

Two distinct chromatin modules regulate proinflammatory gene expression

Corresponding Author: Professor Karsten Rippe

Version 0:

Decision Letter:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Professor Rippe,

Your manuscript, "Two distinct chromatin modules regulate proinflammatory gene expression", has now been seen by 3 referees, who are experts in transcription factor binding (referee 1); chromatin and gene regulation (referee 2); and transcription control (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A- Clarify on the differences between AC and DC modules as well as the details of AC and DC mapping (Rev#1 pts 4-7, Reviewer#2)

B- Analyse how RWireX compares to other tools to calculate co-accessibility (Reviewer#2 and Reviewer#1)

C- Address reviewer#3's point about the physiological relevance of the findings obtained in HUVEC cells and the broad applicability of RWireX to other cell types (Reviewer#3 pt2)

D- All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes, improve the clarity and readability of the manuscript along the lines suggested by reviewers. (reviewer#1 pts 1-3, Reviewer #3 pt 12) should also be addressed.

- Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and <https://www.nature.com/nature/for-authors>).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here <https://www.nature.com/documents/nr-reporting-summary.pdf>). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

When submitting the revised version of your manuscript, please pay close attention to our [Digital Image Integrity Guidelines](https://www.nature.com/nature-portfolio/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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We would like to receive a revised submission within six months.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Sabrya Carim

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Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

Seufert et al. investigate the mechanisms of gene coregulation in human endothelial cells by stimulating an inflammatory response using TNF α . The authors profile genome-wide epigenetic response dynamics at 0, 30 and 240 minutes after TNF α stimulation with scRNA-seq, scATAC-seq, and microscopy using FISH to visualize nascent transcripts in the CXCL gene cluster. To perform a genome-wide analysis of single-cell co-accessibility data, they developed a novel computational

strategy called RWireX which revealed two distinct types of chromatin compartments with the following characteristics:

1. Autonomous links of co-accessibility (AC)
 - a. Long-range enhancer-promoter/CRE interactions
 - b. Regulation via transcriptional bursting
 - c. Protein-coding, downregulated, and late-response TNF α -regulated genes (TRGs)
 - d. Weak correlation, suggesting they may not promote co-expression and potentially transition to a repressive chromatin state by 240 minutes.
2. Domains of contiguous co-accessibility (DC)
 - a. Increased local transcription factor (TF) binding (at 10kb resolution)
 - b. Modulation of transcription burst size
 - c. Clustered, upregulated, early response TRGs.

Together, the goal of this study was to uncover the mechanisms of coordinated gene regulation in response to TNF α stimulation. Although TNF α signaling is well characterized, the authors extend previous findings by connecting chromatin organization to transcriptional bursting, NF- κ B factory formation, while using publicly available data from other biological systems to explore the generalizability of their discoveries. The paper addresses important questions in the field, the authors validate their results across replicates, and the figures look aesthetically pleasing. However, the manuscript was rather difficult to follow as the study is highly complex, and the manuscript would therefore benefit from improved methodological transparency, additional quantitative assessments, benchmarking, and clearer explanations of key results. In addition, the results presented in the manuscript are often scattered, and are not consistently contextualized within the established terminology of the field. Finally, the conclusions presented in the study rely on specific parameters used for chromatin module mapping with RWireX, which, without benchmarking, limits the generalizability of the methodology and weakens confidence in the main findings. Below, we provide suggestions of how the authors could enhance the clarity of the manuscript and make the results more accessible and convincing to readers.

Major comments

1. The study would benefit from more thoroughly contextualizing the results within existing literature to better highlight their relevance and novelty. Specifically, the manuscript should provide a clearer introduction to the RWireX method, explaining its novelty and the motivation behind its development. It is especially important to place the method in the context of existing single-cell approaches to justify its use, i.e., why is a new computational framework needed? How does it compare to existing tools which use single-cell data to map co-accessible regions, such as ArchR and Cicero (Pliner et al., Molecular Cell 2018)? Benchmarking RWireX against these tools and demonstrating its advantages would underscore its utility, most importantly because the choice of thresholding highly influences the results and conclusions.
2. Expanding on the previous point, the manuscript should provide readers with an understanding of how the tool can be applied. For example, what is the required quality of the data? What parameters should be used and when? Adding such quantitative assessments and discussing the optimal conditions for using the tool would greatly enhance its applicability.
3. In Figure 2A, and in line with the previous point, the manuscript would benefit from a detailed description of the parameter selection strategy for clustering. A quantitative evaluation of how different parameters influence compartment properties would be crucial for supporting the statements made in the manuscript.
4. Despite the authors' efforts to explain the differences between AC and DC compartments, the information is scattered throughout the manuscript, making it difficult to form a cohesive understanding of the two types of modules. First, we suggest adding more details on the properties of these compartments to the 'highlights' section (e.g. by providing a comprehensive list of features for both ACs and DCs). Next, we suggest to begin by introducing the current state of the field, followed by a detailed explanation of AC modules, DC modules and their properties, and then building on top of that. This restructuring would enhance clarity and provide better context by linking new concepts to established terminology already familiar within the field.
5. Conceptually, it is unclear what the gene regulatory assumptions are behind mapping AC modules in a homogeneous population? What is the source of variation, considering that only the G1 phase was selected, so it cannot be the cell cycle? If co-accessibility changes at peaks are purely stochastic, how can their seemingly 'random appearance' reflect any underlying provide any functional insight?
6. It is also important to elaborate on DC mapping. What was the rationale for using 10kb tiles? How might the results change with different tile resolutions? What is the upper limit? If DCs were mapped per time point, would it be possible to capture alternations in chromatin activity between A/B compartments over time?
7. It would be helpful to provide a clear, quantitative evaluation of the total number of modules based solely on AC, solely on DC, or both. Currently, this information is discussed rather late in the text (Supplementary Figure 6B). Additionally, the low correlations shown in Figure 6C are rather unconvincing, making it difficult to draw any overarching conclusions from them. Are such low correlations expected? Are they driven by the sparsity of scRNA-seq data? Was expression data imputed using MAGIC (van Dijk et al., Cell, 2018)?
8. The manuscript would benefit from further elaboration and contextualization of the observed TAD overlaps with AC, DC, and AC/DC modules, as it is unclear at this time what can be really concluded here.
9. We believe that the single-cell data is not used to its full potential and that the results are dominated by batch effect, which was not regressed out during the preprocessing step (Figure 1B). Quality control metrics for single-cell data preprocessing, such as those provided by Scanpy or Seurat pipelines, should be included. Pseudo-bulking could support the results obtained from the single cell modality, yet we would envision it as a secondary/complementary approach to confirm findings from single cell analysis. Why were the time points at 30 minutes and 240 minutes not compared?
10. A clearer rationale for examining transcriptional bursting is needed: what was the motivation and reasoning for the

suggested analyses? Given the long intervals between time points, how do the results of the transcriptional bursting analysis (Figure 8) align with the expected “on” times of transcriptional bursts (typically ranging from minutes to hours in mammalian cells)? Similarly, how does this analysis fit with the transient residence times of TF binding to DNA (Figure 5), which usually last only a few seconds?

11. Do the “waves” in Figure 5B suggest the presence of other TFs binding in proximity or cooperative binding by TFs? Can the authors explain the higher accessibility upstream of the focal binding point? Why were only DCs used for the footprint analysis?

12. Would it be possible to include data tracks for the three time points to show differential accessibility at distal CREs and TF binding sites? Including expression values for each time point would provide an informative visual.

13. The illustration in Figure 8B, which is also used as the graphical abstract, is too complex. We suggest simplifying it to emphasize the distinct properties of the modules at different scales.

Minor comments

1. When referring to AC/DC modules, it would be important to talk about types of chromatin modules rather than “two chromatin modules”. In addition, it seems there may be three distinct types of chromatin modules, i.e., AC, DC and AC/DC. Or could these be understood as a continuum?

2. The illustration in Figure 1A is unclear. What do the panels with genes on the left and right represent? Are these isolated TRGs and clusters of TRGs?

3. What are “all peaks” compared to in Supplementary Figure 1G?

4. Figure 2A is not particularly informative in its current format. We suggest either replacing it with bar plots or supplementing it with a more insightful quantitative plot that captures both the temporal and structural aspects of TRG clusters.

5. Consider using eCDF plots instead of density plots, where applicable (e.g., Figure 2B).

6. Sorting the UpSet plot by overlap sizes in Figure 2D would make it easier to read.

7. In Figure 3, the text labeling “clusters 1-3” overlaps with the panel.

8. Replace lowercase “Ac” instances with uppercase “AC” for consistency.

Reviewer #2 (Remarks to the Author):

Seufert et al. analyse in their study titled “Two distinct chromatin modules regulate proinflammatory gene expression” the proinflammatory gene expression program induced by TNF α in human endothelial cells. Using a newly developed bioinformatic tool, RWireX, for single-cell ATAC-seq analysis they identify two types of chromatin modules termed autonomous links of co-accessibility (AC) and regions of contiguous co-accessibility (DC). They show that AC are associated with transcriptional burst frequency and DC with transcriptional burst size.

This is a well-executed and presented study. The findings about gene expression control using TNF α treatment as a model might indeed present a generalizable framework which is transferable to other stimuli in different contexts. The AC/DC concept is interesting; however I am not yet fully convinced about the novelty rather than having a different view on what is already known about gene expression control complexity. The link of the different module types to burst frequency/size is exciting.

I have several comments:

How is the overlap between DCs A/B compartments from HiC (e.g. increased ATAC-signal outside peaks is increased in A compartments, e.g. PMID: 37452040)?

Are DC regions regions that switch from B to A or B to A in Hi-C data after TNF α in HUVECs?

How does RWireX compare to other tools to calculate co-accessibility? Regarding the stochastic accessibility changes in homogenous cells. How much of it is due to missing values? What is the sensitivity to detect an accessible 1kb window? Metacell co-accessibility workflow on 10 cells of similar profiles. How is similar defined? What is the rationale for 10kb windows? Why can the dynamics between timepoints not be analyzed at 1 kb resolution with the presented deep scATAC data?

It is well established that gene expression is controlled by distal enhancers and that correlation between gene expression and chromatin accessibility at distal sites can be low as evidenced also here in Fig S3. Fig 1: Are chromatin accessibility changes linked to gene expression changes of predicted target genes? Fig. 6D What about gene expression of the predicted target genes of dynamic AC behavior? How does AC overlap with loops in Hi-C?

Fig 2D: 30-50% in scRNA for all 4 genes being co-expressed, but only ~20% in padFISH. Is this difference technical?

The changes in bursting frequency between AC and DC overlapping TRG clusters seem to be mild and it seems that TRG cluster overlap with DC is required for increase in size and frequency (this in combination with AC).

Reviewer #3 (Remarks to the Author):

In “Two distinct chromatin modules regulate proinflammatory gene expression”, Seufert et al present a study aimed at

unifying the different observations from genomics- and imaging-based analyses of chromatin features. The authors propose autonomous links of chromatin co-accessibility (AC) and domains of contiguous co-accessibility (DC) to explain the distinct relationships between these features and the regulation of constituent genes. The study is based on an extensive dataset, including smFISH, scRNA-seq, snATAC-seq, H3K27ac ChIP-seq, as well as utilizing published bulk RNA-seq and Hi-C data. The chromatin features are nicely visualized in the arc plots for AC, overlaid over other epigenomic profiles such as ATAC-seq, ChIP-seq, and Hi-C contact map, highlighting the multi-scale profiles. A nice correlation is shown between snRNA-seq and padFISH analyses of intronic transcripts to support that the AC and DC modules correspond to distinct transcriptional bursting properties. While the comprehensive analysis using several different experimental platforms is commendable, perhaps it also made the paper more prone to contain missing information about some data analyses and plots, as pointed out below. The poor descriptions make it difficult to verify and follow the authors' flow of reasoning throughout the paper. The first four comments below are major concerns I have found.

1. While the authors focused on the dynamics of chromatin modules, another aspect of cellular responses to TNF-alpha was not considered at all: oscillatory signaling dynamics of TNF-induced NF-kappaB with a period of about 90 minutes. It is well known that NF-kappaB is a nucleocytoplasmic shuttling protein whose steady state nuclear abundance rises and falls within single live cells, often several times, in multiple cell types examined (Tay S et al, PMID: 20581820; Sung MH et al, PMID: 19787057; Luecke S et al, PMID: 38872050; Sun L et al, PMID: 18384744). Some of the referenced groups have also identified specific features of NF-kappaB dynamics that correlate with the induction kinetics of target genes. Therefore, the authors should incorporate the TNF-induced signaling dynamics of NF-kappaB in the design and interpretation of their experiments.

2. HUVECs are not exactly a relevant cell type for investigating physiological pro-inflammatory responses, due to their umbilical origin. The authors may have historical reasons for the continued use of the source cell, for example to leverage the accumulated data from previous studies. However, to produce broadly impactful findings going forward, I strongly recommend the authors to consider using a more inflammation-relevant cell type. In this regard, it was prudent to include partial analysis of other cell types. However, only certain aspects of the AC/DC were analyzed, and thus it is insufficient to conclude how much of the findings from HUVECs apply to other cell types.

3. It is not clear where the multiomic data was used and how the analysis took advantage of the simultaneous profiling. Only Fig S3H-I seems to show results from the multiomic data. How concordant were the ATAC-seq profiles from the multiomic data with the independently performed scRNA-seq and snATAC-seq? What additional information was gleaned from the multiomic data?

4. In the results about DC, authors mention "nuclear foci" of NF-kappaB (Fig S5C) even in unstimulated cells. Have the authors checked whether it may be a fixation artifact? To demonstrate that these are true foci of concentrated NF-kappaB proteins, these structures need to be shown in native conditions, e.g. live cells expressing or transduced with fluorescent fusion, or at least cells with varying degrees of mild fixation. No information was provided in the Methods section on immunofluorescence about the fix agent, concentration, duration of fixation.

5. Is the red/blue color assignment correct in Fig S1F? It's opposite from panel E. The legend for Fig S1H is missing.

6. The plot in Fig S3I is not intuitive to understand. Why not show the AC arcs, gene expression tracks and annotations (similar to Fig S3A)?

7. Please describe in the legend of Fig S4D, exactly how the correlation coefficients were computed. Correlation over what entities? How was "DC accessibility" was defined? TRG expression was taken from which time point? In Fig S4E, it would be good to add the locations of genes and H3K27ac peaks on top of the DC track.

8. In Figure S5A, the three violins per time point are from the 3 replicates?

9. Does PRDM1 have a sequence-specific binding preference? I suggest the authors should check the sequence logo of the motif used in the TF binding analysis.

10. In figure 6, many panels lack sufficient descriptions in the legend. For example, in (A), what are the definitions of "Cluster (AC/DC)" and "Cluster (genome)"? In (B) bottom, please explain the four groups in the "TRG fraction" plot. In (D), the arc panels seem to be missing the labels of time points? In the legend for panel (E), "annotation in the middle shows DCs (black)..." seems like it should be "... shows DCs (red)..."

