

Review History

First round of review Reviewer 1

Were you able to assess all statistics in the manuscript, including the appropriateness of statistical tests

used? Yes, and I have assessed the statistics in my report.

Comments to author:

This manuscript describes a multi-omic investigation of early neutrophil differentiation, using RNA-seq, ATAC-seq and a variant of the Hi-C assay called Ocean-C. Prior work in this area had focused on early versus late stage neutrophils, whereas this study focuses on characterizing dynamic changes throughout the differentiation process. The approach is quite broad, albeit relatively straightforward. Notably, the authors have done a good job of employing a wide array of analysis techniques to investigate their data. On the other hand, some of the exposition of exactly what they did is frequently unclear. The last section of the results, which does multi-omic integration, is not very compelling.

Figure 1d is hard to interpret without some kind of comparison. Please show the pattern from doing this analysis on other time points. Without that, the claim that the short interactions were "dramatically increased" at this time point is not supported.

Line 166: What value of k was used in the k -means clustering and how are the two major clusters defined? Related to that, it is unclear what genes are included for Figure 2b (visually the plot does not seem to include all genes in the two major clusters).

In Figure 2c, what GO enrichment test is used and what is the gene ratio on the x axis? In Figure 2d, only a small set of aging and cell cycle genes are shown here. How were these genes selected?

In Figure 3b, the peak numbers for each time point (shown on y-axis) are different from the unique peaks numbers. How was this subset of peaks selected?

In Figure 3c, what is the absolute number of genome annotations per category across time?

What is the y-axis in Figure 4b,d and Figure 5b?

Line 340: What is the statistical significance for RARA binding site's enrichment in TSS of discovered TFs?

The networks shown in Figure 6 by themselves are not very informative, I would include GO terms enriched in each cluster (with added information on what enrichment test is used).

Could the short-range vs long-range proportion change across time and be related with cell cycle stage proportion difference?

Right now, the strongest evidence to support the important claim of "changes in chromatin accessibility, but not transcriptomics, occur at the early stage of neutrophil differentiation" is Figure 3e, but only two two gene examples are shown there. Can you show the overall trend across all genes in the up and down clusters? On a related note, can you show a systematic comparison of activation time of ChrInt, ChrAcc and gene expression across?

Line 300 claims that Figure 4d shows "clear association between ChrInt and gene expression changes across time," but I cannot see the association that based on the figure.

I found the last section of the results section pretty underwhelming. In particular, the graph shown in Figure 6a is not obviously useful -- it's merely a way to visually represent the coexpression network. I am not sure what we learn from this graph. Similarly, the multi-omics integration section is hard to evaluate. I can't definitely say that the technique the authors have used to create a multi-omic network is incorrect; indeed, on the face of it the method seems somewhat sensible. But I also don't see any evidence that this method is better than, say, creating a matrix of Pearson correlations separately from each of the three data types, summing those matrices, and then running k-means clustering on it. The end goal here seems to be to create a clustering, and there are many ways to do that from heterogeneous data. If the authors want to invent a new method, then I think they have to explain (and demonstrate) why it's better than other methods for clustering heterogeneous data.

Reviewer 2

Were you able to assess all statistics in the manuscript, including the appropriateness of statistical tests

used?

No, I do not feel adequately qualified to assess the statistics.

Comments to author:

An integrated multi-omics and network analysis of neutrophil 1 differentiation from early to late-stage

While the development of mononuclear cells is well understood in terms of genetic and epigenetics, the differentiation of polymorphonuclear cells remains in a rudimentary stage. In this manuscript the authors have examined for alteration in genome topology, transcription signatures and chromatin accessibility during the course of neutrophil development. Specifically, the authors show that exposure to all-trans-retinoic acid (ATRA) treatment instructs large-scale alteration in nuclear architecture to swiftly orchestrate a neutrophil-specific transcription signature. The authors identify a transcription factor network that underpins neutrophil activation.

The development of polymorphonuclear cells is an important topic in the general area of hematology. The data point to a critical role for genome topology in orchestrating neutrophil differentiation. Additionally, data is presented indicating a critical role for transcription factor activity in modulating neutrophil differentiation. A problem with the manuscript is that previous studies already have demonstrated a potential role for chromatin architecture in neutrophil differentiation. In my opinion the authors need to go beyond those previous observations in order to make the paper suitable for Genome Biology. For example, it would be important to link changes in genome topology to alterations in the transcriptome. This should be done quantitatively (genome-wide) and at the level of individual loci. In particular a correlation should be made between chromatin folding at genomic loci that encode for key transcription factors that establish neutrophil identity, including Spi1, Cebpa, Cebpb, Id1 and so on. It would also be important to relate changes in chromatin accessibility to the assembly of de novo loops. One question that comes to mind is whether the activation of gene expression involving transcription factors instructs the assembly of a neutrophil-specific chromatin architecture. The paper would benefit greatly from experiments that examine whether ablation of Spi1 or Cebp members affect neutrophil-specific nuclear architecture.

Additional comments:

(i) The authors state: The contributions of genome conformational changes towards early stages of neutrophil differentiation have not been studied. This is not correct and appropriate references should be included throughout the manuscript.

(ii) Wright-Giemsa staining should be performed for the different differentiation end-points. (iii) Intra-TAD frequency interactions should be provided for the various conditions.

(iv) Could the authors please show in quantitative terms how changes in looping correlate with gene expression. Examples should be provided.

Authors Response

Dear Claudia,

We would like to thank you, your colleagues at the Genome Biology editorial team, and the anonymous reviewers for your understanding and accommodation for our revision, which has been delayed.

We sincerely appreciate the reviewers' expert evaluation and comments. We have revised our manuscript extensively based on these comments. Major revisions include:

- Performed the first transcription factor-mediated 3DG experiments in neutrophils using CEBPA HiCut.
- Monitored HL60 differentiation using Wright-Giemsa staining.
- Re-analyzed RARA ChIP-seq data to investigate the effect of ATRA induction.
- Performed Diffhic analysis to identify pairwise differentially interacting chromatin regions.
- Analyzed intra-TAD interaction frequencies.
- Extended TAD+N analysis to other time points.
- Performed GO analysis on all GRN clusters.
- Improved manuscript clarity by incorporating focused introductory and concluding remarks for each section.

A point-to-point response to the comments of the reviewers is also included below.

Reviewer 1:

Comments:

This manuscript describes a multi-omic investigation of early neutrophil differentiation, using RNA-seq, ATAC-seq and a variant of the Hi-C assay called Ocean-C. Prior work in this area had focused on early versus late stage neutrophils, whereas this study focuses on characterizing dynamic changes throughout the differentiation process. The approach is quite broad, albeit relatively straightforward. Notably, the authors have done a good job of employing a wide array of analysis techniques to investigate their data. On the other hand, some of the exposition of exactly what they did is frequently unclear. The last section of the results, which does multi-omic integration, is not very compelling.

Response: We thank the reviewer for the thoughtful summary highlighting the importance of understanding neutrophil biology. To address the comments regarding unclear exposition and the less compelling final section, we have implemented the following changes:

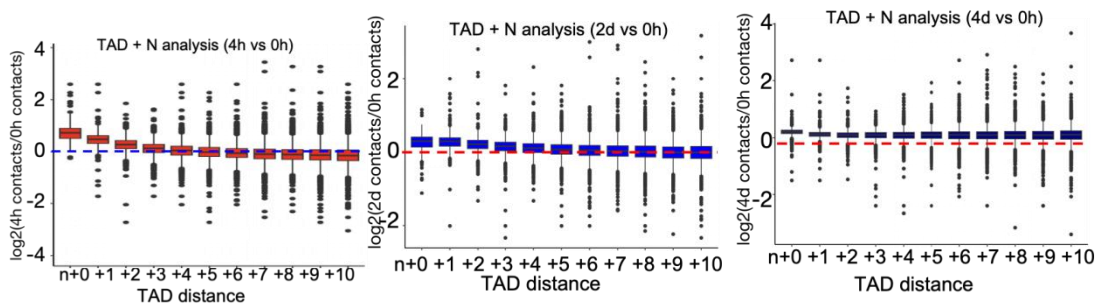
Enhanced Clarity and Conclusion Integration: We have significantly improved the clarity of the manuscript by adding a concise summary and clear conclusions after each individual study/analysis. This ensures that the key findings and contributions of each section are explicitly highlighted and easily understood by the reader.

Integrated GRN and TF Dynamics: We have merged the Gene Regulatory Network (GRN) analysis section with the transcription factor (TF) dynamics discussion. This

integration allows for a more cohesive narrative, and strengthens the overall argument by presenting these interconnected aspects in a unified manner.

