Lv, X., Liu, G., Wang, L. Y., Hao, D.-L., Wang, J., Liu, D.-P. and Liang, C.-

C. (2014). CTCF controls HOXA cluster silencing and mediates PRC2-repressive higher-order chromatin structure in NT2/D1 cells. *Mol. Cell. Biol.* **34**, 3867–3879.

Second decision letter

MS ID#: JOCES/2021/259307

MS TITLE: Nup93 and CTCF modulate spatiotemporal dynamics and function of the HOXA gene locus during differentiation

AUTHORS: Ajay Labade, Adwait Salvi, Saswati Kar, Krishanpal Karmodiya, and Kundan Sengupta
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

I am satisfied with the authors detailed responses to my queries.

Comments for the author

I am satisfied with the authors detailed responses to my queries.