11. Results in Fig S6A seem important to warrant a more prominent discussion in the main paper.

12. I found the "AC and DC modules correlate with distinct 3D chromatin organization features" section the most vague and confusing. Please improve readability by justifying each statement (e.g. "...TAD boundaries appeared weaker when overlapping with DCs, ..." with corresponding patterns in Figures 7 and S7 (e.g. indicating specific "junctions", "stripes", and "borders" in the figure) and clarify descriptions of analysis. For example, justify the statement "...Hi-C contact maps further confirmed their coincidence with stripes of increased chromatin interactions" by showing data. What is "stacking of loops/TAD boundaries"? The interpretation of Figure 7 (especially panels C and F) is not intuitive; please explain. The colors in panel E are not visible.

13. In Figure 8 and associated text, the three examples genes NFKBIA, SELE, BIRC2 should be clearly labeled with their

AC, AC/DC, DC categories.

Reviewer #3 (Remarks on code availability):

We had a partial testing. Some plots could be produced as expected with RWireX.

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ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see <https://www.nature.com/licenceforms/nrg/competing-interests.pdf>.

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Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and

any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

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We recommend that you upload the step-by-step protocols used in this manuscript to [protocols.io](http://www.protocols.io). More details can found at <https://www.protocols.io/help/publish-articles>.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs \$600 for the first, and \$300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.
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- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the "unprocessed scans" Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial <http://www.nature.com/ncb/authors/submit/index.html#suppinfo>; <http://www.nature.com/ncb/journal/v14/n3/index.html#ed>). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos must be accompanied by a separate Word document including titles and legends.

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STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used

to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from $n < 3$. For sample sizes of $n < 5$ please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

----- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements -----

Version 1:

Decision Letter:

Our ref: NCB-A55703A

15th September 2025

Dear Dr. Rippe,

Thank you for submitting your revised manuscript "Two distinct chromatin modules regulate proinflammatory gene expression" (NCB-A55703A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines. Thank you very much for providing a point by point response to the pending reviewer comments. Please address the remaining referee#1 and referee#3 points in the final version. We would overrule on referee#3's point about validating the framework with another immune-relevant physiological cell system.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

Please also note that our articles must have 5 to 8 main figures and they can have up to 10 ED figures. If there are more figures than that, they become supplementary figures. Supplementary materials are less accessed than our main and ED figures so we try to limit the use of supplementary figures as much as we can. Please ensure that all figures fit into a single page (not multiple pages) and adhere to a maximum page size of roughly 180mm wide x 200mm high and use a font size of no smaller than 6pt Arial or Helvetica throughout the figures, to ensure legibility of the figures once resized for publication. Also, please use the full page space to fill the figure and remove the figure labels from the Figures.

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Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

Best wishes,
Sabrya

Sabrya Carim, PhD
(she/her/hers)
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Nature Portfolio

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Reviewer #1 (Remarks to the Author):

We have reviewed the revised manuscript from Seufert et al. and acknowledge substantial improvements in analytical clarity and presentation. The distinction between AC/DC modules has been made clearer, and the expanded statistics and supplementary tables are helpful. That said, several issues remain that, in our view, continue to limit the impact and novelty of the study.

1. Definition of ACs and DCs

The manuscript still lacks a clear and consistent explanation of how ACs and DCs are defined and computed. The current approach appears to mix two types of variation (within-population vs. across-perturbation) and two types of resolution (1kb peaks vs. 10kb bins), which complicates direct comparisons between ACs and DCs.

If we understood correctly:

- ACs are defined via correlations between 1kb peak-pairs across individual cells from a homogeneous population (e.g., G1-phase HUVECs at a single time point). They reflect stochastic chromatin interactions and are derived from cell-to-cell variability. These ACs are weakly anti-associated with co-expression while being linked to a tiny burst in transcriptional activity (e.g. Figure 7e), which suggests they may represent highly transient, non-committed contacts.
- DCs, in contrast, are derived from 10kb binned co-accessibility correlations across heterogeneous cell populations (e.g., multiple TNF α timepoints or cell types (PBMCs)). These modules seem to represent dynamically regulated chromatin domains activated or repressed in response to a perturbation.

Please note that this is our own interpretation as we were unable to find a clearer definition in the manuscript itself. Thus, this distinction, based on both biological input and analytical resolution, should be clearly and explicitly stated in the main text, not left to inference from figure legends or methods. At present, the use of different resolutions and populations introduces interpretive ambiguity.

Furthermore, the manuscript suggests a dichotomy between long-range (AC) and local (DC) interactions. However, this distinction appears to stem from methodological differences rather than intrinsic biological properties. Because ACs are based on non-contiguous peak pairs, they naturally span broader distances, while DCs, which are computed from contiguous genomic bins, appear more local. Yet this is a byproduct of the chosen resolution, not a principled biological distinction. For example, Figure 5a and the graphical abstract show DCs linking distant loci (e.g., IFNGR1 and TNFAIP3), which appears to undermine the claim that DCs are strictly local.

In short, the definition of ACs as “long-range” and DCs as “local” as well as the underlying biological interpretation (e.g. long-range interaction vs. local TF binding activity) are potentially misleading, as it may reflect how the modules were computed rather than what they biologically represent. Unless DCs are explicitly defined as contiguous blocks of highly correlated bins (which, again, is not clearly stated), the rationale for these methodological asymmetries remains unclear. Could DCs be computed at peak-level resolution? Could ACs be computed using binned data? If not, why?

We strongly recommend that the authors clarify the motivations and consequences of using different computational frameworks and avoid mixing analytical artifacts with biological interpretations. A more unified rationale, or at least a justified divergence, would significantly strengthen the conceptual clarity of the study.

2. Functional relevance of ACs and relationship to gene regulation

The functional interpretation of ACs remains vague. While DCs are more convincingly linked to transcriptional bursting size and co-expression, the role of ACs is less clear. They appear to correspond to low-frequency, stochastic interactions with weak correlation to gene expression. Given this, the claim that ACs represent a novel regulatory module seems overstated. If ACs are primarily anti-correlated with co-expression and show only very modest effects on bursting frequency, what is then the rationale for identifying and interpreting them as functionally relevant modules?

Thus, we strongly encourage the authors to:

- Reassess their claims regarding the regulatory relevance of ACs.
- Consider whether the observed ACs might reflect structural or architectural background noise, rather than specific regulatory mechanisms.
- Add visual overlays of gene expression (e.g., KLF10 and GASAL1) to Figure 3b to substantiate claims about transcriptional associations (i.e. to provide a clear visual representation of an association between changes in % accessible cells and gene expression).

Moreover, the manuscript's comparative analysis with PBMC data focuses almost exclusively on DCs. If ACs are a meaningful class of chromatin modules, how do they behave across systems? Are they conserved, context-specific, or reproducible?

3. Figures and visualization

Several figures are visually appealing but difficult to interpret. We suggest the following improvements:

- Extended Data Fig. 3f appears blank. Please confirm whether this is intentional.
- Figures 2a and 7d: While informative, they are hard to read. A histogram or bar plot format could improve interpretability.
- Figure 3c: Include a legend or scale for circle size to indicate overlap/strength.
- Figure 2d: Define “Abundance (%)” clearly in the caption.

- Figure 4b: The matrix plot lacks clear axis labels. Are these genome coordinates? Why are ACs plotted on both axes, and why is the matrix not symmetrical?
- Figure 5d and supplements: Similarly, axes need clearer definitions, and it should be easier to locate the DCs and TADs.
- Graphical Abstract: The current version lacks polish and clarity. Consider improving the cartoon quality and labels to better communicate the AC/DC model.

4. Other minor points

- Page 21: The sentence beginning with “Interestingly [...] and/or DC features” is incomplete.
- Data Integration: A dedicated method section describing how datasets (e.g., snATAC, snRNA, Hi-C, ChIP-seq) were integrated would be valuable.

Summary

While the manuscript has improved in clarity and organization, it remains unfortunately unclear what this study substantively contributes to our understanding of gene regulatory mechanisms. The distinction between ACs and DCs still rests on computational differences rather than on clearly defined biological principles, and the functional relevance of ACs, in particular, is not convincingly established. As a result, the broader conceptual impact of the AC/DC framework remains limited. At a minimum, clearer definitions, justification of analytical choices, and more cautious interpretation of the regulatory significance, especially of ACs, are essential to support the reported claims. Additional effort to align computational methods with biological rationale would also considerably strengthen the manuscript's value to the field.

Reviewer #2 (Remarks to the Author):

My comments have been addressed in the revision.

Reviewer #3 (Remarks to the Author):

Regarding the statement “... immunostaining of NF- κ B revealed the presence of some nuclear NF- κ B foci, which were already visible in the uninduced state before...”, the images in Ext Data Fig 6d and in Fig R4 are not convincing for the uninduced cells to support this conclusion. The small foci in “0 min” cells are hardly specific to the nucleus against the cytoplasmic background. Also, what is the last (4th) image of Fig R4e? Is the upper set of images from uninduced cells? Importantly, the p65-EGFP in live cells don't show any nuclear foci in uninduced condition. Overall, without a rigorous background correction and foci quantification, I suggest the statement should be limited to the stimulated conditions after TNF treatment.

“...we observed homogeneous populations for nuclear NF- κ B/p65 and its activity in HUVEC cells at all time points studied, with no evidence of oscillation ...” is not a fair description of Ext Data Fig 6e-g. While unimodal, these distributions have large variances at all time points, suggesting heterogeneity in individual cells or asynchronous behavior, which are indistinguishable in snapshot samples. Some live-cell imaging of fluorescently labeled p65 was attempted (Fig R2 showing two cells), but the result is inclusive without a quantification and statistical analysis. Therefore, the statement should be adjusted to match the data shown.

Although the authors have added RWireX analysis of additional cell types (Fig 8c-e) from published datasets, these do not approach a comparable level of examination, especially in relation to gene expression, because they did not include correlative analysis of matching scRNA-seq datasets. The added section seems to simply describe the results from applying RWireX to other snATAC-seq datasets. I suggest authors acknowledge that the same in-depth analysis framework should be validated with another immune-relevant physiological cell system, due to the exclusive reliance on HUVECs.

For future revisions, the authors should help reviewers to quickly identify the changes by marking the modified text in color or tracked changes.

Version 2:

Decision Letter:

Dear Dr Rippe,

I am writing on behalf of my colleague Dr Sabrya Carim, who has been out of the office.

I am pleased to inform you that your manuscript, "Two distinct chromatin modules regulate proinflammatory gene expression", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Cell Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Please feel free to contact us if you have any questions.

With kind regards,

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Response to reviewers for manuscript NCB-A55703-T

"Two distinct chromatin modules regulate proinflammatory gene expression" by Seufert et al.

We thank the expert reviewers for their insightful, constructive, and very helpful assessment of our study and their overall positive evaluation. In **General Comments 1-4** below, we summarize how we addressed recurring issues and then reply to all the specific reviewers' points. We have included additional validations and analyses addressing the various points raised and thoroughly revised the manuscript as described below. The reviewer comments are highlighted in blue.

General Comment 1. Differences between AC and DC modules

Our study identifies two distinct yet complementary types of regulatory chromatin modules that reflect different scales and mechanisms of transcriptional control: autonomous links of co-accessibility (ACs), representing long-range regulatory chromatin interactions, and domains of contiguous co-accessibility (DCs), which reflect regions of locally increased transcription factor activity. In several comments (Reviewer 1, pts 4-7, Reviewer 2 pts 1, 3, 4) we were asked to clarify the differences between AC and DC modules as well as provide details of AC and DC mapping. In the revised manuscript, we now provide this information, encompassing computational parameters, statistical thresholds, and quality control metrics with the following changes:

- We provided additional data that illustrate reproducibility and deep coverage of the snATAC-seq data, allowing us to distinguish critical co-accessibility features (**Fig. R1**).
- Expanded the Methods section and added a new **Supplementary Information RWireX** file that provides further details on RWireX, including guidelines on how to apply RWireX to other data, parameter optimization for DC detection, construction of meta cells, selection of genomic tile size and a comparison to other methods that evaluate co-accessibility.
- We added two new supplementary tables: **Supplementary Table S4** provides an overview of the key properties distinguishing AC and DC features, and **Supplementary Table S5** summarizes the distribution of TRGs associated with AC, DC, and AC/DC regulation.

Our high-quality scATAC-seq dataset, acquired with the optimized TurboATAC protocol ¹, enables reliable identification of both stable and dynamic co-accessibility patterns for characterizing AC and DC features. The relevant QC metrics are now listed in **Supplementary Table S1**.

High-quality cells were selected using thresholds of >30,000 unique fragments per cell and TSS enrichment scores >7 (**Fig. R1a-c**). This is over 10-fold higher than recent benchmarks reporting 1,000-10,000 fragments/cell². The resulting dataset had a median of 150,000 unique fragments/cell and TSS enrichment scores of ~11, with three true biological replicates for all three time points of excellent quality. The individual replicates were used to compute ACs and DCs with RWireX-sc and RWireX-meta, respectively. As described in the manuscript, 75% of ACs at TRGs, identified by RWireX-sc, are reproducibly detected across replicates (**Fig. 3c**). Furthermore, we only use ACs found in at least two replicates for further analyses (**Extended Data Fig. 3a-c**). Consensus DCs were called from the consensus metacell co-accessibility of replicates using RWireX-meta. Here, the overlap with DCs called from individual replicates was at >60% (**Extended Data Fig. 5a, b**; previous Fig. S4A, B). Thus, we conclude that we have implemented a robust approach to identify patterns of co-accessibility and their changes reflecting true biological features and not random noise (s. also **General Comment 2**).

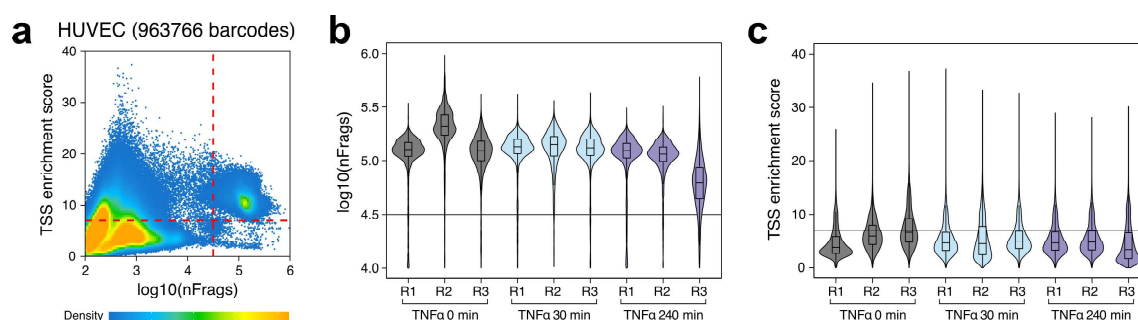


Fig. R1 Data quality of snATAC-seq data. (a) TSS enrichment score over the number of unique fragments (\log_{10}) of cell barcodes from all conditions and replicates. The color of points reflects the density of cell barcodes. The red lines mark the lower thresholds for selecting high-quality cell barcodes. (b) Number of unique fragments (\log_{10}) per cell. The black line marks the lower threshold for selecting high-quality cell barcodes. (c) TSS enrichment score per cell. The black line marks the lower threshold for selecting high-quality cell barcodes.