Q1: Figure 1d is hard to interpret without some kind of comparison. Please show the pattern from doing this analysis on other time points. Without that, the claim that the short interactions were "dramatically increased" at this time point is not supported.

Response: We agree with the reviewer and appreciate his/her suggestion to include additional comparisons to consolidate our findings. As requested, we performed the TAD+N analysis on the other two time points (2d and 4d) comparing with 0h, and added the corresponding figures to Supplementary Figure 2f. For your reference, we also present the three comparisons at the Review-Only Figure 1 below.

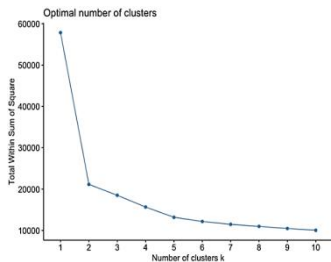


Review-Only Figure 1. TAD+N analysis on three time points including 4h, 2d and 4d.

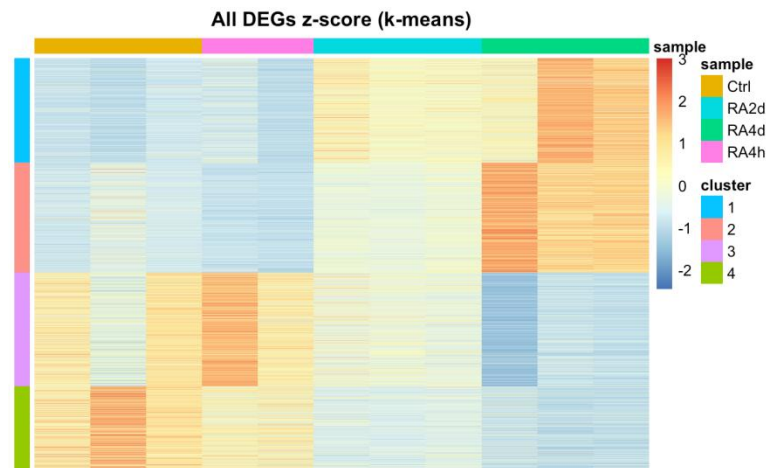
The results clearly indicate, when compare with 2d and 4d, the short interactions were dramatically increased at 4h, as is indicated by the overall positive log2 fold change within n+0, n+1, n+2 categories.

Q2: Line 166: What value of k was used in the k-means clustering and how are the two major clusters defined? Related to that, it is unclear what genes are included for Figure 2b (visually the plot does not seem to include all genes in the two major clusters).

Response: We have rephrased the text to clarify this part. First, k=2 was used in the Heatmap (figure 2b). The value of k was chosen using the function 'fviz_nbclust' from R package factoextra, which determines the optimal number of clusters based on the within-cluster sums of squares. As shown in the



following plot, $k = 2$ was the inflection point and was thus chosen for the clustering. In fact, different k values do not change the clustering results (i.e., the two main clusters) but only lead to sub-clusters within them. For example, the following heatmap shows the clustering pattern for $k=4$, where the structure of two major clusters is still maintained.



Reviewer-Only Figure 3.

Second, for the genes used in Figure 2b, we combined all the pairwise DEGs among the four conditions (total of six pairs), yielding a total of 5929 unique genes. These genes were used for k-means clustering, resulting in two clusters (2833 and 3096 genes, respectively, adding up to 5929). We have added these number to the manuscript (page 7 line159).

Q3: In Figure 2c, what GO enrichment test is used and what is the gene ratio on the x axis?

Response: clusterProfiler is used for enrichment test. Gene Ratio, calculated by cluster Profiler, indicates the ratio of input genes that are annotated in a specific term. We have added the explanation to the manuscript Method part(page 21 line 507).

Q4: In Figure 2d, only a small set of aging and cell cycle genes are shown here. How were these genes selected?

Response: Aging and cell cycle genes are selected from the literature: Xie X, Shi Q, Wu P, Zhang X, Kambara H, Su J, et al. Single-cell transcriptome profiling reveals neutrophil heterogeneity in homeostasis and infection. Nat Immunol.

2020;21:1119–33 We have added a method section to clearly describe how genes are selected from the literature (page 21 line 512).

Q5: In Figure 3b, the peak numbers for each time point (shown on y-axis) are different from the unique peaks numbers. How was this subset of peaks selected?

Response: The different numbers (0h: 23,690; 4h: 46,022; 2d: 36,881; 4d: 28,727) represent the total ChrAcc peak count in each time point. After identifying all pair-wise differential ChrAcc sites using EdgeR, we found a total of 18,014 unique differential ChrAcc peaks. These were then clustered, resulting in 2,131, 6,494, 2,200, and 7,189 specific ChrAcc sites in 0h, 4h, 2d, and 4d, respectively, as shown in Figure 3b ($2,131 + 6,494 + 2,200 + 7,189 = 18,014$). We have revised the text to clarify this point (page 9 line 194). Additionally, we have corrected a typo in the main figure; "2123" should be "2131".

Q6: In Figure 3c, what is the absolute number of genome annotations per category across time?

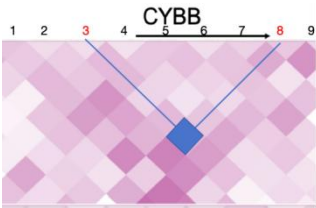
Response: We summarized the absolute number of genome annotations per category across time, as shown in the following table. Additionally, we have provided detailed annotations for each peak along with the summary table in Supplementary Table S2.

Number of annotation	0h	4h	2d	4d
3' UTR	20	146	20	95
5' UTR	10	35	5	33
Distal Intergenic	1200	2790	1362	1595
Downstream (<=300bp)	1	10	1	12
Exon	58	206	31	194
Intron	630	2884	695	952
Promoter	212	423	86	4308
Total	2131	6494	2200	7189

Reviewer-Only Table 1. The number of annotations per category.

Q7: What is the y-axis in Figure 4b,d and Figure 5b?

Response: There are no “y-axis” in these figures.

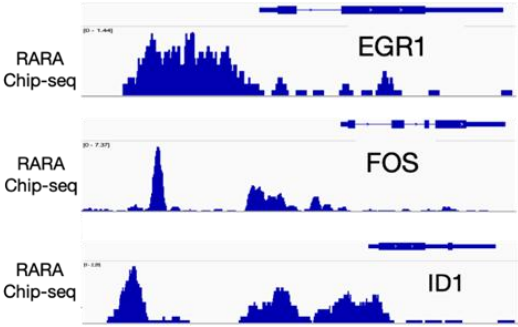


Instead, they are 2D displays of chromatin interactions (Chrlnt) around specific genes, where x-axis represents the linear chromosome segment around the gene. Each pixel in the heatmap represents the Chrlnt between two specific locations.

An illustrative example is shown at Reviewer-Only Figure 4. For simplicity, we took out the genome locations around the CYBB gene and named each bin from 1 to 9. The highlighted square pixel shows the Chrlnt between bins 3 and 8.

Q8: Line 340: What is the statistical significance for RARA binding site's enrichment in TSS of discovered TFs?

Response: We downloaded the original RARA ChIP-seq data and reanalyzed it to determine the statistical significance of RARA binding site enrichment around the TSS. As shown below, the peaks around the TF's TSS all exhibit significant RARA binding enrichment (Reviewer-Only Figure 5; Supplementary Fig S7f). We have also added the corresponding $-\log_{10}(p \text{ value})$: EGR1: 12.5, ID1: 11.9, FOS:46.2.



chr	start	end	length	abs_summit	pileup	$-\text{LOG}_{10}(p\text{value})^1$	fold_enrich	$-\text{LOG}_{10}(q\text{value})^1$	name	Asso_gene
chr4	48272013	48272227	215	48272085	16	12.4852	6.98956	9.26901	SRR032074_peak_15967	EGR1
chr20	30190722	30190921	200	30190849	16	11.9262	6.65258	8.73469	SRR032074_peak_13146	ID1
chr14	75741617	75741875	259	75741754	53	46.1838	13.1855	41.9954	SRR032074_peak_6261	FOS
chr14	75743624	75743948	325	75743726	22	11.9895	5.41253	8.79516	SRR032074_peak_6262	FOS

Q9: The networks shown in Figure 6 by themselves are not very informative, I would include GO terms enriched in each cluster (with added information on what enrichment test is used).

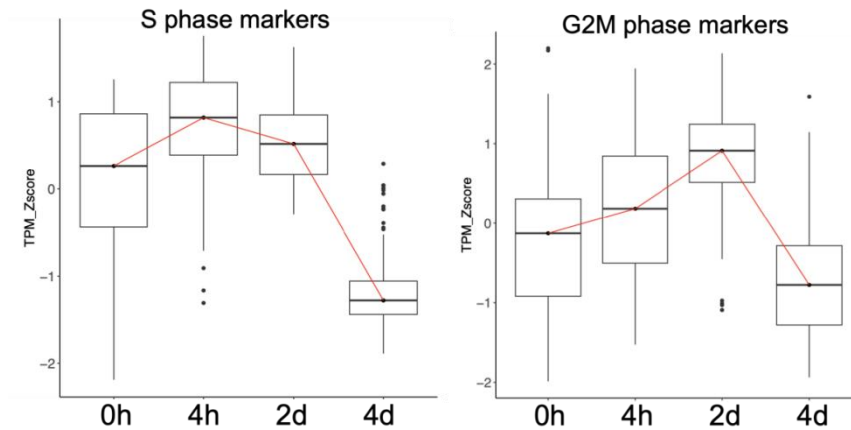
Response: As requested, clusterProfiler is used for enrichment test. we added the enriched GO terms of each cluster as additional file 6. An illustrative example of cluster 3 and 7 is added to Supplementary Fig. S8b.

Q10: Could the short-range vs long-range proportion change across time and be related with cell cycle stage proportion difference?

Response: We would like to thank the reviewer for the insightful question. Based on our analysis (Figure 1a), we observed that the proportion of short-range interactions increased, while the proportion of long-range interactions decreased at 4 hours compared to 0 hours. Whether this is related to cell cycle stage proportion difference is worth further investigation. Indeed, a previously published study (by Nagano et al. 2018 Nature) using single-cell Hi-C revealed the cell-cycle dynamics of chromosomal organization, which showed a smooth, cyclical transition of the distribution of genomic contact distances. This cyclical pattern implies that the difference in the cell cycle stage proportion could affect the distribution of genomic contact distances.

In our data, we investigated the proportion of the cell cycle stage based on the known cell-cycle stage-specific genes (provided by the Seurat R package) including S phase markers and G2M phase markers. The gene expression patterns (Reviewer-Only Figure 6 below) across the differentiation time points clearly indicate the differences at different cell cycle stages, which may affect the distribution of genomic contact distances. We have added a paragraph in the discussion section to reflect the possible effects of cell cycle stages as below (page 18 line 419).

“A clear observation we made is that the proportion of short-range interactions increased, while the proportion of long-range interactions decreased at 4 hours compared to 0 hours. While this pattern is less pronounced at 2 and 4 days, the overall dynamics of genomic contact distances could be influenced by several factors. These include nucleus condensation, and importantly, variations in cell cycle stage proportions. Our analysis of cell-cycle stage-specific gene expression (S-phase and G2M-phase markers) across different time points indicates a shift in cell cycle distribution: S-phase markers are relatively more abundant at the early 4-hour stage, while G2M-phase markers show increased prominence at the later 2-day stage. These differences in cell cycle stage proportions within the population could contribute to the observed changes in the distribution of genomic contact distances. Despite the complexity arising from such influencing factors, the significant and specific changes in chromatin accessibility (ChrAcc) observed at 4 hours further reinforce the notion of active chromatin reorganization at this particular time point, which aligns with the observed increase in short-range interactions.”



Reviewer-Only Figure 6.

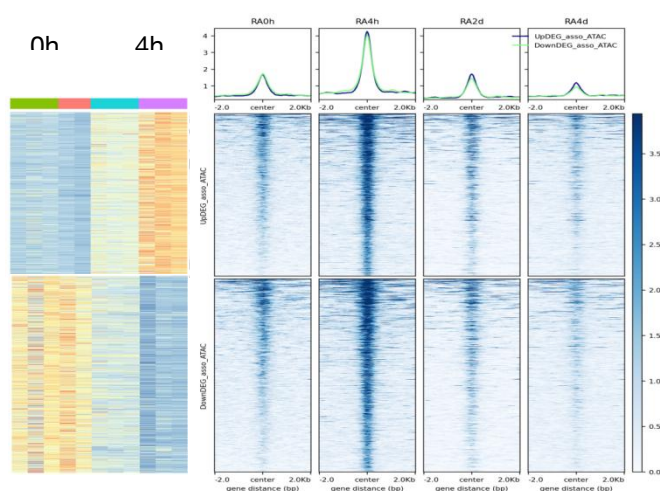
Q11: Right now, the strongest evidence to support the important claim of "changes in chromatin accessibility, but not transcriptomics, occur at the early stage of neutrophil differentiation" is Figure 3e, but only two two gene examples are shown there. Can you show the overall trend across all genes in the up and down clusters? On a related note, can you show a systematic comparison of activation time of ChrInt, ChrAcc and gene expression across?

Response: We greatly appreciate the reviewer's insightful request to present the overall trend and a systematic comparison to further substantiate our claim regarding the early kinetics of chromatin remodeling versus gene expression during neutrophil differentiation. This indeed is a core finding of our study.

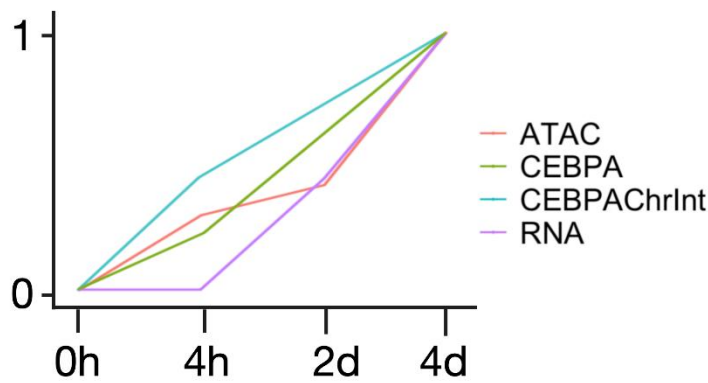
To provide a global perspective on how chromatin accessibility changes precede transcriptional activity, we analyzed the expression of genes proximal to timepoint-specific ChrAcc sites. We found that for both "up-cluster" and "down-cluster" DEGs identified in Figure 2, their associated ChrAcc sites generally reached maximum accessibility at 4h (Supplementary Fig. S4d) (Reviewer-Only Figure 7a below). We hypothesize that the 4h timepoint resembles a stem cell-like stage, where numerous chromatin regions are opened to provide multiple possibilities for subsequent cell fate determination (as seen in Figure 3a, where the PC1 position of 4h ChrAcc precedes that of 0h, suggesting a progenitor-like state). However, whether these opened regions remain accessible depends on the binding of appropriate transcription factors to these newly accessible sites, such as CEBPA. In Supplementary Figure S10, several examples demonstrate that regardless of whether genes are upregulated or downregulated at 4d, their associated ChrAcc sites show increased accessibility at 4h. For genes upregulated at 4d, increased CEBPA binding correlates with enhanced ChrInt and RNA expression, further strengthening chromatin accessibility. Conversely, for genes downregulated at 4d,

reduced CEBPA binding is associated with decreased Chrlnt and RNA expression, leading to diminished chromatin accessibility. These examples help explain why many 4h ChrAcc changes are transient.

To systematically compare the activation timing across chromatin interaction (Chrlnt from CEBPA HiCUT), chromatin accessibility (ChrAcc from ATAC-seq), and gene expression (RNA-seq) at a global level, we developed a unified approach for kinetic analysis. We focused on the upregulated cluster of differentially expressed genes (DEGs) identified in our RNA-seq data. First, these DEGs were filtered based on their association with CEBPA peaks, retaining only those whose TSS $\pm$ 1 kb overlapped with CEBPA peaks. The corresponding CEBPA peak signals linked to these DEGs were also extracted. Next, focusing on these DEGs, we further extracted gene-associated ATAC-seq signals (TSS $\pm$ 1 kb) and CEBPA-associated chromatin interaction (Chrlnt) signals (TSS $\pm$ 5 bin row sums). For each data type, we calculated the overall mean signal across different time points. Finally, individual curves were plotted together after normalizing the y-axis to a scale of 0 to 1 (Page 32 line 761). This normalization allowed us to directly compare the temporal kinetics of changes across these distinct molecular modalities in a quantitative and unbiased manner. Our systematic comparison, which we now present in a new integrative Supplementary Figure S9d (Reviewer-Only Figure 7b below), consistently demonstrates a clear temporal hierarchy: changes in both chromatin interactions (Chrlnt) and chromatin accessibility (ChrAcc) generally initiate and reach their maximum relative change earlier than observable changes in gene expression (RNA). This global kinetic analysis provides compelling evidence that the 'remodeling' or 'opening' of chromatin, both at the local accessible sites and through altered long-range interactions, typically occurs as an initial regulatory event (e.g., as early as 4 hours), effectively priming the genomic landscape for subsequent widespread transcriptional responses that become more evident at later stages (e.g., 2 days and beyond).