Our data identified 201,329 ATAC peaks in the pseudo-bulk analysis (**Extended Data Fig. 1g**, left). We have now included an additional analysis (new **Extended Data Fig. 1b, c**) that estimates how many pseudo-bulk ATAC peaks are simultaneously accessible in a single cell at a given time point. New **Extended Data Fig. 1b** shows the accessibility distribution of these peaks across the ~30,000 high-quality cells: Only 2.6% of peaks were accessible in >80% of cells, while 55% were accessible in 5-20% of cells. Our analysis estimates $N_{\text{peaks}} = 96,000$ peaks that are simultaneously accessible per single cell (new **Extended Data Fig. 1c**). By snATAC-seq, we detect 50,000 peaks/cell on average, which represents ~50% of the accessible peaks/cell. These results lead us to conclude that our data are not sparse and are well-suited to reveal true biological accessibility variation.

General Comment 2. Comparison of RWireX to other tools to calculate co-accessibility

RWireX introduces two key innovations for co-accessibility analysis: the detection of stochastic interactions at single-cell resolution (RWireX-sc workflow for detecting ACs) and the identification of contiguous domains with coordinated accessibility changes using metacells (RWireX-meta workflow for detecting DCs). In multiple comments (Reviewer 1, pts 1; Reviewer 2, pts 2 and 3; Reviewer 3, pt 14) we were asked to analyze how RWireX compares to other tools to calculate co-accessibility. To validate these capabilities and address this request, we compared RWireX to the established co-accessibility analysis tools Cicero (v. 1.24.0) ³ and ArchR (v. 1.03) ⁴ and made the following additions:

- An overview of the differences is given in the new **Supplementary Table S3**.
- A detailed comparison is provided in the **Supplementary Information RWireX** that describes the RWireX workflow in further detail.
- Additionally, the **Supplementary Information RWireX** file includes comparative co-accessibility analysis between Cicero, ArchR, and RWireX for replicate 1 of our high-quality snATAC-seq data.

Based on this comparison, we conclude that RWireX demonstrates distinct advantages over Cicero and ArchR for the co-accessibility analysis conducted in our study. In particular, the RWireX-sc workflow allows to detect stochastic co-accessibility links across single cells. The use of metacells as done in Cicero and ArchR is not suited for this purpose. Aggregating single cells results in a loss of information regarding stochasticity, particularly for less frequent events, and artificially increases correlations between differential sites. Furthermore, for metacell construction, Cicero and ArchR select sets of cells without excluding their presence in multiple metacells, which aggravates this issue. Accordingly, Cicero and ArchR compute what we consider an artificially high number of long-range co-accessibility links, which are difficult to interpret (**Supplementary Information RWireX**). Finally, RWireX-sc calculates the fraction of cells in which one or both of the interacting peaks exhibit an ATAC signal that allows us to differentiate between rarely and frequently occurring ACs. For the DCs called with the RWireX-meta workflow, there is no equivalent in ArchR, which solely reports co-accessibility links from metacells. Cicero identifies so-called *cis*-co-accessibility networks (CCANs), representing clusters of interconnected co-accessible peaks. However, CCANs are distinctly larger than DCs with sizes more comparable to TADs detected from bulk HiC-seq data.

General Comment 3. Physiological relevance of the findings and broader applicability

To address the physiological relevance of the findings obtained in HUVEC cells (Reviewer 3, point 2), we first like to point out that transcriptional regulation of inflammatory responses via TNF α signaling and subsequent NF- κ B activation is a conserved mechanism across diverse

cell types ⁵. While immune cells like macrophages and T cells are classical mediators of inflammation, endothelial cells play a crucial role in these responses by regulating vascular permeability, leukocyte recruitment, and local cytokine production ^{6,7}. We chose HUVECs as our model system because: (i) they are primary human cells that maintain physiological regulation, (ii) they show a robust and representative gene expression program response to TNF α , and (iii) extensive genomic datasets are available to validate and integrate our findings with other readouts (e.g., ^{8,9}).

To demonstrate the broader usefulness of our AC/DC framework beyond HUVECs and the broad applicability of RWireX to other cell types (Reviewer 3, pt 2), including macrophages and T cells, we have now performed detailed analyses across various biological systems and specifically made the following additions:

- We examined human peripheral blood mononuclear cells (PBMCs), comparing regulatory features across four different T cell types and B cells (new **Fig. 8c-e**, new **Extended Data Fig. 9**). The RWireX-sc analysis identified cell-type-specific ACs (12,000-23,000 per cell type) with ~25% overlap between cell types, while RWireX-meta found 4,863 DCs similar in size to those in HUVECs. Cell-type-specific regulatory features were clearly visible at key loci: ACs at the *CD4* promoter showed specific interactions in CD4+ mature T cells versus naïve cells, while the *CD22* DC was mainly active in B cells, and the *TBX21* DC showed high activity in mature CD4+ and CD8+ T cells.
- We demonstrated perturbation-response analysis by examining interferon-induced regulatory changes in mouse embryonic stem cells and fibroblasts, where RWireX-meta successfully identified stimulus-responsive DCs at interferon-stimulated gene clusters (**Extended Data Fig. 10c, d**; previous Fig. S9A, B).
- We used RWireX to explore disease-related regulatory mechanisms by analyzing the effects of T-bet knockout in a chronic lymphocytic leukemia mouse model, revealing how the loss of this transcription factor disrupts both AC and DC modules at target loci (**Extended Data Fig. 10a, b**; previous Fig. S9C, D).

Together, these diverse applications – spanning inflammatory responses, immune cell differentiation, cytokine signaling, and disease models – establish the AC/DC framework as a versatile tool for dissecting chromatin-mediated gene regulation across species, cell types, and experimental conditions. The conservation of AC/DC modules across different cell types, while showing cell-type-specific activities, supports their role as a general organizational principle in gene regulation, with RWireX providing the computational foundation for identifying regulatory modules in any high-quality snATAC-seq dataset.

General Comment 4. Strengthening data and methodology and enhancing clarity

In several comments (Reviewer 1, pts 1-3; Reviewer 3, pt 12), we were asked to strengthen the existing data, provide controls, improve methodological details, and enhance the clarity and readability of the manuscript. In response to this feedback, we have thoroughly revised the manuscript to improve its clarity, strengthen its methodological basis, and enhance data presentation.

A new schematic introduces the different regulatory mechanisms we address with our co-accessibility analysis, providing readers with a clear conceptual framework (**Fig. 1a**). In the Results section, the narrative now logically progresses from the TNF α response through the AC and DC modules to their integration. **Figures 3-6** were rearranged to better connect related findings, including improved integration of the Hi-C analysis with the AC and DC sections. Additionally, we expanded the discussion to better contextualize our findings within the current understanding of nuclear organization and transcriptional regulation. We now consistently use standard terminology when discussing chromatin organization (e.g., "enhancer-promoter interactions," "transcription factor hubs", "nuclear compartments") and more clearly define any new terms we introduce (such as AC and DC modules).

The technical foundation has been strengthened by providing additional information and controls. Method descriptions have been improved to make it easier for other researchers to reproduce and apply them. They now include detailed protocols for all experimental procedures, specifying exact fixation conditions, the immunofluorescence protocol, and comprehensive padFISH probe design details. As explained in the point-by-point response below, several figure legends were revised to offer clearer descriptions of analyses and metrics. Statistical reporting has been improved with precise *p*-values and test selections. New quality control metrics and parameter optimization analyses have been added (see **General Comment 1**), offering transparent validation of our analytical methods. The **Supplementary Information RWireX** now includes additional documentation on the software package to help other researchers use it effectively.

Finally, we have improved data presentation throughout the manuscript to make our findings more accessible. Two new **Supplementary Tables, S4 and S5**, offer quantitative summaries of key findings regarding AC and DC properties. We have also improved the visualization of complex figures, e.g., by using eCDF or stacked bar plots, as suggested by the reviewers.

Reviewer 1

Summary

[Seufert et al. investigate the mechanisms of gene coregulation in human endothelial cells by stimulating an inflammatory response using TNF \$\alpha\$...](#)

Together, the goal of this study was to uncover the mechanisms of coordinated gene regulation in response to TNF α stimulation. Although TNF α signaling is well characterized, the authors extend previous findings by connecting chromatin organization to transcriptional bursting, NF- κ B factory formation, while using publicly available data from other biological systems to explore the generalizability of their discoveries. The paper addresses important questions in the field, the authors validate their results across replicates, and the figures look aesthetically pleasing. **However, the manuscript was rather difficult to follow as the study is highly complex, and the manuscript would therefore benefit from improved methodological transparency, additional quantitative assessments, benchmarking, and clearer explanations of key results.** In addition, the results presented in the manuscript are often scattered, and are not consistently contextualized within the established terminology of the field. Finally, the conclusions presented in the study rely on specific parameters used for chromatin module mapping with RWireX, which, without benchmarking, limits the generalizability of the methodology and weakens confidence in the main findings. Below, we provide suggestions of how the authors could enhance the clarity of the manuscript and make the results more accessible and convincing to readers.

We thank the reviewer for the expert evaluation of our work on transcription factor binding and activity patterns, which has helped us to better integrate TF dynamics with transcriptional regulation. Their insights on footprinting analysis and suggestions improve the link between TF binding dynamics with transcriptional bursting significantly improved our manuscript. The specific technical and conceptual points raised have been addressed and are detailed in **General Comments 1-4**, as well as in our response to the specific comments below.

Major comments

1. The study would benefit from more thoroughly contextualizing the results within existing literature to better highlight their relevance and novelty. Specifically, the manuscript should provide a clearer introduction to the RWireX method, explaining its novelty and the motivation behind its development. It is especially important to place the method in the context of existing single-cell approaches to justify its use, i.e., why is a new computational framework needed? How does it compare to existing tools which use single-cell data to map co-accessible regions, such as ArchR and Cicero (Pliner et al., Molecular Cell 2018)? Benchmarking RWireX against these tools and demonstrating its advantages would underscore its utility, most importantly because the choice of thresholding highly influences the results and conclusions.

Further details on the mapping of AC and DC types of chromatin modules, as well as parameter selection, have been added to the revised manuscript, as noted in **General Comment 1**. We agree that comparing RWireX to existing co-accessibility methods provides valuable insights for assessing our analysis in the context of current studies and have included this in the **Supplementary Information RWireX**. This comparison is now included in the revised manuscript, as explained in **General Comment 2**.

2. Expanding on the previous point, the manuscript should provide readers with an understanding of how the tool can be applied. For example, what is the required quality of the data? What parameters should be used and when? Adding such quantitative assessments and discussing the optimal conditions for using the tool would greatly enhance its applicability.

We have added additional information and guidelines for the RWireX application in the **Supplementary Information RWireX** and the GitHub repository. According to our benchmarking, snATAC-seq data with >30,000 unique fragments per cell provides reliable detection of both ACs and DCs. DC detection remains robust with lower coverage, while AC mapping becomes less dependable. Detailed parameters and quality metrics are now available to assist users in optimizing their analysis for specific datasets (**General Comment 1** and **2**).

3. In Figure 2A, and in line with the previous point, the manuscript would benefit from a detailed description of the parameter selection strategy for clustering. A quantitative evaluation of how different parameters influence compartment properties would be crucial for supporting the statements made in the manuscript.

We evaluated the relationship between genomic distance thresholds and gene cluster properties to identify co-regulated gene clusters. We have added new figure panels of quantitative analyses to further validate our approach to gene cluster identification (new **Extended Data Fig. 2b, c**). The study of gene cluster numbers across different window sizes (100 kb to 2 Mb) revealed a clear inflection point at 500 kb (**Extended Data Fig. 2d**, previous Fig. S2B), which was used as the maximum distance between TRGs assigned to a gene cluster in **Fig. 2a**. At this threshold, we identified 356 TRG clusters containing 1,008 genes, significantly more than expected from random gene sets ($p < 0.001$, **Extended Data Fig. 2f**, previous Fig. S2C). The distribution of pairwise intrachromosomal TRG distances shows a local minimum at ~500 kb, suggesting a natural boundary between co-regulated and independent TRGs (new **Extended Data Fig. 2b**). At this window size, 67% of TRGs were located in a gene cluster, which was well below saturation as window sizes increased (new **Extended Data Fig. 2c**).

The average TRG cluster size of 460 kb aligns well with the typical sizes of regulatory domains observed in the inflammatory response⁹. This size also falls within the expected range for enhancer-promoter interactions¹⁰. Furthermore, the majority of TRG clusters (69%) were found within a single TAD (**Extended Data Fig. 2h**, previous Fig. S2E), which is consistent with established features of regulatory domains¹⁰.

4. Despite the authors' efforts to explain the differences between AC and DC compartments, the information is scattered throughout the manuscript, making it difficult to form a cohesive understanding of the two types of modules. First, we suggest adding more details on the properties of these compartments to the 'highlights' section (e.g. by providing a comprehensive list of features for both ACs and DCs). Next, we suggest to begin by introducing the current state of the field, followed by a detailed explanation of AC modules, DC modules and their properties, and then building on top of that. This restructuring would enhance clarity and provide better context by linking new concepts to established terminology already familiar within the field.

We restructured the manuscript to provide a clearer conceptual framework (**General Comment 1** and **4**). The updated Results section improves understanding by (i) introducing the TNF α response and existing models, (ii) presenting AC and DC modules and their key features more logically alongside the Hi-C comparison, (iii) demonstrating AC and DC presence at TRG clusters, and (iv) linking our AC/DC framework to functional gene regulation features such as induction timing, strength, and bursting kinetics. The extended description of the analysis workflow in **Supplementary Information RWireX**, along with the new **Supplementary Table S4** and **S5**, provides a structured comparison of AC and DC features and quantifies their distribution across TRGs.

5. Conceptually, it is unclear what the gene regulatory assumptions are behind mapping AC modules in a homogeneous population? What is the source of variation, considering that only the G1 phase was selected, so it cannot be the cell cycle? If co-accessibility changes at peaks are purely stochastic, how can their seemingly 'random appearance' reflect any underlying provide any functional insight?

The stochastic nature of ACs reflects established properties of chromatin regulation: TF binding occurs with approximately 10-second residence times ¹¹, enhancer-promoter interactions are inherently dynamic ¹¹, and even "stable" features like CTCF loops represent probabilistic rather than fixed structures ¹². As outlined in **General Comment 1**, these dynamics are best revealed in homogeneous cell populations where variations reflect stochastic molecular changes rather than differences in cell states. Our deep sequencing coverage enables the quantification of these genuine biological fluctuations.