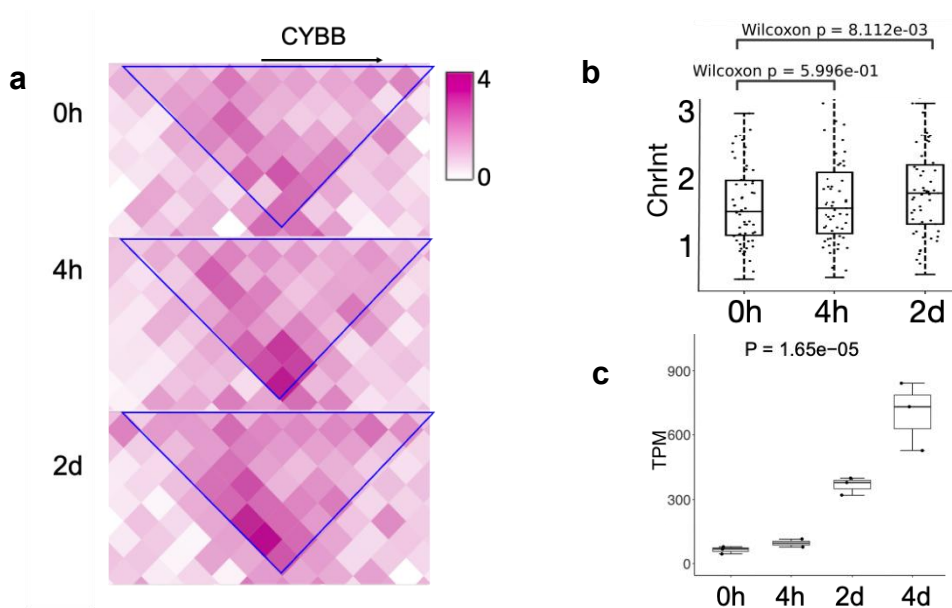
Reviewer-Only Figure 7a.



Reviewer-Only Figure 7b.

Q12: Line 300 claims that Figure 4d shows "clear association between ChrInt and gene expression changes across time," but I cannot see the association that based on the figure.

Response: We agree with the reviewer that the association is not clearly evident from the figure. To address this point, we conducted further analysis. We performed a more detailed examination of the overall ChrInt in the highlighted region around the gene CYBB in figure 4, and examined the ChrInt and gene expression changes from 0h to 2d, but not 4d, as 4d data ChrInt is significantly affected by nuclear condensation. The results, shown in Figure 4b-d (Reviewer-Only Fig 8 below) and also indicate the same trend of ChrInt and gene expression changes as shown by the boxplots. The mean ChrInt from 0h to 2d are 1.571372, 1.679624, and 1.799064.



Reviewer-Only Figure 8 a, 2D display of changes in chromatin interactions (ChrInt) around the CYBB gene (gene shown with arrow and region that spanned the gene as marked with blue boxes). b, Boxplot showing the ChrInt strength changes within the highlighted regions in a, statistical differences were determined using paired Wilcoxon test, c, CYBB gene expression changes shown in boxplot, Statistical differences were determined using ANOVA.

Q13: I found the last section of the results section pretty underwhelming. In particular, the graph shown in Figure 6a is not obviously useful -- it's merely a way to visually represent the coexpression network. I am not sure what we learn from this graph. Similarly, the multi-omics integration section is hard to evaluate. I can't definitely say that the technique the authors have used to create a multi-omic network is incorrect; indeed, on the face of it the method seems somewhat sensible. But I also don't see any evidence that this method is better than, say, creating a matrix of Pearson correlations separately from each of the three data types, summing those matrices, and then running k-means clustering on it. The end goal here seems to be to create a clustering, and there are many ways to do that from heterogeneous data. If the authors want to invent a new method, then I think they have to explain (and demonstrate) why it's better than other methods for clustering heterogeneous data.

Response: We greatly appreciate the reviewer's candid and insightful feedback regarding the gene regulatory network (GRN) analysis. The primary objective of our GRN analysis was indeed to elucidate the hierarchical importance and coordinated action of various TFs during neutrophil differentiation. However, we acknowledge that the complexity of the inferred TF network, as initially presented, did not yield a particularly clear or digestible conclusion for the reader.

Given this valuable feedback, we have significantly revised this section to enhance its clarity and focus. Specifically, we have streamlined the presentation of the GRN analysis: some content has been removed, while other relevant parts have been relocated to a supplementary file (Supplementary Figure S8). Crucially, the most pertinent findings are now integrated with and serve as a supplement to the analysis in Figure 5, allowing for a more targeted discussion.

Critically, to provide a more definitive understanding of TF function, we have performed CEBPA HiCut experiments. This cutting-edge technique allowed us to directly link CEBPA binding sites with chromatin accessibility, 3D genomic organization, and gene expression information. This dedicated analysis for CEBPA provides stronger and more direct evidence to explore this crucial TF's role in triggering neutrophil differentiation, moving beyond broad co-expression patterns to specific mechanistic insights. The results from the CEBPA HiCut analysis are now

prominently featured and discussed in the revised manuscript to underscore the specific regulatory functions of this key TF.

Reviewer 2:

Comments :

While the development of mononuclear cells is well understood in terms of genetic and epigenetics, the differentiation of polymorphonuclear cells remains in a rudimentary stage. In this manuscript the authors have examined for alteration in genome topology, transcription signatures and chromatin accessibility during the course of neutrophil development. Specifically, the authors show that exposure to all-trans-retinoic acid (ATRA) treatment instructs large-scale alteration in nuclear architecture to swiftly orchestrate a neutrophil-specific transcription signature. The authors identify a transcription factor network that underpins neutrophil activation.

The development of polymorphonuclear cells is an important topic in the general area of hematology. The data point to a critical role for genome topology in orchestrating neutrophil differentiation. Additionally, data is presented indicating a critical for transcription factor activity in modulating neutrophil differentiation.

Response: We thank the reviewer for the thoughtful summary and for highlighting the critical insights into understanding neutrophil differentiation.

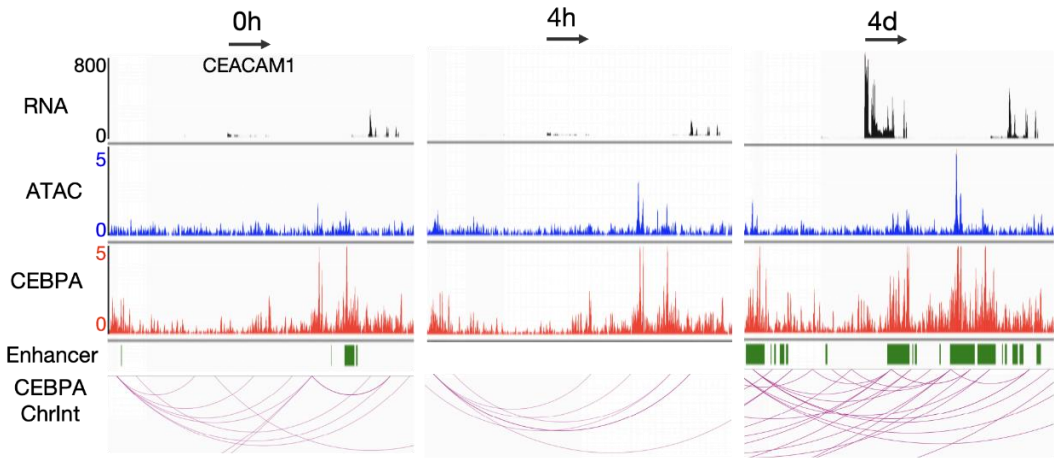
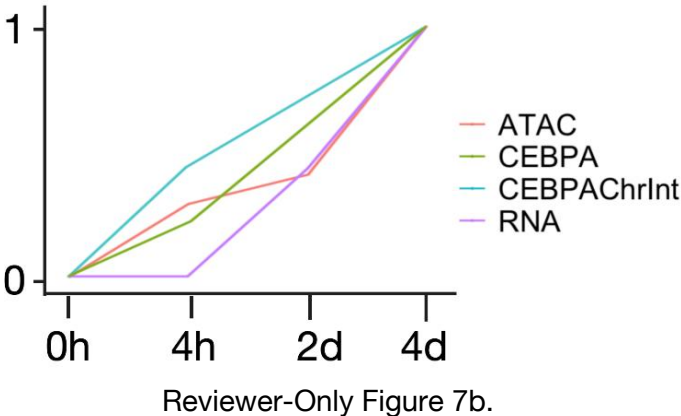
Q1 A problem with the manuscript is that previous studies already have demonstrated a potential role for chromatin architecture in neutrophil differentiation. In my opinion the authors need to go beyond those previous observations in order to make the paper suitable for Genome Biology. For example, it would be important to link changes in genome topology to alterations in the transcriptome. This should be done quantitatively (genome-wide) and at the level of individual loci. In particular a correlation should be made between chromatin folding at genomic loci that encode for key transcription factors that establish neutrophil identity, including Spi1, Cebpa, Cebpb, Id1 and so on.