6. It is also important to elaborate on DC mapping. What was the rationale for using 10kb tiles? How might the results change with different tile resolutions? What is the upper limit? If DCs were mapped per time point, would it be possible to capture alternations in chromatin activity between A/B compartments over time?

The choice of 10 kb resolution for DC mapping was based on systematic optimization, as shown in the new **Supplementary Information RWireX** and discussed in **General Comment 1**. This resolution strikes an optimal balance between signal-to-noise ratio and feature detection. It also demonstrates that DC mapping necessitates a heterogeneous signal and does not perform well for isolated time points.

7. It would be helpful to provide a clear, quantitative evaluation of the total number of modules based solely on AC, solely on DC, or both. Currently, this information is discussed rather late in the text (Supplementary Figure 6B). Additionally, the low correlations shown in Figure 6C are rather unconvincing, making it difficult to draw any overarching conclusions from them. Are such low correlations expected? Are they driven by the sparsity of scRNA-seq data? Was expression data imputed using MAGIC (van Dijk et al., Cell, 2018)?

We now present the quantitative analysis of AC and DC modules earlier in the text, along with the new Supplementary Table S5. Our scRNA-seq data are of excellent quality. Across all conditions and replicates, we achieve a median of 20k-30k UMI counts and a median of 5,200-6,000 detected genes. The correlations in Fig. 7e (previous Fig. 6C), $R = -0.18$ for ACs and $R = 0.19$ for DCs, are moderate but highly significant statistically ($p < 0.001$). Data imputation would artificially inflate these correlations, so we prefer to analyze our raw data to preserve biological authenticity. We also expect only a moderate correlation. The TRG clusters labeled as AC or DC contain only about one-third of clusters that are exclusively AC or DC (Fig. 7d), but often include one or more TRGs without any annotation. Additionally, many regulatory steps occurring directly at the promoter are likely partly independent of mechanisms affecting the entire cluster and are not captured by our AC/DC annotation.

8. The manuscript would benefit from further elaboration and contextualization of the observed TAD overlaps with AC, DC, and AC/DC modules, as it is unclear at this time what can be really concluded here.

We have restructured the manuscript in **Fig. 3, 4, 5** and **6** to have a systematic comparison with the Hi-C data and included comprehensive metrics in **Supplementary Table S4**. Our analysis revealed distinct patterns: ACs can exist within TADs but also across boundaries (**Extended Data Fig. 3g**, previous Fig. S3F), with ACs showing significantly higher Hi-C contact frequencies than non-AC linked sites (**Fig. 7a, b**; previous Fig. 6A, 6D). DCs tend to be more confined within TADs (**Fig. 5c**, previous Fig. 4C); however, those that cross boundaries correlate with a weakened TAD structure (**Fig. 7c**, previous Fig. 6E). These findings suggest that while TADs provide a framework for regulatory interactions, AC and DC modules can operate independently of, and even modify, TAD structure, aligning with recent evidence that TADs represent probabilistic rather than fixed structures¹² and can be dynamically modified during gene activation¹³.

9. We believe that the single-cell data is not used to its full potential and that the results are dominated by batch effect, which was not regressed out during the preprocessing step (Figure 1B). Quality control metrics for single-cell data preprocessing, such as those provided by Scanpy or Seurat pipelines, should be included. Pseudo-bulking could support the results obtained from the single cell modality, yet we would envision it as a secondary/complementary approach to confirm findings from single cell analysis.

We appreciate the reviewer's concern regarding data quality and batch effects. Our analysis shows that the apparent batch effects are minor, with biological replicates demonstrating high reproducibility (**Extended Data Fig. 1a**, previous Fig. S1A-C; new **Extended Data Fig. 1b, c**; **Fig. R1b-c**). More importantly, our optimized TurboATAC protocol achieves unprecedented coverage of ~150,000 unique fragments/cell, which is over 10-fold higher than recent

benchmarks², as discussed in more detail in **General Comment 1**. This deep coverage allows for the detection of ~50,000 peaks per cell (new **Extended Data Fig. 1c**).

Quality metrics for all samples are now provided in **Supplementary Table S1**, including TSS enrichment scores and unique fragment counts. The high quality of our data is further validated by the reproducibility of both ACs (75% overlap between replicates at TRGs) and DCs (>60% overlap genome-wide) (**Fig. 3c**; **Extended Data Fig. 3a-c**; **Extended Data Fig. 5a, b**, previous Fig. S4A, B). While we utilize pseudo-bulk analysis where appropriate, our single-cell resolution is essential for detecting stochastic regulatory interactions, as demonstrated in **General Comment 1**.

Why were the time points at 30 minutes and 240 minutes not compared?

The high-resolution Hi-C data from Rao et al used in our study are only available for the uninduced conditions at the 0 min TNF α treatment time point⁸.

10. A clearer rationale for examining transcriptional bursting is needed: what was the motivation and reasoning for the suggested analyses? Given the long intervals between time points, how do the results of the transcriptional bursting analysis (Figure 8) align with the expected “on” times of transcriptional bursts (typically ranging from minutes to hours in mammalian cells)? Similarly, how does this analysis fit with the transient residence times of TF binding to DNA (Figure 5), which usually last only a few seconds?

Our motivation for the transcriptional bursting analysis was to understand how AC and DC modules contribute to different steps of transcriptional regulation. The analysis of intronic RNA reads at 0, 30, and 240 minutes of TNF α treatment offers snapshots of the distribution of bursting states rather than direct measurements of bursting kinetics. Nevertheless, the results for burst size and frequency are consistent between snRNA-seq and padFISH measurements. These findings support our conclusions that ACs predominantly affect burst frequency while DCs have a larger effect on burst size (new **Fig. 8a**, new **Extended Data Fig. 8c-d**).

11. Do the “waves” in Figure 5B suggest the presence of other TFs binding in proximity or cooperative binding by TFs? Can the authors explain the higher accessibility upstream of the focal binding point? Why were only DCs used for the footprint analysis?

The ATAC-seq footprint patterns are highly specific to transcription factor (TF) motifs and result from a complex interplay of TF binding that simultaneously induces (i) increased accessibility adjacent to the TF motif through nucleosome displacement or translocation, (ii) reduced accessibility at the TF motif where the TF interacts with the DNA, (iii) effects on nucleosome positioning in the areas flanking the TF binding site, and (iv) sequence-specific cleavage by the Tn5 enzyme¹⁴⁻¹⁶. Therefore, we would not know how to infer specific molecular details about TF cooperativity from the footprints. Instead, we focus on analyzing TF occupancy using the approach implemented in the TOBIAS software¹⁴. We utilize it to evaluate whether the

occupancy of a given TF is increased within DCs compared to their surrounding regions. A systematic analysis of motifs and footprints at ACs would also be intriguing but considerably more complex due to the stochastic and long-range nature of these interactions.

12. Would it be possible to include data tracks for the three time points to show differential accessibility at distal CREs and TF binding sites? Including expression values for each time point would provide an informative visual.

We show the pseudo-bulk ATAC tracks in a number of instances, e.g., at the top of **Fig. 3b** and **Fig. 5a** (previous **Fig. 4A**) for each condition (replicates merged) and provide the data on the number and location of differential ATAC peaks (**Extended data Fig. 1g** and Source data). We agree that a more comprehensive analysis of the differential pseudo bulk data would provide interesting information. We did not include it, however, as its integration with our co-accessibility data in a meaningful manner would add another layer of complexity. It is noted that for ACs (e.g., **Fig. 3b**), the aggregated ATAC profiles are very similar, as explained in **General Comment 2**. Thus, in this context, a main point is that we map long-range interactions from differences in single cells that are averaged out and not seen as differential ATAC peaks in the pseudo bulk. DCs, on the other hand, are computed across all three time points (**General Comment 1, Fig. R5**). Thus, presenting them alongside differential pseudo-bulk tracks computed between individual time points may be confusing. Finally, visuals for the TRG expression values could be helpful; however, since multiple TRGs exist within a locus, we believe it is clearer to mention only the log fold changes in the text and provide a table and plots of all gene expression data in the supplementary materials for all TRGs.

13. The illustration in Figure 8B, which is also used as the graphical abstract, is too complex. We suggest simplifying it to emphasize the distinct properties of the modules at different scales.

We agree and have simplified the illustration in **Fig. 8b** to more clearly emphasize the distinct properties of AC and DC modules and included TRGs regulated by promoter binding as an additional category. The revised figure now highlights the key mechanistic differences between these two types of regulatory modules, focusing on: (i) the long-range, stochastic nature of ACs compared to the contiguous domain structure of DCs; (ii) their different effects on transcriptional bursting (frequency versus size); and (iii) their unique relationships with spatial genome organization.

Minor comments (renumbered)

14. When referring to AC/DC modules, it would be important to talk about types of chromatin modules rather than “two chromatin modules”. In addition, it seems there may be three distinct types of chromatin modules, i.e., AC, DC and AC/DC. Or could these be understood as a continuum?

We have revised our terminology throughout the manuscript to refer to "types of chromatin modules" instead of simply "chromatin modules," which more accurately reflects their distinct regulatory mechanisms. We have also clarified that while AC and DC represent two primary types of modules, the AC/DC classification indicates regions where both types of regulation coexist rather than suggesting a third, discrete category. In the revised manuscript, we discuss how these module types may represent points along a continuum of regulatory mechanisms, with some regions exhibiting primarily one type of regulation while others display characteristics of both.

15. The illustration in Figure 1A is unclear. What do the panels with genes on the left and right represent? Are these isolated TRGs and clusters of TRGs?

Thank you for pointing out this lack of clarity. We have redesigned **Fig. 1a** to offer a better introduction to the different regulatory mechanisms that we address with our co-accessibility analysis, together with the different methods and readouts used in our study. This redesigned figure provides a clearer entry point to our study and helps readers grasp the key concepts before diving into the more complex analyses.

16. What are "all peaks" compared to in Supplementary Figure S1G?

We have revised the legend for **Extended Data Fig. 1g** to make clear that the genomic distribution of all 201,329 ATAC peaks detected in the pseudo-bulk analysis of is compared to the differential ATAC peaks after 30 min or 240 min of TNF α treatment compared to the 0 min time point.

17. Figure 2A is not particularly informative in its current format. We suggest either replacing it with bar plots or supplementing it with a more insightful quantitative plot that captures both the temporal and structural aspects of TRG clusters.

We would like to retain the network visualization in **Fig. 2a** as it effectively illustrates the spatial organization of TRG clusters along the genomic coordinate. We have enhanced it with additional quantitative analyses presented as the suggested bar plots in new **Extended Data Fig. 2e**. This updated figure includes bar plots that capture both the temporal dynamics (early, late, and persistent response TRGs) and structural characteristics (up/down-regulation patterns, protein-coding vs. lncRNA distribution) of TRG clusters.

18. Consider using eCDF plots instead of density plots, where applicable (e.g., Figure 2B).

As suggested, we replaced the density plots in **Fig. 2b** and **Extended Data Fig. 2i** (previous Fig. S2F) with empirical cumulative distribution function (eCDF) plots. We agree that this change offers a clearer quantitative representation of co-expression differences between conditions and TRG groups.

19. Sorting the UpSet plot by overlap sizes in Figure 2D would make it easier to read.

We have sorted the UpSet plot in **Fig. 2d** as requested.

20. In Figure 3, the text labeling “clusters 1-3” overlaps with the panel.

This error has been corrected.

21. Replace lowercase “Ac” instances with uppercase “AC” for consistency.

This inconsistency has been corrected.

Reviewer 2

Summary

Seufert et al. analyse in their study titled “Two distinct chromatin modules regulate proinflammatory gene expression” the proinflammatory gene expression program induced by TNF α in human endothelial cells. Using a newly developed bioinformatic tool, RWireX, for single-cell ATAC-seq analysis they identify two types of chromatin modules termed autonomous links of co-accessibility (AC) and regions of contiguous co-accessibility (DC). They show that AC are associated with transcriptional burst frequency and DC with transcriptional burst size.

This is a well-executed and presented study. The findings about gene expression control using TNF α treatment as a model might indeed present a generalizable framework which is transferable to other stimuli in different contexts. The AC/DC concept is interesting; however I am not yet fully convinced about the novelty rather than having a different view on what is already known about gene expression control complexity. The link of the different module types to burst frequency/size is exciting. I have several comments:

We appreciate the reviewer's positive and insightful evaluation of our study, which has helped us to better position our findings within the established framework of enhancer-promoter interactions and regulatory domains. We have now articulated more clearly how the AC/DC framework provides new mechanistic insights into their integration and have addressed the specific comments outlined below.

Comments

1. How is the overlap between DCs A/B compartments from HiC (e.g. increased ATAC-signal outside peaks is increased in A compartments, e.g. PMID: 37452040)? Are DC regions regions that switch from B to A or B to A in Hi-C data after TNFa in HUVECs?

As detailed in **General Comment 1** and in the response to reviewer 1, pt 6, our analysis focused on the relationship between DCs and TADs rather than A/B compartments. We used Hi-C data from Rao et al. ⁸ primarily to identify TADs in unstimulated HUVECs, as shown in **Fig. 5d** (previous Fig. 7C) and **Extended Data Fig. 4c** (previous Fig. S7A). However, our analysis showed that DCs (average size 162 kb) are significantly smaller than typical TADs and A/B compartments (usually ~1 Mb), indicating they represent a distinct level of chromatin organization. The overlap between DCs and TADs (64% within TADs, 18% across TAD boundaries, 18% outside TADs; **Fig. 5c**, previous Fig. 4C; **Supplementary Table S4**) further supports that DCs act as regulatory units capable of functioning independently of the higher-order chromatin structure of TADs, setting them apart from the assembly of TADs into A/B compartments. While exploring this relationship further would be interesting, we chose to focus on the numerous other aspects we needed to address for the revisions but mention this point in the discussion.

2. How does RWireX compare to other tools to calculate co-accessibility? Regarding the stochastic accessibility changes in homogenous cells. How much of it is due to missing values? What is the sensitivity to detect an accessible 1kb window?

To better assess our results, we have included a comparison of RWireX with two other tools, Cicero and ArchR, as outlined in **General Comment 2**. The issue of stochastic accessibility changes and missing values/data sparsity is discussed in **General Comment 1**. Briefly, we conclude that RWireX, unlike Cicero and ArchR, can reliably identify stochastic co-accessibility links (**General Comment 2**). Moreover, we estimate that the extensive coverage of our snATAC-seq data enables us to detect 50% of all accessible peaks present in a given cell (**General Comment 1**, **Extended Data Fig. 1b, c**).

3. Metacell co-accessibility workflow on 10 cells of similar profiles. How is similar defined? What is the rationale for 10kb windows? Why can the dynamics between timepoints not be analyzed at 1 kb resolution with the presented deep scATAC data?

These important technical questions regarding metacell construction and resolution selection are discussed in detail in **General Comment 1** and the **Supplementary Information RWireX**. In summary, our parameter choices were based on systematic optimization: metacells were constructed using ArchR's iterative LSI dimensionality reduction along with k-nearest neighbor clustering. The 10 kb resolution was selected through systematic optimization as now described in the **Supplementary Information RWireX**. It is important to note that co-accessibility analysis necessitates biological variation across metacells; this signal is

insufficient when analyzing individual time points, requiring integration across the TNF α time course.

4. It is well established that gene expression is controlled by distal enhancers and that correlation between gene expression and chromatin accessibility at distal sites can be low as evidenced also here in Fig S3. Fig 1: Are chromatin accessibility changes linked to gene expression changes of predicted target genes? Fig. 6D What about gene expression of the predicted target genes of dynamic AC behavior? How does AC overlap with loops in Hi-C?

Notably, while 91% of all TRGs had an open chromatin site at the promoter, only 9% of these were linked to significant changes in promoter accessibility upon TNF α treatment (**Extended Data Fig. 2a**). In contrast, the analyses of multiome data presented in **Extended Data Fig. 3h, i** and **Extended Data Fig. 5d, e** (previous Fig. S4D, E) show a direct correlation between chromatin accessibility at ACs or DCs with target gene expression at the single-cell level for some genes. The relationship between AC link activity and TRG expression is generally modest, though certain AC-TRG pairs exhibit distinctly stronger correlations. This overall modest correlation aligns with the established understanding that distal enhancer accessibility does not always directly correlate with target gene expression, due to the complex interplay of gene regulation, including time delays, additional co-factors, and combinatorial effects. We have revised **Fig. 3** and **Fig. 4** and the associated text in the Results section to provide a clearer description of the relation between our AC data and the Hi-C contact frequencies.

5. Fig 2D: 30-50% in scRNA for all 4 genes being co-expressed, but only ~20% in padFISH. Is this difference technical?

In our view, the difference primarily arises from the differences between the two techniques, which is now mentioned in the revised manuscript in the Methods section. In the scRNA-seq data, gene expression is assessed at the cellular level, with transcripts from both alleles combined. In contrast, our padFISH analysis evaluates the co-occurrences of nascent transcripts at the same site within the nucleus, providing allele-level resolution. Therefore, a lower co-expression percentage in padFISH compared to scRNA-seq is anticipated, which is consistent with the experimental observations. Despite this technical distinction, the correlation between the co-expression patterns measured by both methods remains remarkably high, with correlation coefficients of 0.83 at 30 minutes and 0.79 at 240 minutes (**Extended Data Fig. 2k**, previous Fig. S2H).

6. The changes in bursting frequency between AC and DC overlapping TRG clusters seem to be mild and it seems that TRG cluster overlap with DC is required for increase in size and frequency (this in combination with AC).

We appreciate the reviewer for highlighting this point. The differences in burst frequency between AC and DC modules are indeed less pronounced than those in burst size, which has now been clarified in the manuscript. They are statistically significant, as illustrated in **Extended Data Fig. 8a, b**. We agree that the increase in burst size for DC type of TRGs has a stronger effect size as evident from the new **Fig. 8a**, which also illustrates that the highest change in burst frequency is observed for the mixed AC/DC type. These points have been clarified in the revised manuscript.

Reviewer 3

Summary

In “Two distinct chromatin modules regulate proinflammatory gene expression”, Seufert et al present a study aimed at unifying the different observations from genomics- and imaging-based analyses of chromatin features. The authors propose autonomous links of chromatin co-accessibility (AC) and domains of contiguous co-accessibility (DC) to explain the distinct relationships between these features and the regulation of constituent genes. The study is based on an extensive dataset, including smFISH, scRNA-seq, snATAC-seq, H3K27ac ChIP-seq, as well as utilizing published bulk RNA-seq and Hi-C data. The chromatin features are nicely visualized in the arc plots for AC, overlaid over other epigenomic profiles such as ATAC-seq, ChIP-seq, and Hi-C contact map, highlighting the multi-scale profiles. A nice correlation is shown between snRNA-seq and padFISH analyses of intronic transcripts to support that the AC and DC modules correspond to distinct transcriptional bursting properties. While the comprehensive analysis using several different experimental platforms is commendable, perhaps it also made the paper more prone to contain missing information about some data analyses and plots, as pointed out below. The poor descriptions make it difficult to verify and follow the authors’ flow of reasoning throughout the paper. The first four comments below are major concerns I have found.

We are very grateful for the reviewer's thorough assessment of our study, which highlighted various aspects of transcriptional regulation dynamics that were missing and/or unclear. We have addressed these concerns as described in **General Comments 1-4** and in our responses to the specific reviewer comments below. The reviewer’s comments also prompted us to expand our analysis to immune cells, revealing that the AC/DC framework identifies both conserved and cell-type-specific regulatory features across different human cell types. This highlights a new and exciting aspect of our approach.

Major comments

1. While the authors focused on the dynamics of chromatin modules, another aspect of cellular responses to TNF-alpha was not considered at all: oscillatory signaling dynamics of TNF-induced NF-kappaB with a period of about 90 minutes. It is well known that NF-kappaB is a nucleocytoplasmic shuttling protein whose steady state nuclear abundance rises and falls within single live cells, often several times, in multiple cell types examined (Tay S et al, PMID: 20581820; Sung MH et al, PMID: 19787057; Luecke S et al, PMID: 38872050; Sun L et al,

PMID: 18384744). Some of the referenced groups have also identified specific features of NF-kappaB dynamics that correlate with the induction kinetics of target genes. Therefore, the authors should incorporate the TNF-induced signaling dynamics of NF-kappaB in the design and interpretation of their experiments.

We thank the reviewer for pointing to the potentially important role of oscillatory NF-kB signaling dynamics in TNF responses. We acknowledge that NF-kB oscillations have been observed in various cell types and can influence the expression of target genes. However, these oscillations are not universal. They are context-dependent, varying significantly across cell types, stimulation conditions, and even among individual cell clones^{17,18}. The oscillatory behavior described in the literature is mainly observed in fibroblasts and epithelial cells, while endothelial cells like HUVECs tend to show more sustained NF-kB activation profiles.

To address the reviewer's concern, we analyzed our data for evidence of NF-kB oscillations using three complementary approaches: (i) genome-wide NF-kB/p65 motif accessibility from our snATAC-seq data (new **Extended Data Fig. 6e**) at the three standard time points, (ii) nuclear NF-kB/p65 intensities from immunofluorescence at multiple time points of 0, 30, 100, 170, and 240 min (new **Extended Data Fig. 6f, g**), and (iii) live-cell imaging of NF-kB dynamics (**Fig. R2**). In analyses (i) and (ii), we observed unimodal distributions with similar widths with no increase of the variance at the later time points. From the 30 min time point onward, we do not detect cells indicating a population with reduced nuclear NF-kB activity or protein localization. We conclude that within our experimental framework, NF-kB activity in HUVECs does not show detectable population-level oscillations that would need to be considered in the analysis.

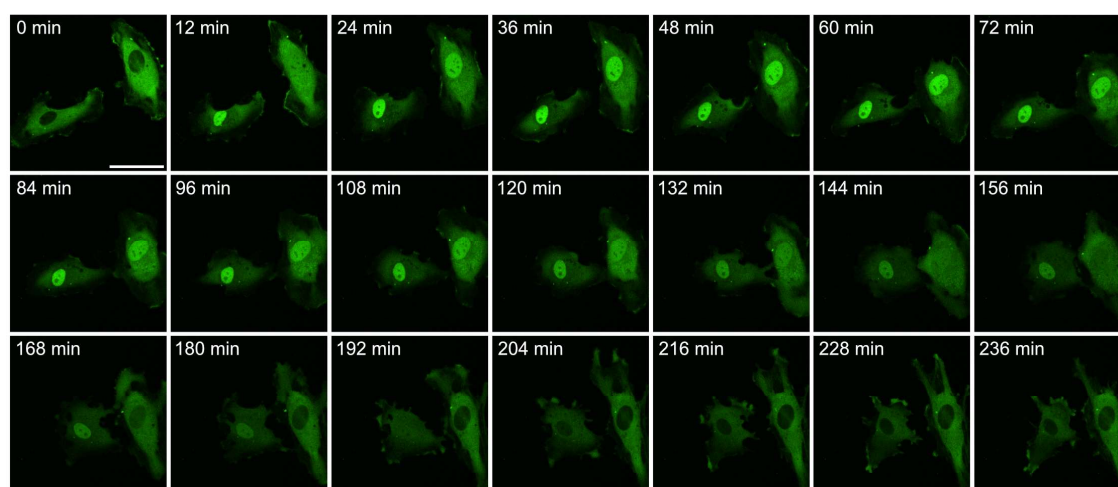


Fig. R2 NF-kB oscillations. Dynamics of HUVECs transfected with a pCMV plasmid expressing hRelA/p65-EGFP via electroporation (0.4 ug/ 1×10^5 cells). Cells were stimulated at t_0 (0 min) with 10 ng/mL TNF α and imaged every 4 minutes for a total time series of 240 min. Snapshots of representative cells at different time points are displayed. Scale bar: 50 μ m.

We further investigated the oscillatory behavior of NF- κ B by transfecting HUVEC cells with GFP-tagged p65 and imaging the cells over a 240-minute time series at 4-minute intervals. In these experiments, we observed rapid shuttling of NF- κ B into the nucleus upon TNF treatment (10-15 minutes), followed by slower shuttling back to the cytoplasm (**Fig. R2**). Thus, upon over-expression of p65 with the transfected GFP construct, we find some evidence for oscillation. However, as stated above, the shuttling of NF- κ B/p65 from the nucleus back to the cytoplasm was absent for the endogenous protein as judged from the immunostaining (new **Extended Data Fig. 6f, g**). These findings are in line with a previous report that points to the effect of protein over-expression on the NF- κ B oscillations ¹⁹.

2. HUVECs are not exactly a relevant cell type for investigating physiological pro-inflammatory responses, due to their umbilical origin. The authors may have historical reasons for the continued use of the source cell, for example to leverage the accumulated data from previous studies. However, to produce broadly impactful findings going forward, I strongly recommend the authors to consider using a more inflammation-relevant cell type. **In this regard, it was prudent to include partial analysis of other cell types. However, only certain aspects of the AC/DC were analyzed, and thus it is insufficient to conclude how much of the findings from HUVECs apply to other cell types.**

We thank the reviewer for raising this important point regarding the physiological relevance of our findings. Our rationale for conducting the study with HUVECs is detailed in **General Comment 3**, which also explains how we have expanded the applicability of our findings beyond HUVECs, a well-established model system for primary human cells. In summary, we included a comprehensive analysis of RWireX from human peripheral blood mononuclear cells (PBMCs), focusing on B cells, CD4+, and CD8+ T cells (new **Fig. 8c-e**, new **Extended Data Fig. 9**). This analysis demonstrates that (i) specific AC and DC modules are detected across B cells, CD4+, and CD8+ T cells; (ii) both shared and cell-type specific regulatory features are identified; and (iii) AC and DC properties are comparable to those observed in HUVECs. Thus, the AC/DC framework identifies conserved regulatory principles across diverse cell types while capturing cell-type-specific features.

3. It is not clear where the multiomic data was used and how the analysis took advantage of the simultaneous profiling. Only Fig S3H-I seems to show results from the multiomic data. **How concordant were the ATAC-seq profiles from the multiomic data with the independently performed scRNA-seq and snATAC-seq?** What additional information was gleaned from the multiomic data?

The multiome data (simultaneous RNA and ATAC from the same nucleus) were used to compute the correlation between AC link activity and TRG expression (**Extended Data Fig. 3h, i**) and the correlation between DC accessibility and TRG expression (**Extended Data Fig. 5d, e**, previous Fig. S4D, E) in the same cell. They provide direct evidence for the functional significance of AC and DC modules by showing correlations between regulatory elements and

their target genes. We also demonstrate in **Fig. R3** that the chromatin accessibility profiles from our multiome ATAC-seq exhibit strong concordance regarding peak locations and relative intensities compared to those from the independently performed snATAC-seq. We have clarified the use of the snMultiome-seq data in the legends to **Extended data Fig. 3h, I**, and **Extended data Fig. 5d, e**, and the Methods section of the revised manuscript.

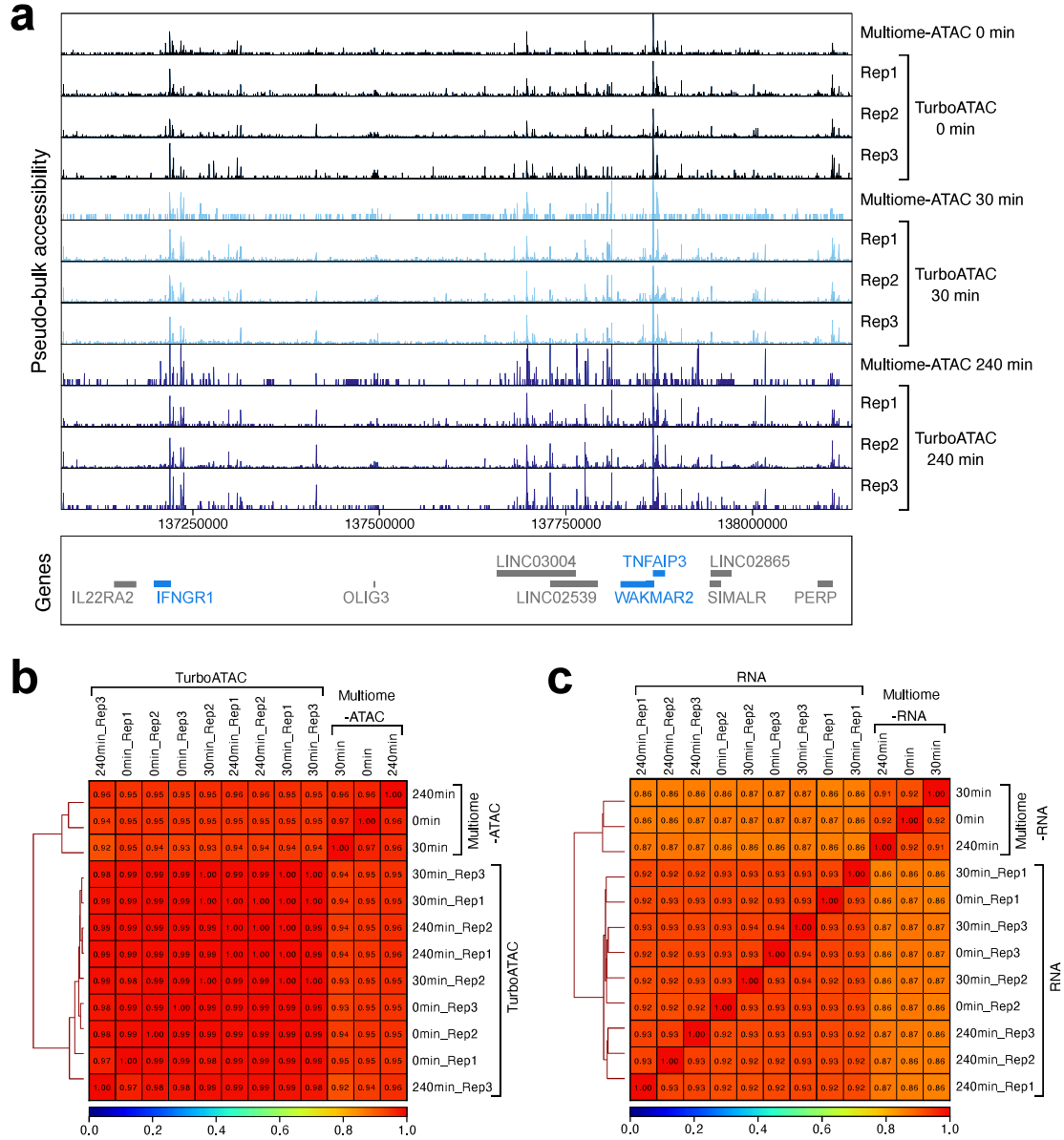


Fig. R3 Comparison of multiome and separate snATAC-seq and snRNA-seq data. (a) snMultiome snATAC-seq and single-omic snATAC-seq for the *TNFAIP3/IFNGR1/WAKMAR2* TRG cluster. Top: pseudo-bulk chromatin accessibility. Bottom: TRGs (blue) and other genes (gray). (b) Genome-wide correlation of pseudo-bulk snATAC-seq and multiome snATAC-seq profiles. Pairwise Spearman correlation coefficients for 10 kb genomic tiles are displayed and ordered by hierarchical clustering. (c) Genome-wide correlation of pseudo-bulk scRNA-seq and Multiome snRNA-seq profiles. Pairwise Spearman correlation coefficients for 10 kb genomic tiles are shown and ordered by hierarchical clustering.