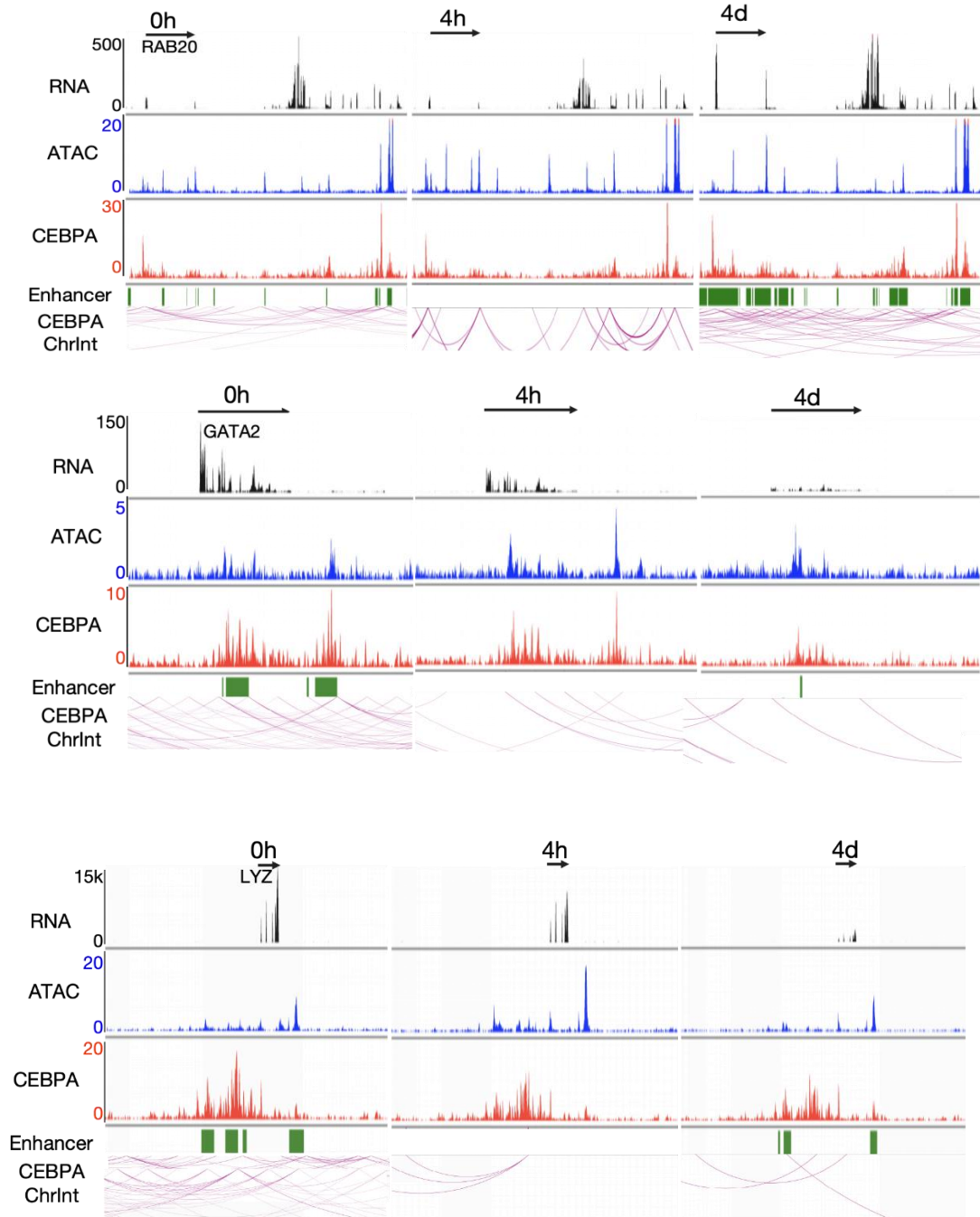
Q7:(iv) Could the authors please show in quantitative terms how changes in looping correlate with gene expression. Examples should be provided.

Response: We appreciate the reviewer's astute observation that prior studies have demonstrated a role for chromatin architecture in neutrophil differentiation. To unequivocally address the reviewer's call to "go beyond those previous observations" and more directly link changes in genome topology to alterations in the transcriptome, we performed the first transcription factor-mediated 3D genomic experiments in neutrophils using CEBPA HiCut. This cutting-edge technique allowed

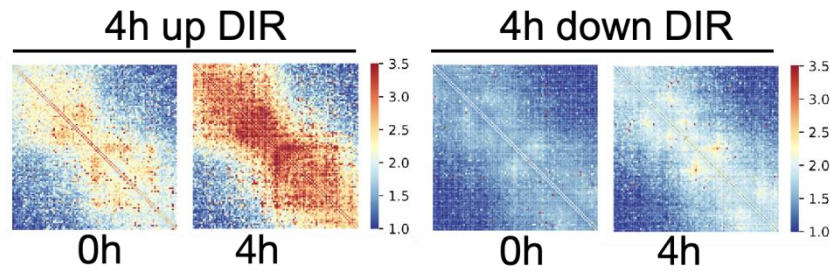
us to directly connect CEBPA binding sites with chromatin accessibility, 3D genomic organization, and gene expression information.

Our CEBPA HiCut analysis revealed a sequential regulatory cascade during neutrophil differentiation. For DEGs that associated with CEBPA peaks in the up regulated clusters (in Figure 2b), we observed that chromatin interactions and accessibility changes occurred first (at 4h), followed by enhanced CEBPA binding, and finally increased RNA expression (Supplementary Fig. S9d; Reviewer-Only Figure 7b). Representative examples such as CEACAM1 and RAB20 showed early accessibility increases and refined CEBPA-mediated interactions at 4h, with significant binding and interaction increases by 4d (Fig. 6e, Supplementary Fig. S10a, also shown below). Conversely, for downregulated genes like GATA2 and LYZ, we observed reduced CEBPA binding accompanied by corresponding decreases in chromatin interactions (Supplementary Fig. S10b-c, also shown below). These findings demonstrate the intricate coordination between 3D genome reorganization, chromatin accessibility, transcription factor binding, and gene expression during neutrophil differentiation. The comprehensive results from this CEBPA HiCut analysis are now prominently featured and discussed in the revised manuscript.