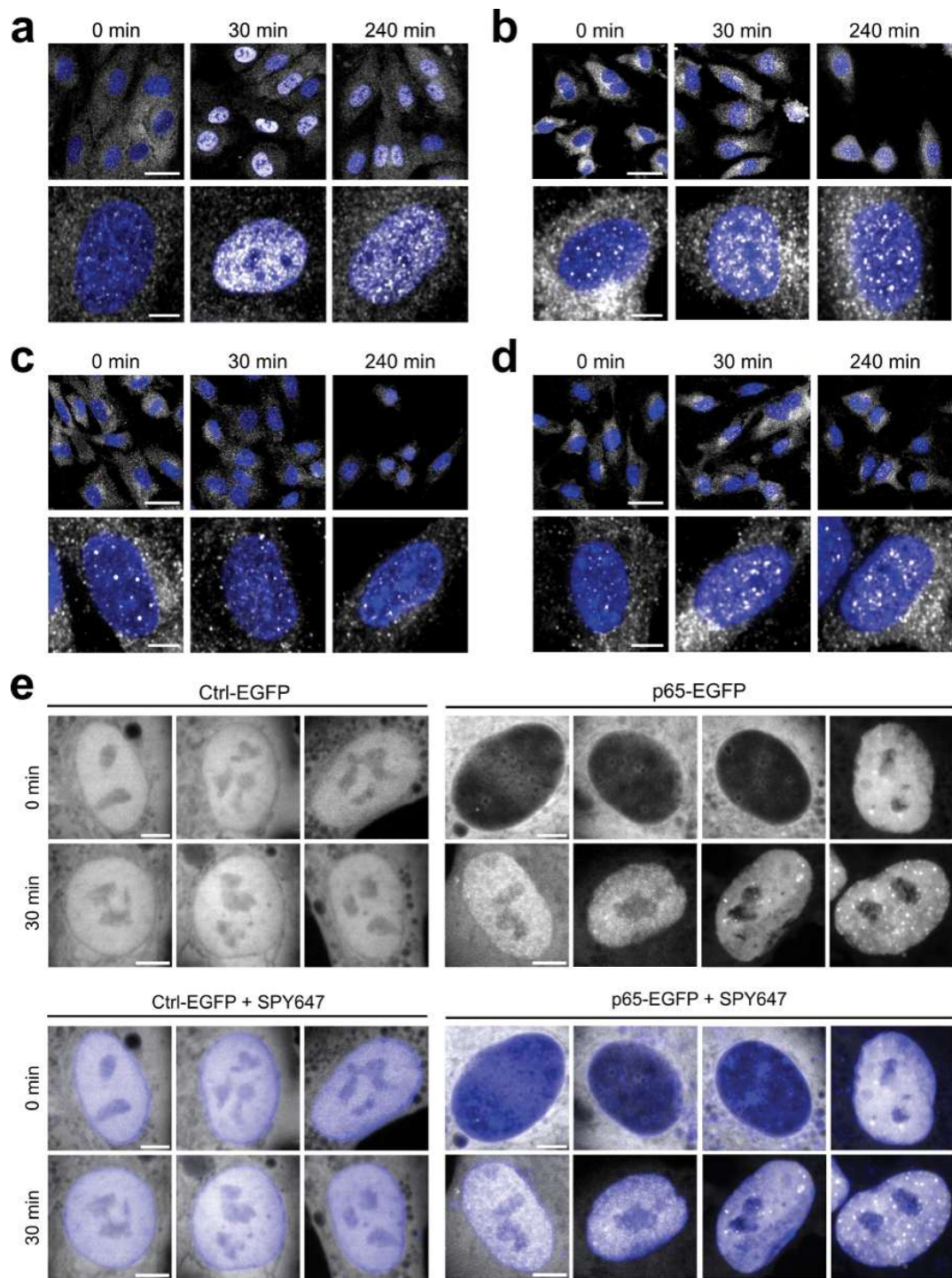


Fig. R4. Detection of NF-κB/p65 nuclear foci in HUVECs. (a) IF on fixed cells with the standard protocol using the antibody #8242 from Cell Signaling Technologies. (b) IF on cells fixed in methanol using ab32536 from Abcam (PFA fixation with this antibody was used in Fig. S5C of our previous submission). (c) Same as panel B but with ethanol fixation. (d) Same as panel B but with cells fixed with methanol:acetic acid (3:1). (e) Nuclear localization of NF-κB/p65 from live cell imaging before (0 min) and after 30 min stimulation with $TNF\alpha$. Zoom-ins of representative cell nuclei are shown. Left: GFP control construct. Right: hRelA/p65-GFP. Top: GFP signal (grey color). Bottom merged images of GFP (grey) and DNA staining with SPY647 (blue). Transfection was performed by electroporation with 0.8 or 1.6 μ g DNA per 1×10^5 cells. Scale bar: 5 μ m.

4. In the results about DC, authors mention “**nuclear foci**” of **NF-kappaB** (Fig S5C) even in unstimulated cells. Have the authors checked whether it may be a fixation artifact? To demonstrate that these are true foci of concentrated NF-kappaB proteins, these structures need to be shown in native conditions, e.g. live cells expressing or transduced with fluorescent fusion, or at least cells with varying degrees of mild fixation. No information was provided in the Methods section on immunofluorescence about the fix agent, concentration, duration of fixation.

We have conducted several controls and conclude that the “nuclear foci” of NF-κB (previous Fig. S5C) are not artifacts: (i) They are detected with another antibody against p65 (**Fig. R4a**, now included in the revised manuscript, see **Extended Data Fig. 6d, f, g**). (ii) They are present across a wide range of fixation conditions, including PFA (previous **Fig. S5C**), methanol, ethanol, and methanol-acetic acid fixation (**Fig. R4b-d**). (iii) They are also detected with GFP-tagged p65 in living cells (**Fig. R4e**) in line with a previous study reporting on nuclear p65 foci²⁰. Thus, we conclude that the assembly of p65 protein into nuclear foci of 0.1-1 μm size is not a fixation artefact.

Minor comments

5. Is the red/blue color assignment correct in Fig S1F? It's opposite from panel E. The legend for Fig S1H is missing.

We thank the reviewer for spotting these inconsistencies. The color scheme in **Extended Data Fig. 1f** has been corrected to match panel E (red for upregulated genes, blue for downregulated genes). We have added the legend for **Extended Data Fig. 1h** (previous Fig. S1H) and removed the coloring since it did not provide additional information.

6. The plot in Fig S3I is not intuitive to understand. Why not show the AC arcs, gene expression tracks and annotations (similar to Fig S3A)?

We have revised the figure legend of **Extended Data Fig. 3h, i** to make the approach easier to understand. We agree that the visualization of **Extended Data Fig. 3h** could be clearer but we are already at the maximum of 10 extended data figures and cannot fit in another large panel like **Extended Data Fig. 3a**.

7. Please describe in the legend of Fig S4D, exactly how the correlation coefficients were computed. Correlation over what entities? How was “DC accessibility” was defined? TRG expression was taken from which time point? In Fig S4E, it would be good to add the locations of genes and H3K27ac peaks on top of the DC track.

We have expanded the Method section and the legend of **Extended Data Fig. 5d** (previous Fig. S4D) to clarify our analysis: Spearman correlation coefficients were calculated between TRG expression (UMI counts) and DC accessibility (aggregated ATAC signal within DC boundaries) using single-cell resolution data from our multiome experiments.

8. In Figure S5A, the three violins per time point are from the 3 replicates?

Yes, each time point shows three violin plots that represent TF binding activity scores from our three biological replicates. We have revised **Extended Data Fig. 6a** (previous Fig. S5A) to clarify this and added labels to identify each replicate distinctly.

9. Does PRDM1 have a sequence-specific binding preference? I suggest the authors should check the sequence logo of the motif used in the TF binding analysis.

We used the established sequence-specific binding motif GAAAGTGAAAGT for PRDM1 (also known as BLIMP1) from the Homer database (Fig. R5). This motif was used for calculating PRDM1 binding activity scores in our transcription factor footprinting analysis of DC regions. Thus, the statement in the Methods is correct that TF activity was calculated with Homer universal motifs for the TFs identified in Fig. 1^J, which includes PRDM1.

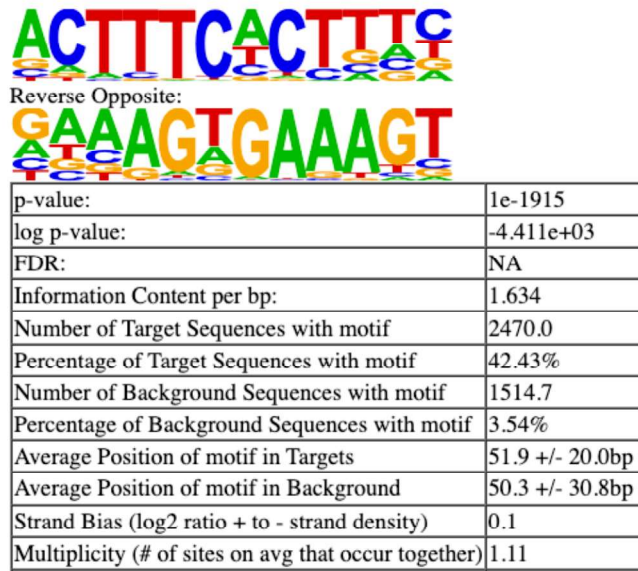


Fig. R5. PRDM1 sequence-specific binding motif used in transcription factor binding analysis. Sequence logo of the PRDM1 (BLIMP1) transcription factor binding motif obtained from the Homer motif database (<http://homer.ucsd.edu/homer/motif/HomerMotifDB/homerResults/motif295.info.html>).

10. In figure 6, many panels lack sufficient descriptions in the legend. For example, in (A), what are the definitions of “Cluster (AC/DC)” and “Cluster (genome)”? In (B) bottom, please explain the four groups in the “TRG fraction” plot. In (D), the arc panels seem to be missing the labels of time points? In the legend for panel (E), “annotation in the middle shows DCs (black)...” seems like it should be “... shows DCs (red)...”.

We thank the reviewer for pointing to the missing information. We have revised **Fig. 7** (previous Fig. 6) and its legend to clearly define all terms and categories, added missing time point labels, and corrected color references in the annotations.

11. Results in Fig S6A seem important to warrant a more prominent discussion in the main paper.

We agree that the differential enrichment of AC and DC modules across various TRG categories demonstrated in **Extended Data Fig. 7a** (previous Fig. S6A) represents an important finding. We now include this observation in the main text within the Results section. Furthermore, we refer to the thorough quantification of these patterns in the new **Supplementary Table S5**, which offers detailed statistics on the distribution of AC and DC features across different TRG categories (clustered vs. isolated, protein-coding vs. lncRNA, early vs. late response, etc.).

12. I found the “AC and DC modules correlate with distinct 3D chromatin organization features” section the most vague and confusing. Please improve readability by justifying each statement (e.g. “...TAD boundaries appeared weaker when overlapping with DCs, ...”) with corresponding patterns in Figures 7 and S7 (e.g. indicating specific “junctions”, “stripes”, and “borders” in the figure) and clarify descriptions of analysis. For example, justify the statement “...Hi-C contact maps further confirmed their coincidence with stripes of increased chromatin interactions” by showing data. What is “stacking of loops/TAD boundaries”? The interpretation of Figure 7 (especially panels C and F) is not intuitive; please explain. The colors in panel E are not visible.

We have revised and reorganized **Fig. 3-7** and the corresponding **Extended Data Fig. 3-7** to address these issues as described in **General Comment 4**. The different parts of the Hi-C analysis were moved to facilitate the comparison with AC and DC features. We have also improved the figure panels by adding clear labels for specific features and enhancing the visibility of interaction patterns.

13. In Figure 8 and associated text, the three example genes NFKBIA, SELE, BIRC2 should be clearly labeled with their AC, AC/DC, DC categories.

We have revised **Fig. 8** and **Extended Data Fig. 8c, d** (previous Fig. S8C-H) and added the requested information to **Extended Data Fig. 8c, d**. To simplify the analysis of bursting kinetics for AC vs DC type TRGs, we have now focused on the maximum log2FC change after 30 or 240 min in burst size and frequency in the revised manuscript (**Fig. 8a, Extended Data Fig. 8a, b**). Representative genes for the AC and DC type TRGs for this analysis are shown in **Extended Data Fig. 8c, d** to illustrate that the padFISH analysis yields similar changes in bursting parameters than the scRNA-seq analysis (**Fig. 8a**).

14. Remarks on code availability: We had a partial testing. Some plots could be produced as expected with RWireX.

We appreciate the testing of our code and are pleased to learn that it works as described. We will continue to expand the documentation and add tutorials to illustrate further applications.

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Response to reviewers for revised manuscript NCB-A55703A

"Two distinct chromatin modules regulate proinflammatory gene expression" by Seufert et al.

We are pleased to learn that reviewer #2 is now satisfied with the revised manuscript and are also very grateful for the additional comments of reviewers #1 and #3 (shown in blue below), which helped us to further improve the clarity and impact of our work. We address their remaining concerns as described in detail below. These revisions significantly strengthen our manuscript by addressing the core methodological and interpretive concerns raised by the reviewers.

Reviewer 1

We have reviewed the revised manuscript from Seufert et al. and acknowledge substantial improvements in analytical clarity and presentation. The distinction between AC/DC modules has been made clearer, and the expanded statistics and supplementary tables are helpful. That said, several issues remain that, in our view, continue to limit the impact and novelty of the study.

1. Definition of ACs and DCs

The manuscript still lacks a clear and consistent explanation of how ACs and DCs are defined and computed. The current approach appears to mix two types of variation (within-population vs. across-perturbation) and two types of resolution (1kb peaks vs. 10kb bins), which complicates direct comparisons between ACs and DCs.

If we understood correctly:

- ACs are defined via correlations between 1kb peak-pairs across individual cells from a homogeneous population (e.g., G1-phase HUVECs at a single time point). They reflect stochastic chromatin interactions and are derived from cell-to-cell variability. These ACs are weakly anti-associated with co-expression while being linked to a tiny burst in transcriptional activity (e.g. Figure 7e), which suggests they may represent highly transient, non-committed contacts.
- DCs, in contrast, are derived from 10kb binned co-accessibility correlations across heterogeneous cell populations (e.g., multiple TNF α timepoints or cell types (PBMCs)). These modules seem to represent dynamically regulated chromatin domains activated or repressed in response to a perturbation.

Please note that this is our own interpretation as we were unable to find a clearer definition in the manuscript itself. Thus, this distinction, based on both biological input and analytical resolution, should be clearly and explicitly stated in the main text, not left to inference from figure legends or methods. At present, the use of different resolutions and populations introduces interpretive ambiguity.

We appreciate the reviewer's request for clearer definitions. Our dual-workflow approach is designed to capture two fundamentally different mechanisms of chromatin-mediated gene

regulation: (1) stochastic enhancer-promoter interactions that occur transiently between discrete regulatory elements (captured by ACs), and (2) coordinated transcription factor activity within contiguous chromatin domains (captured by DCs). These represent distinct biological phenomena that require different analytical approaches to detect reliably.

The reviewer's description of the RWireX method is mostly accurate. We had included such a description in the main text of our manuscript in the Results section titled "Co-accessibility analysis with RWireX reveals features of gene regulation". Additionally, the **Supplementary Information** and **Supplementary Table S4** offer more detailed descriptions and extra information. We agree that providing a clearer definition of our approach in the main text is essential and have addressed this point in the revised manuscript. In particular, we have thoroughly revised the Results section "Co-accessibility analysis with RWireX reveals features of gene regulation". It introduces and describes RWireX now more precisely in the two paragraphs that start with "The RWireX co-accessibility analysis was performed in a dual-workflow approach..." and better explains the logic behind it.

Furthermore, the manuscript suggests a dichotomy between long-range (AC) and local (DC) interactions. However, this distinction appears to stem from methodological differences rather than intrinsic biological properties. Because ACs are based on non-contiguous peak pairs, they naturally span broader distances, while DCs, which are computed from contiguous genomic bins, appear more local. Yet this is a byproduct of the chosen resolution, not a principled biological distinction. For example, Figure 5a and the graphical abstract show DCs linking distant loci (e.g., IFNGR1 and TNFAIP3), which appears to undermine the claim that DCs are strictly local.

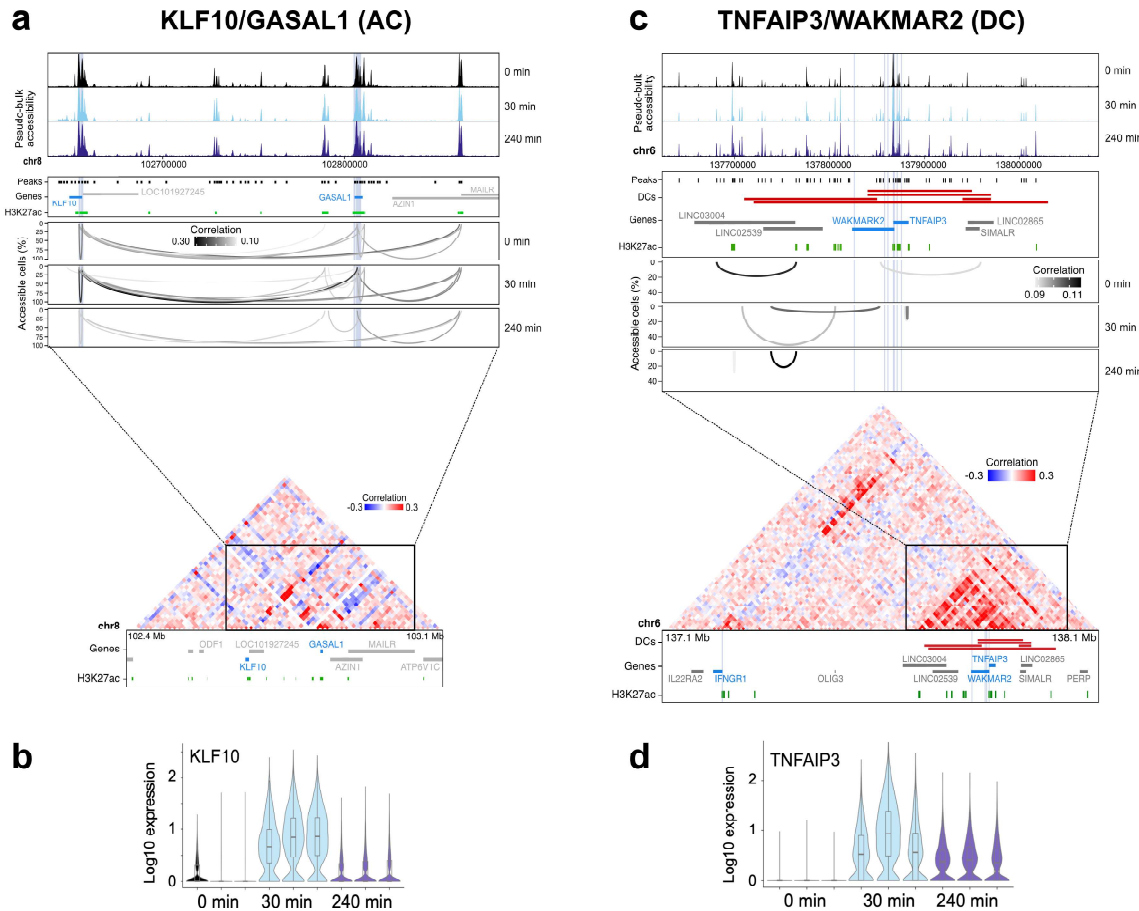
In short, the definition of ACs as "long-range" and DCs as "local" as well as the underlying biological interpretation (e.g. long-range interaction vs. local TF binding activity) are potentially misleading, as it may reflect how the modules were computed rather than what they biologically represent. Unless DCs are explicitly defined as contiguous blocks of highly correlated bins (which, again, is not clearly stated), the rationale for these methodological asymmetries remains unclear. Could DCs be computed at peak-level resolution? Could ACs be computed using binned data? If not, why?

We strongly recommend that the authors clarify the motivations and consequences of using different computational frameworks and avoid mixing analytical artifacts with biological interpretations. A more unified rationale, or at least a justified divergence, would significantly strengthen the conceptual clarity of the study.

We are grateful for this input from the reviewer and realize that the description of ACs as "long-range" vs. DCs as "local" interaction is indeed misleading, as a large fraction of both ACs and DCs falls within a 50-200 kb range. We have now revised our terminology throughout the manuscript, replacing 'long-range vs local' with 'autonomous interactions vs contiguous domains' to reflect the underlying biology better. The key difference to be made clear and emphasized is that AC interactions occur between more isolated and autonomous sites, which require looping of the intervening chromatin to enable spatial proximity and interactions. In contrast, the DCs represent contiguous coordinated accessibility changes of a whole domain

along the genomic coordinate. According to our analysis, they are linked to cooperative changes in transcription factor activity within this region.

The reviewer raises the important concern that our findings might stem from different visualizations of the same underlying biological process and requests clarification of the underlying rationale. We want to emphasize that our approach of using two different workflows simultaneously is designed to assess biologically distinct regulatory principles: ACs capture both rare and frequent stochastic interactions that are best detected at high resolution in a homogeneous population of individual cells. Specifically, ATAC peaks from the pseudo-bulk represent a well-defined, data-driven atomic unit for these transitions. Conversely, the DC analysis minimizes stochasticity by using metacells and larger genomic bins of 10 kb to detect coordinated co-accessibility changes over broader regions. For developing our analysis framework, we have explored the parameter space, including the comparison of bins of different sizes vs. peaks, which is illustrated in part in **Supplementary Fig. 5**. It can be seen that the 10 kb bin size optimizes the balance between signal-to-noise ratio and spatial resolution. Additionally, the figure demonstrates that detecting DCs requires heterogeneity across metacells to reveal cooperative fluctuations in accessibility levels.



Supplementary Fig. 3. Comparison of RWireX-sc and RWireX-meta co-accessibility analyses at the KLF10/GASAL1 and the TNFAIP3/WAKMAR2 TRG clusters. (a) RWireX co-accessibility of the KLF10/GASAL1 TRGs (blue) together with pseudo-bulk ATAC chromatin accessibility and H3K27ac peaks at 30 min (green). Top: RWireX-sc analysis to map ACs at TRG promoters. Multiple promoter-linked ACs are present for KLF10 and GASAL1. Bottom: RWireX-meta co-accessibility showing that no DC is detected for these TRGs. (b) Gene expression of *KLF10* TRG. (c) RWireX co-accessibility of the *TNFAIP3*, *IFNGR1*, and *WAKMAR2* TRGs (blue) together with pseudo-bulk ATAC chromatin accessibility and H3K27ac peaks at 30 min (green). Top: RWireX-sc analysis to map ACs at TRG promoters. No promoter-linked ACs were detected. Bottom: RWireX-meta co-accessibility showing with annotation of DCs (red), showing that *TNFAIP3* and *WAKMAR2* lie within a DC. (d) Gene expression of *TNFAIP3* TRG.

A simple argument showing that our approach reveals biologically relevant differences is now illustrated more clearly in the new **Supplementary Fig. 3**, which is included above. When applying our AC vs. DC annotation to TNF α -induced genes (TRGs), we observe genes like KLF10 and GASAL1 that have multiple promoter-linked ACs but are not located in a DC. In contrast, *TNFAIP3* and *WAKMAR2* lie within a DC, but do not have any promoter-linked ACs, as shown in the figure below.

2. Functional relevance of ACs and relationship to gene regulation

The functional interpretation of ACs remains vague. While DCs are more convincingly linked to transcriptional bursting size and co-expression, the role of ACs is less clear. They appear to correspond to low-frequency, stochastic interactions with weak correlation to gene expression. Given this, the claim that ACs represent a novel regulatory module seems overstated. If ACs are primarily anti-correlated with co-expression and show only very modest effects on bursting frequency, what is then the rationale for identifying and interpreting them as functionally relevant modules?

Thus, we strongly encourage the authors to:

- Reassess their claims regarding the regulatory relevance of ACs.

Current studies point to highly dynamic architectural interactions that have lifetimes in the minute range ^{1,2}, with enhancer-promoter interactions being even more dynamic and occurring at low frequency ³⁻⁵. Thus, ACs as stochastically occurring rare interactions are fully in line with current findings on promoter-enhancer interactions. Furthermore, the functional relevance of ACs includes the following specific points that we have highlighted in our revised manuscript text:

- (i) For 529 TRGs, we find that ACs from TRG promoters link to distant sites enriched in H3K27ac that point to putative enhancers, indicating their involvement in regulating gene expression. These TRGs show no changes in promoter accessibility and are not located within a DC. Furthermore, TRGs are defined by a significant change in gene expression upon TNF α treatment, and identifying potential enhancer interactions is an important aspect of understanding the underlying gene regulation mechanism.

(ii) ACs show a bimodal distribution in their abundance among the cell populations, differentiating rare and frequent ones (**Fig. 4a**). The pile-up plots of contact frequency enrichment for frequent ACs show that the regions between the two peaks linked via the ACs have a higher contact frequency enrichment than the flanking regions (**Fig. 4b**), indicating that they are more frequently at TAD borders with higher contact probabilities within the TADs than beyond. Furthermore, as described in the context of this figure in the Results section, frequently observed ACs could involve chromatin looping as inferred from a direct comparison with Hi-C contacts (see also **Fig. 3f**). Contributing to a certain 3D genome structure would also be linked to function and direct more transient contacts like those between promoters and enhancers. This is similar to the general consensus in the field that TADs are structurally relevant for gene regulation. However, one would not claim that each link detected in a Hi-C map can be assigned to a gene regulatory function.

(iii) We find statistically significant differences of TRGs with ACs in comparison to those annotated as promoter-regulated or within DCs with respect to induction strength, induction time, co-expression, and transcription bursting kinetics; these are summarized in **Fig. 8b**. Thus, they likely represent a signature of a distinct gene induction mechanism and also are associated with functionally relevant features. In addition, our analysis of PBMCs (**Figure 8c-e, Extended Data Figure 9**) shows that a fraction of 20-30% of ACs are conserved across different cell types while the other fraction is cell-type-specific, further supporting their functional importance.

- Consider whether the observed ACs might reflect structural or architectural background noise, rather than specific regulatory mechanisms.

We appreciate this important consideration. Our reproducibility analysis across replicates (**Extended Data Fig. 3a-c**) demonstrates that ACs represent consistent interaction links rather than random noise. In addition, we think that the implicit distinction of functional vs structural-only roles is too deterministic (as discussed above). Our findings on ACs as transient interactions that occur stochastically in single cells are fully in line with the highly dynamic nature of long-range promoter-enhancer interactions, as well as architectural loops that are emerging from recent studies³.

- Add visual overlays of gene expression (e.g., KLF10 and GASAL1) to Figure 3b to substantiate claims about transcriptional associations (i.e. to provide a clear visual representation of an association between changes in % accessible cells and gene expression).

The AC pattern changes related to gene regulation are complex, involving the gain or loss of activating and repressive links, changes in the strength of correlations, and variations in the number of accessible cells. These changes are evident in **Fig. 3b** when comparing the AC pattern for KLF10 and GASAL1 at 30 minutes, where these genes are induced, to the 0 and

240 min time points. While creating a single composite metric is technically challenging, we have improved the description of **Fig. 3b** to better emphasize the key regulatory changes at 30 minutes. These include the appearance of a new connection from a putative enhancer to the GASAL1 promoter, the strengthening of the link from this enhancer to the KLF10 promoter, and a significantly increased correlation of accessibility between the GASAL1 and KLF10 promoters. Therefore, our AC analysis indicates that the interaction among these three loci is the main factor driving the induction of KLF10 and GASAL1 expression at 30 minutes.