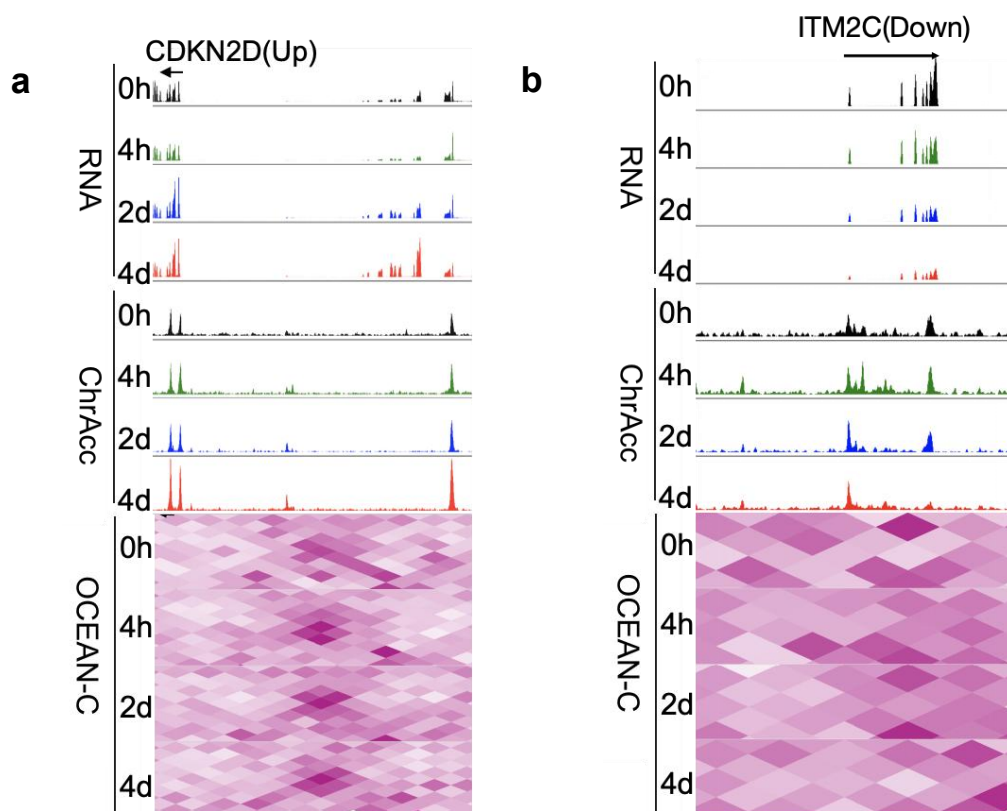


To link changes in genome topology to alterations in the transcriptome genome-wide, we perform diffHic to study changes in chromatin organization by identifying pairwise differentially interacting chromatin regions (DIRs) between two distinct genomic loci. We identified 15,577 up DIRs and 24,926 down DIRs. The aggregated OCEAN-C interaction contact heatmap, centered on these DIR sites, revealed significantly enhanced interactions towards up DIRs when comparing 4h with 0h. The results are presented in Reviewer-Only Figure 9 below and in the newly added Supplementary Fig S6a.



Reviewer-Only Figure 9. Heatmap showing differentially interacting chromatin regions (DIRs) (4h vs 0h) through OCEAN-C pile-up analysis centered DIR.

Next, we associated the differentially interacting chromatin regions (DIRs) with two clusters of differentially expressed genes (DEGs) that exhibited consistent directional behavior. We applied strict criteria, requiring that the transcription start site (TSS) of the DEG lies within the DIR anchor. We identified 326 up DIRs associated with up-regulated DEGs and 347 down DIRs associated with down-regulated DEGs. Two examples, *CDKN2D* (up-regulated) and *ITM2C* (down-regulated), with highlighted DIRs. The results are presented in Reviewer-Only Figure 10 below and in the newly added Supplementary Fig S6b.

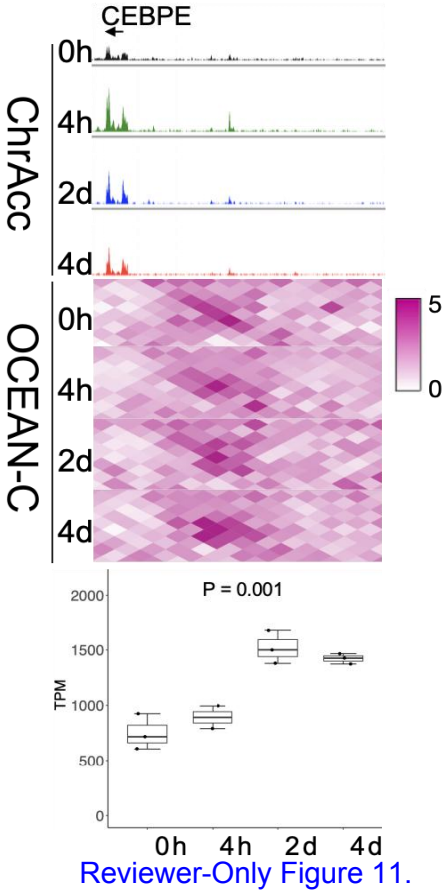


Reviewer-Only Figure 10.1D display of changes in gene expression and chromatin accessibility (ChrAcc) together with 2D display of changes in ChrInt around the *CDKN2D*(a) and *ITM2C*(b).

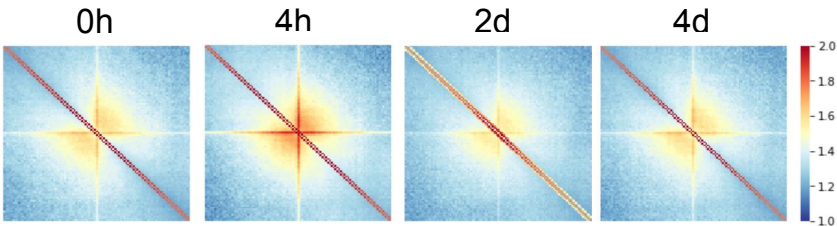
Additionally, Reviewer 1's Q11 raised similar concerns, and our response to that question may also address your valuable suggestions.

Q2: It would also be important to relate changes in chromatin accessibility to the assembly of de novo loops.

Response: We thank the reviewer for highlighting the importance of relating chromatin accessibility changes to the assembly of de novo loops. We then address this relationship through genome-wide analyses and at the level of individual loci. The aggregated OCEAN-C interaction heatmap, centered on chromatin accessibility (ChrAcc) sites, revealed that 4h-specific ChrAcc sites were significantly enriched for 4h-specific OCEAN-C interactions (Reviewer-Only Figure 12; Fig. 4a). We further validated this relationship at individual loci critical for neutrophil function, particularly at the CEBPE locus (Reviewer-Only Figure 11; Supplementary Fig. S5b), which shows striking coordination between chromatin accessibility and the formation of de novo chromatin loops during differentiation, with similar patterns also observed at CYBB (Fig. 4b) and ALOX5AP (Supplementary Fig. S5a) loci. These examples collectively demonstrate the strong correlation between chromatin accessibility changes and the establishment of de novo loops during neutrophil differentiation.



ChrAcc sites centered OCEAN-C interactions



Reviewer-Only Figure 12. Aggregated OCEAN-C interaction contact heatmap for 4h-specific chromatin accessibility (ChrAcc) peaks among all the samples

Q3: One question that comes to mind is whether the activation of gene expression involving transcription factors instruct the assembly of a neutrophil specific chromatin architecture. The paper would benefit greatly from experiments that examine whether ablation of Spi1 or Cebp members affect neutrophil specific nuclear architecture.

Response: We agree with the reviewer regarding the importance of validating transcription factors on neutrophil-specific nuclear architecture, and we greatly appreciate this valuable suggestion.

First, we conducted experiments involving EGR1 knockout. However, these experiments showed limited impact on cell differentiation and overall neutrophil architecture. This observed limited effect is likely attributable to EGR1's relatively low expression level (TPM around 10, compared to hundreds for key transcription factors like CEBPA, CEBPE, and SPI1) and the potential for cellular compensatory mechanisms. This preliminary finding underscored the necessity of investigating a more central regulatory factor.

Consequently, recognizing CEBPA as a well-established pioneer transcription factor for neutrophil differentiation, we performed CEBPA HiCut experiments to mechanistically dissect its role in orchestrating neutrophil differentiation. This cutting-edge technique allowed us to directly link CEBPA binding sites with chromatin accessibility, 3D genomic organization, and gene expression information. This approach was favored over a CEBPA knockout (KO) strategy, as KO of such a pivotal upstream factor would likely result in widespread, indirect downstream effects, rather than pinpointing CEBPA's direct instructive role in shaping chromatin architecture.

Our CEBPA HiCut data demonstrate an initial enhancement of its DNA binding activity and corresponding, dynamic changes in DNA looping at 4 hours. These architectural effects become more pronounced by 4 days and persist even as mRNA levels subsequently decrease, robustly promoting the expression of neutrophil-specific genes (Figure 6).

Additional comments:

Q4: (i)The authors state: The contributions of genome conformational changes towards early stages of neutrophil differentiation have not been studied. This is not correct and appropriate references should be included throughout the manuscript.