Moreover, the manuscript's comparative analysis with PBMC data focuses almost exclusively on DCs. If ACs are a meaningful class of chromatin modules, how do they behave across systems? Are they conserved, context-specific, or reproducible?

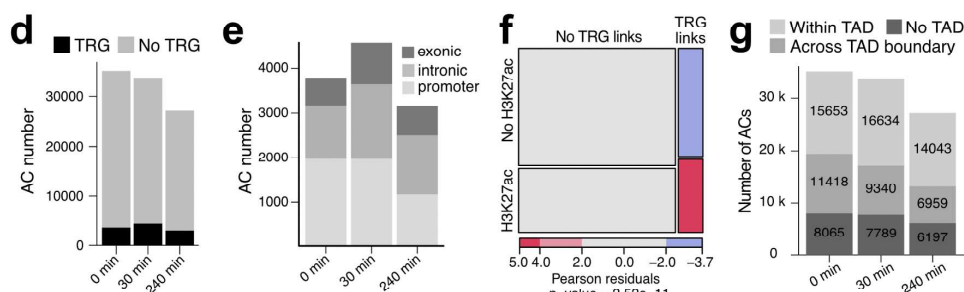
We show in **Extended Data Fig. 9c-e** that valuable information on cell-type-specific gene regulation can also be obtained by mapping ACs across cell types. Our current analysis is limited by space constraints as well as technical issues. The RWireX-sc analysis profits largely from deeper snATAC-seq coverage, which is now stated in the Methods. It is about 5-fold lower for the PBMCs than for the HUVECs, where we have an unprecedented deep scATAC-seq coverage of ~150,000 unique integrations per single cell. It is straightforward to generate such data with our enhanced scATAC-seq protocol and sufficiently deep sequencing so that this issue can be addressed in future studies.

3. Figures and visualization

Several figures are visually appealing but difficult to interpret. We suggest the following improvements:

- Extended Data Fig. 3f appears blank. Please confirm whether this is intentional.

We confirmed that the mosaic plot in **Extended Data Fig. 3f** displays correctly in the submitted file with Acrobat as pdf viewer and contains the intended correlation analysis data. The figure panel should look like it is displayed below.



- Figures 2a and 7d: While informative, they are hard to read. A histogram or bar plot format could improve interpretability.

We would like to keep **Fig. 2a** and **Fig. 7d** but we have added representations of the gene cluster composition as stacked bar plots in **Extended Fig. 2e** and **Fig. 7d**. In addition, the numbers on AC, DC, AC/DC, and clusters without annotation are given in **Supplementary Table 5**.

- **Figure 3c:** Include a legend or scale for circle size to indicate overlap/strength.

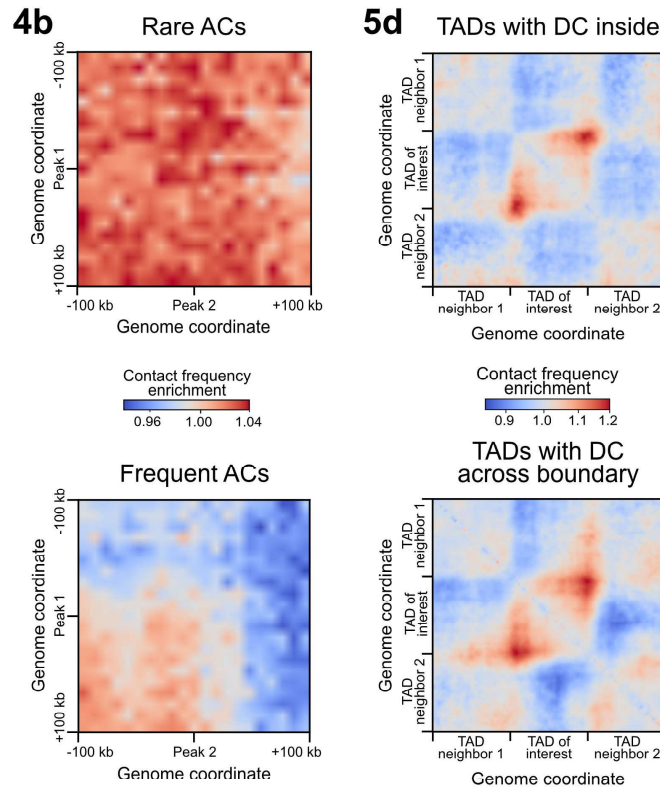
We have added additional information to the legend and size scales for circle overlaps/number of links to the panel.

- **Figure 2d:** Define “Abundance (%)” clearly in the caption.

The “Abundance (%)” as y-axis label in Fig. 2d has been replaced by “Cell fraction (%)” and is defined in the caption as the percentage of cells that displayed the indicated combination of co-expressed genes among the total population.

- **Figure 4b:** The matrix plot lacks clear axis labels. Are these genome coordinates? Why are ACs plotted on both axes, and why is the matrix not symmetrical?

We apologize for the lack of clear axis labels (genomic coordinates) and have added them (s. below). One peak of the AC link is at the center of the x-axis and the other at the center of the y-axis. Rare and frequent ACs are centered, scaled, and averaged, and the contact frequency enrichment is visualized in this pile-up representation, respectively. The pile-up plot becomes asymmetric if the regions between the two peaks linked via the ACs have a different contact frequency enrichment than the flanking regions. This is the case for the frequent ACs, which leads to the conclusion that they are more frequently at TAD borders with higher contact probabilities within the TADs than beyond.



Centered, scaled, and averaged pileups of ACs and DCs in relation to Hi-C contact frequencies from untreated HUVECs generated with coolpup.py⁶. **Fig. 4b.** Chromatin contact frequency enrichment at rare (top) and frequent ACs (bottom). **Fig. 5d.** Chromatin contact frequency enrichment at TADs with DC within (top left) or across TAD boundary (bottom).

- Figure 5d and supplements: Similarly, axes need clearer definitions, and it should be easier to locate the DCs and TADs.

Again, we apologize for the incomplete figure panel labeling. Enhanced axis definitions and clearer localization of DCs and TADs are now provided as shown above.

- Graphical Abstract: The current version lacks polish and clarity. Consider enhancing the cartoon quality and labels to improve communication of the AC/DC model.

The graphical abstract and "Highlights" section have been removed per NCB journal style.

4. Other minor points

- Page 21: The sentence beginning with "Interestingly [...]" and/or DC features" is incomplete.

Thank you for noticing this. We have corrected the incomplete sentence on pg. 21 regarding AC/DC features. It now reads *"Interestingly, we identified a subset of TRGs with direct promoter regulation that either was present independently or occurred in combination with AC and/or DC features (Extended Data Fig. 8e)."*

- **Data Integration:** A dedicated method section describing how datasets (e.g., snATAC, snRNA, Hi-C, ChIP-seq) were integrated would be valuable.

We have now added a dedicated section describing integration protocols for snATAC and scRNA-seq in the context of the cell cycle annotation. For the multiome data ATAC and RNA data are acquired from the same cell and no integration is needed. For Hi-C and ChIP-seq no specific integration with other datasets was conducted as our analysis used the shared genome coordinate to align the different readouts.

Summary

While the manuscript has improved in clarity and organization, it remains unfortunately unclear what this study substantively contributes to our understanding of gene regulatory mechanisms. The distinction between ACs and DCs still rests on computational differences rather than on clearly defined biological principles, and the functional relevance of ACs, in particular, is not convincingly established. As a result, the broader conceptual impact of the AC/DC framework remains limited. At a minimum, clearer definitions, justification of analytical choices, and more cautious interpretation of the regulatory significance, especially of ACs, are essential to support the reported claims. Additional effort to align computational methods with biological rationale would also considerably strengthen the manuscript's value to the field.

We sincerely appreciate reviewer #1's thorough engagement with our work. The concerns raised have helped us clarify fundamental aspects of our approach and strengthen our biological interpretations. As detailed above, our AC/DC framework captures distinct regulatory mechanisms that complement existing models of chromatin organization and gene regulation.

Reviewer 2

My comments have been addressed in the revision.

We thank the reviewer for assessing our revised manuscript and are glad to learn that the comments have been addressed in the revised manuscript.

Reviewer 3

Regarding the statement "... immunostaining of NF-kB revealed the presence of some nuclear NF-kB foci, which were already visible in the uninduced state before...", the images in Ext Data Fig 6d and in Fig R4 are not convincing for the uninduced cells to support this conclusion. The small foci in "0 min" cells are hardly specific to the nucleus against the cytoplasmic background. Also, what is the last (4th) image of Fig R4e? Is the upper set of images from uninduced cells? Importantly, the p65-EGFP in live cells don't show any nuclear foci in uninduced condition. Overall, without a rigorous background correction and foci quantification, I suggest the statement should be limited to the stimulated conditions after TNF treatment.

We acknowledge the reviewer's concerns regarding the visibility of nuclear p65 foci in uninduced conditions. We have revised our statement to clarify that these foci are mainly observed under stimulated conditions, while noting that some assemblies might also be

present in unstimulated cells. We apologize for the lack of clarity in **Fig. R4e** from the previous revision letter. The updated version below now includes labels for the right panels as well, to specify which figure panels correspond to the uninduced (0 min) and induced (30 min) conditions.

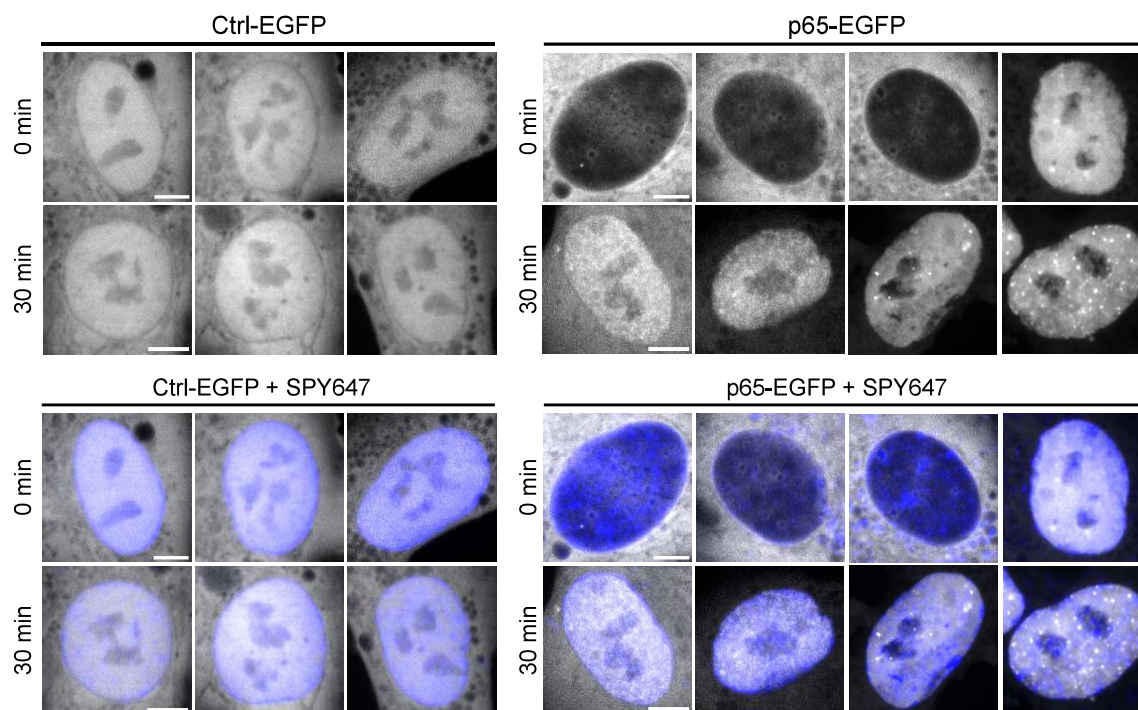


Fig. R4e revised. Nuclear localization of NF- κ B/p65 from live cell imaging before (0 min) and after 30 min stimulation with TNF α . Zoom-ins of representative cell nuclei are shown. Top panels: GFP signal (grey) from the Ctrl-GFP construct (left) and the p65-EGFP construct (right). Bottom panels: same as top panels, but with merged images of GFP (grey) and DNA staining with SPY647 (blue). Transfection was performed by electroporation with 0.8 or 1.6 μ g DNA per 1×10^5 cells. Scale bar: 5 μ m.

“...we observed homogeneous populations for nuclear NF- κ B/p65 and its activity in HUVEC cells at all time points studied, with no evidence of oscillation ...” is not a fair description of Ext Data Fig 6e-g. While unimodal, these distributions have large variances at all time points, suggesting heterogeneity in individual cells or asynchronous behavior, which are indistinguishable in snapshot samples. Some live-cell imaging of fluorescently labeled p65 was attempted (Fig R2 showing two cells), but the result is inclusive without a quantification and statistical analysis. Therefore, the statement should be adjusted to match the data shown.

We agree that analyzing snapshots at different time points cannot distinguish between heterogeneous steady states and asynchronous dynamics. However, as the reviewer mentioned, the data in **Fig. 6e-g** clearly show that the distribution of p65 in the cell nucleus is unimodal at all timepoints. It peaks after 30 minutes of treatment and then declines linearly at later times. Therefore, there is no need to consider the coexistence of two distinct induced states in our analysis of the single-cell sequencing data. To address the reviewer’s concern, we replaced "homogeneous populations" with "unimodal distributions with similar cell-to-cell

variability." We also recognize that the large variances truly reflect biological heterogeneity in NF- κ B responses.

It is noted that the analysis of living cells cannot be done with the primary HUVEC cells used in our study without ectopic protein expression. The admittedly limited analysis of transiently transfected cells included in the previous revision letter in the context of **Fig. R2** was included to illustrate that the introduction of fluorescently-tagged p65 affects inflammatory response kinetics.

Although the authors have added RWireX analysis of additional cell types (Fig 8c-e) from published datasets, these do not approach a comparable level of examination, especially in relation to gene expression, because they did not include correlative analysis of matching scRNA-seq datasets. The added section seems to simply describe the results from applying RWireX to other snATAC-seq datasets. I suggest authors acknowledge that the same in-depth analysis framework should be validated with another immune-relevant physiological cell system, due to the exclusive reliance on HUVECs.

We acknowledge this limitation and point out that our detailed RWireX methodology and parameter optimization in HUVECs provide the foundation for future, more comprehensive studies in other systems. It is clarified that our PBMC analysis demonstrates proof-of-principle for AC/DC conservation across cell types. At the same time, the HUVEC system remains our primary model for an in-depth analysis. An additional application introduced in **Fig. 8c-e** for the analysis of PBMCs is to call DCs across a heterogeneous population and then compute an accessibility score for these domains for each single cell. We believe that this approach has great potential for further applications in which accessibility patterns between different cell clusters are compared.

For future revisions, the authors should help reviewers to quickly identify the changes by marking the modified text in color or tracked changes.

We acknowledge this helpful suggestion and have now included a version with tracked changes.

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