Response: We rephased statement in page 3 line 63 and added relevant references accordingly, especially the 2024 Nature paper which demonstrated that halting loop extrusion was sufficient to bring about the assembly of polymorphonuclear structures[Patta I, Zand M, Lee L, Mishra S, Bortnick A, Lu H, et al. Nuclear morphology is shaped by loop-extrusion programs. *Nature*. 2024;627:196–203], Other references added include Zhu et al. *Genes Dev* 2017 also demonstrated the

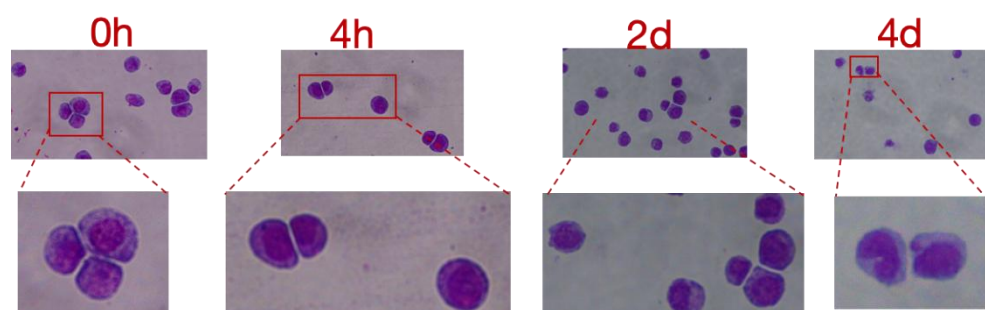
genome conformational changes like supercontraction and recruitment of heterochromatin and nucleoli to the nuclear lamina towards neutrophil differentiation.[Zhu Y, Gong K, Denholtz M, Chandra V, Kamps MP, Alber F, et al. Comprehensive characterization of neutrophil genome topology. *Genes Dev.* 2017;31:141–53.]

The revised statement in page 3 line 64 as follows:

The contributions of genome conformational changes towards neutrophil differentiation have been studied especially by Cornelis Murre's group, particularly their most recent paper in *Nature*, which demonstrated that halting loop extrusion was sufficient to bring about the assembly of polymorphonuclear structures. They also demonstrated the genome conformational changes like super-contraction and recruitment of heterochromatin and nucleoli to the nuclear lamina towards neutrophil differentiation.

Q5: (ii) Wright-Giemsa staining should be performed for the different differentiation end-points.

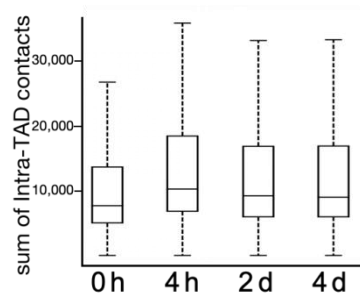
Response: We performed Wright-Giemsa staining to illustrate the dynamic nuclear morphology changes across the differentiation program. We have added the method to the manuscript (page 20. Line481) The results have been added to Supplementary Fig. S1a and Reviewer-Only Figure 13 below. The nucleus shape using Hoechst staining figure was also updated in Supplementary Fig. S1b.



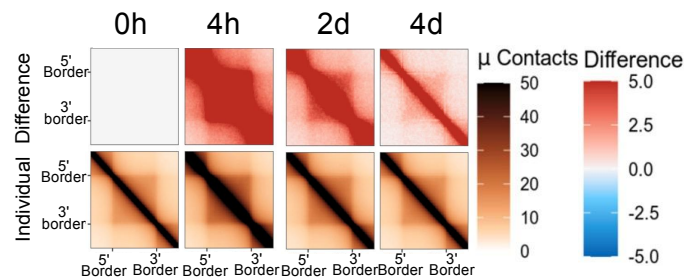
Reviewer-Only Figure 13. Wright-Giemsa staining of the neutrophil in different time point.

Q6:(iii) Intra-TAD frequency interactions should be provided for the various conditions.

Response: We added intra-TAD frequency interactions in Reviewer-Only Fig. 14 below, as well as in supplementary Figure S2e. The sum of normalized intra-TAD contacts was calculated for each condition, and the pattern is consistent with Figure 1C(also shown below), which shows highest intra-TAD interactions at 4h.



Reviewer-Only Figure 14. Intra-TAD frequency interactions across time



Second round of review

Reviewer 1

Overall, the manuscript is much improved. I particularly appreciate the overhaul of the final section and the focus on the HiCut results. I do have a number of additional suggestions, as outlined below.

In reviewer-only Figure ,1 why are the vertical axis ranges different? Please make them consistent. Also, fi you want to claim an enrichment, then you should report ni the text the median values of the log fold- changes in the different plots, so the reader doesn't have to visually compare between the main text figure and the supplementary figures in order to verify your claim. The claim should also be accompanied by a p-value from an appropriate statistical test.

Figure 4b requires better labeling. The response claims that the figure has no y-axis, but that seems odd to me. Clearly, there is a vertical axis on this figure, and ti seems that it corresponds to the genomic distance between the associated pairs of loci. This should be made clear with labeled axes, both -x and .y- The color scale also needs a label to explain what exactly the values are.

Please clarify what is the statistical test that you used to obtain the RARA enrichment p-value.

The new, systematic analysis of the timing of chromatin accessibility and gene expression appears to be well done, but I had trouble interpreting the reviewer-only Figure 7b, which lacks axis labels or any caption. Ialso wonder whether, given theapparent importance of this result (you said it was "a core finding of(y)our study," some version of this figure belongs in the main text.

Typo at line 356: "This analysis revealed consisting of trends:"

Reviewer 2

The data presented in the manuscript are overall compelling and well presented. The topic is of interest to the readership of Genome Biology. The authors have addressed, albeit not all, the comments raised by the reviewers. Hence, in the opinion of this reviewer the manuscript is now suitable for publication in Genome Biology.

An integrated multi-omics and network analysis of neutrophil differentiation from initial to late-stage

Previous studies revealed that the differentiation of neutrophils is closely associated with the induction of large-scale changes in genome topology. However, insights into the interplay involving changes in chromatin folding and gene expression remain tentative at best. In this manuscript the authors seek to determine how gene expression is linked with alterations in chromatin structure. They show the onset of neutrophil differentiation is closely linked with changes in chromatin topology is associated with the assembly of a dynamic transcription network that orchestrates a neutrophil-specific program of gene expression.

The data presented in the manuscript are overall compelling and well presented. The topic is of interest to the readership of Genome Biology. The authors have addressed, albeit not all, the comments raised by the reviewers. Hence, in the opinion of this reviewer the manuscript is now suitable for publication in Genome Biology.

Authors Response

Point-by-point responses to the reviewers' comments:

Dear Editor,

We are pleased to submit the 2nd revised version of our manuscript for your consideration. We have addressed and responded to each of the comments raised by Reviewer 1, which are detailed below. We sincerely appreciate the valuable feedback from the reviewers. Thank you for considering our submission. Sincerely,
Wei Li

Point-By-Point Response

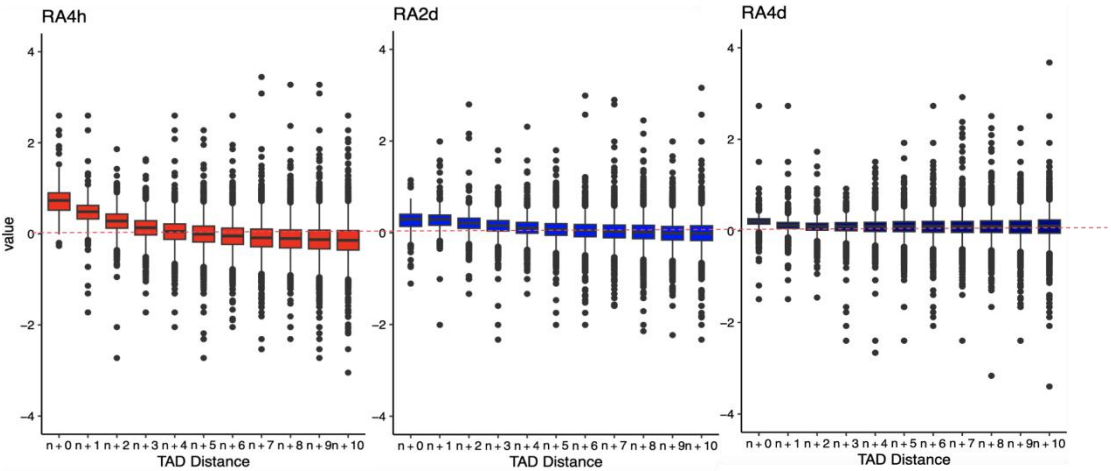
Reviewer #1: Overall, the manuscript is much improved. I particularly appreciate the overhaul of the final section and the focus on the HiCut results. I do have a number of additional suggestions, as outlined below.

Response: Thank you so much for your insightful feedback last time. It really helped

guide the revisions for this article.

In reviewer-only Figure 1, why are the vertical axis ranges different? Please make them consistent. Also, if you want to claim an enrichment, then you should report in the text the median values of the log fold-changes in the different plots, so the reader doesn't have to visually compare between the main text figure and the supplementary figures in order to verify your claim. The claim should also be accompanied by a p-value from an appropriate statistical test.

Response: Thank you for this excellent suggestion. We have revised the figures as recommended to ensure a consistent y-axis range, which we agree significantly improves clarity. The main text (Fig. 1d) and supplementary materials (Fig. S2f) have been updated accordingly. For your reference, we have also created the new figure below (Reviewer-only Figure 1) to illustrate this change.



Reviewer only Figure 1: TAD+N analysis (4h vs 0h, 2d vs 0h and 4d vs 0h)

Second, we have calculated the median value for each boxplot in the figure above. These values are provided in the following table. We also conducted a t-test and confirmed that the changes are statistically significant (Reviewer-only Table 1).The log2 fold change levels in TAD+0 showed statistically significant differences across the three time points (4h, 2d, and 4d), with median values of 0.73, 0.29, and 0.21, respectively. This trend was also observed in neighboring TADs. For example, TAD+1 had median levels of 0.48, 0.29, and 0.12, while TAD+2 had levels of 0.28, 0.21, and 0.09. These results indicate that both intra-TAD and neighbor TAD interactions were more frequent at the 4h time point compared to 2d and 4d.The main text has been updated as well to show the changes.

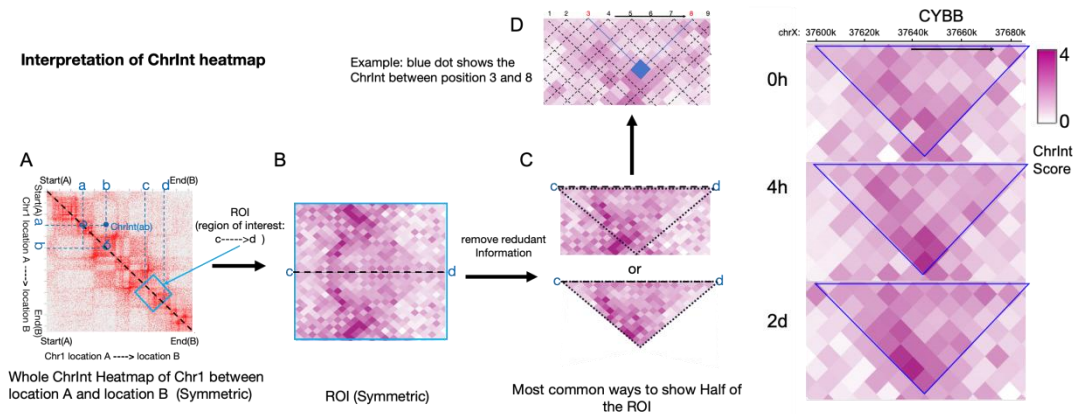
TAD N	RA4h_median	res_2d_median	res_4d_median	4hVS2d P value	4hVS4d P value	2dVS4d P value
0	0.732172394	0.295442089	0.206588434	2.22e-16	2.22e-16	2.22e-16
1	0.485011912	0.29142252	0.120405019	2.22e-16	2.22e-16	2.22e-16
2	0.280793154	0.212784778	0.095381692	2.22e-16	2.22e-16	2.22e-16
3	0.135045797	0.157079435	0.096842508	2.70E-05	2.22e-16	2.22e-16
4	0.052305366	0.107342107	0.095396156	2.22e-16	1.40-12	1.90E-07
5	-0.007701944	0.070876282	0.10028075	2.22e-16	2.22e-16	2.22e-16
6	-0.047230308	0.050042778	0.099770173	2.22e-16	2.22e-16	2.22e-16

7	-0.087872292	0.029368411	0.096113729	2.22e-16	2.22e-16	2.22e-16
8	-0.102412906	0.010342654	0.092933928	2.22e-16	2.22e-16	2.22e-16
9	-0.126586107	-0.008850911	0.092390643	2.22e-16	2.22e-16	2.22e-16
10	-0.142969968	-0.008850911	0.104806333	2.22e-16	2.22e-16	2.22e-16

Reviewer only Table 1: Median level of log fold-changes in the 3 different plots.

Figure 4b requires better labeling. The response claims that the figure has no y-axis, but that seems odd to me. Clearly, there is a vertical axis on this figure, and it seems that it corresponds to the genomic distance between the associated pairs of loci. This should be made clear with labeled axes, both x- and y-. The color scale also needs a label to explain what exactly the values are.

Response: We apologize for not explaining this clearly before. In general, this matrix represents the interaction frequencies between adjacent or distal genomic regions. We created an diagram to illustrate how to interpret ChrInt matrix heatmap (Reviewer only figure 2). As such, the coordinates of genomic regions in the matrix could be read from the horizontal axis located on the top line, as indicated by the arrows. Therefore, the y-axis does not necessarily need to be annotated. Based on this reason, we have annotated the x axes and updated the main Figure 4b accordingly. Similar changes has been updated to the supplementary figures as well.



Reviewer only figure 2: Interpretation of ChrInt matrix heatmap

Please clarify what is the statistical test that you used to obtain the RARA enrichment p-value.

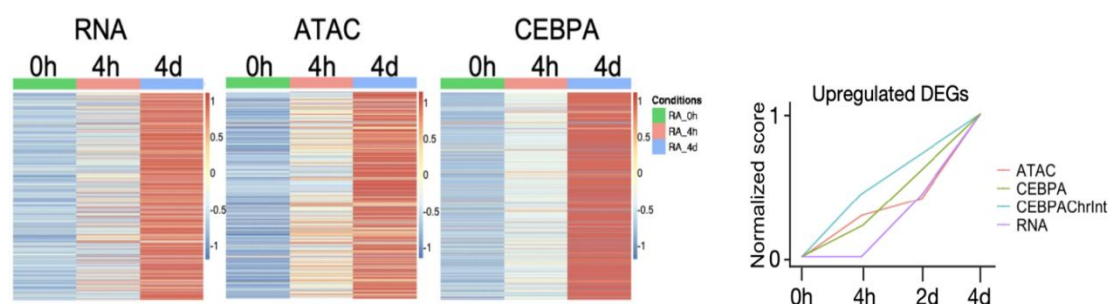
Response: We use Macs2 software to get RARA enrichment p-value. MACS2 calculates p values for identified peaks based on a dynamic Poisson distribution, which accounts for local biases in read background levels. The relevant content has been added to the main text.

The new, systematic analysis of the timing of chromatin accessibility and gene expression appears to be well done, but I had trouble interpreting the reviewer-only Figure 7b, which lacks axis labels or any caption. I also wonder whether, given the apparent importance of this result (you said it was "a core finding of [y]our study," some version of this figure belongs in the main text.

Response: Thank you for your appreciation and interest in this part of our research. We are pleased to have moved the previous reviewer-only Figure 7a and b to the main text as Figure 6e and f, with some adjustments, to underscore the importance of these findings.

In Figure 6e, we illustrate that for the RNA expression of upregulated DEGs that associated with CEBPA peaks, as well as the ATAC and CEBPA binding peaks within TSS $\pm$ 1 kb of those DEGs, there is a noticeable increase in RNA expression, chromatin accessibility, and CEBPA binding signals at 4 hours, with further enhancement observed at 4 days. To effectively compare the kinetics of these different datasets, we calculated the overall mean signal across various time points. The individual curves were then plotted together after normalizing the y-axis to a scale of 0 to 1. This analysis revealed a sequential regulatory cascade: chromatin interactions and accessibility changes occurred first at 4 hours, followed by CEBPA binding, and finally, RNA expression (Figure 6f).

We also added labels and captions for your reading convenience.



Reviewer only Figure 3: Dynamic analysis of CEBPA centered gene regulation during neutrophil differentiation.

a, Overall pattern of RNA expression of upregulated DEGs that associated with CEBPA peaks, as well as the ATAC and CEBPA binding peaks within TSS $\pm$ 1 kb of those DEGs.

b, A variation of normalized CEBPA peaks, CEBPA-chromatin interactions (CEBPA-ChrInt), chromatin accessibility, and RNA expression for upregulated DEG associated with CEBPA peaks during neutrophil differentiation.

Typo at line 356: "This analysis revealed consisting of trends:"

Response: Thank you for your correction. We have now revised it to: This analysis revealed consistent trends.

Reviewer #2: The data presented in the manuscript are overall compelling and well presented. The topic is of interest to the readership of Genome Biology. The authors have addressed, albeit not all, the comments raised by the reviewers. Hence, in the opinion of this reviewer the manuscript is now suitable for publication in Genome Biology.

Response: Thank you very much for your valuable suggestions during the last review. They significantly helped us enhance the quality of the article.