nature portfolio

Peer Review File

RB loss modulates chromatin organization by regulating cohesin-dependent loops and enhancer-promoter interactions

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
RB is a well-established cell cycle regulator whose canonical mechanism of action is to bind E2F transcription factors at the promoters of cell cycle genes and recruit chromatin regulatory complexes to suppress transcription. RB has been implicated in many other cellular functions beyond the cell cycle and E2F, however, including cellular differentiation and cancer lineage infidelity. The mechanisms underlying such non-canonical functions are largely unknown. This manuscript investigates whether RB influences chromatin looping dynamics and gene expression independent of E2F. This work extends the groups prior Mol. Cell paper that used engineered immortalized cells to perform high quality RB Chip-seq to demonstrate Rb binds enhancers and insulators independent of E2F, as well as promoters in an E2F dependent fashion. The current paper utilizes Micro-C to analyze the same engineered cell system to assess the impact of Rb on higher order chromatin structure. A key finding in this paper is that RB loss prevents normal removal of CTCF sites during mitosis. This results in an elevated number of cohesion-defined chromosome loops with stronger chromatin interactions within them. Another important finding is that RB-bound CTCF sites show increased chromatin accessibility, transcriptional activity, and stronger insulator boundary strength compared to CTCF sites not bound by RB. This suggests a novel transcriptional co-activator mechanism for RB at these regions. Overall, this work represents an important and significant area of inquiry as it has potential to shift paradigms in understanding RB function and the impact of RB deficiency in cancer.

There are some conceptual and technical limitations, however, that should be addressed before publication in Nature Communications. The authors demonstrate RB loss increases the number of chromosome loops and strengthens interactions within those loops. To a first approximation this may be expected to increase the number of enhancer/promoter interactions within a larger number of TADs, increasing the fraction of genes expressed per cell. A standard scRNA-seq analysis could be used to test this. The data would also have the benefit of testing whether RB affects molecular heterogeneity between cells. Molecular heterogeneity could confound interpretation of peak calls in bulk Micro-C, Hi-C, and ChIP-seq data if a larger number of potential genome interactions sites are spread across a larger fraction of the genome. This could explain why the strength of H3K27ac HiChIP peaks is reduced upon RB loss despite a stronger H3K27ac ChIP-seq signal. Another issue that is not directly addressed is the stability of higher order chromatin structures across cell cycles. This chromatin structure presumably needs to be recreated with some fidelity after every cell division cycle to maintain stable phenotypes. It would be interesting to know if RB loss decreases the fidelity of this process as the findings may be relevant to important non-canonical RB functions like cancer lineage fidelity.

Additional Strengths:
1) The generation and analysis of data is robust and well controlled, with exceptions noted below. The experimental approaches and methodology used are appropriate.
2) The computational approaches for all omics analyses were sound and justified based on the methodology and hypotheses. Gold-standard chromatin conformation pipelines such as ‘Juicer/JuiceBox’, ‘cooltools’, ‘HiCCUPS’ were described appropriately for reproducible analyses.
3) Complementary RB knockout and RB activation models are employed in a non-transformed, genetically stable cell line so RB-dependent effects can be cleanly identified.
4) Use of Micro-Capture technology, which provides nucleotide-resolution and higher sensitivity for chromatin interactions, offered a distinct advantage in detecting the subtle (but significant) genome-wide RB1-dependent chromatin organization

effects while minimizing digestion bias which can sometimes hinder interpretations when other chromatin conformation assays are used. The use of Hi-ChIP (H3K27ac) is also appropriate for investigating RB's impact on enhancer-promoter interactions.

Additional Weaknesses:
1) It would be nice to see the distribution of mapped sequencing reads across the genome to get a sense of how the MicroC, HiC, and ChIP signals are distributed in the presence and absence of RB.
2) The authors claim that RB promotes removal of cohesion, a significant finding, but no complementary or orthogonal experiments were performed to confirm this.
3) The mechanisms by which RB removes cohesion or co-occupies SMC3 sites independent of E2F is unknown, but the authors could speculate in the discussion section. Potential interactions with Wapl could also be discussed,
4) The figures are difficult to interpret easily given their size and resolution. For example, it was hard to discern the subtle differences in TAD boundaries between RB KO and RB WT (Figure 1H and 2F).
5) The descriptions provided in the figure legends are cursory, making it difficult to interpret figures without frequent consultation of the main text.
6) The deltaCDK RB activation experiments do not control for cell cycle phase distribution differences as far as I can tell. Pre and post Dox induction time points are compared, but only the Dox induced cell will undergo cell cycle arrest. This could be controlled by comparing confluent, G1 arrested Dox induced and Dox uninduced cells.
7) While the engineered cell model is a strength, it is the only model employed so the generality of the findings are unclear. Whether findings are applicable in the more clinically relevant context of RB deficiency in cancer is unclear. Targeted experiments to validate some key observations in other cell lines would be helpful.
8) The manuscript would benefit from a clearer and more detailed graphical abstract which depicts the RB-dependent effects on TAD boundaries, enhancer-promoter interactions, and gene expression. The current graphical abstract does not clearly convey the high-level chromatin organizational changes that the paper is demonstrating.
9) The gene set enrichment and functional analysis of EMT needs clarification. The PI focuses on 181 genes showing an increase in SMC3 binding in the absence of RB. These genes are enriched for EMT genes and the PI provides functional evidence that RB loss causes EMT properties. However, the authors also conclude that RB depletion significantly decreases expression of the "cohesion gain" gene set. These conclusions seem at odds with one another.

(Remarks on code availability)
My co-reviewer reviewed the code and felt it appropriate based on standards in the field. The paper makes use of publicly available code and thus is generally available to the community.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In this manuscript, Lee et al. investigate Retinoblastoma protein (RB) dependent effects on chromatin and chromosome organization. They utilize comparisons between control and knock out cells as well as an exogenous expression system to induce a non-phosphorylatable version of RB. They use chromosome conformation capture based methods to investigate chromosome organization in the different genotypes of cells under varying conditions related to RB phosphorylation or cell cycle position. The also undertake SMC3 ChIP-seq and H3K27ac HiChIP experiments to further elucidate RB status effects on chromosome organization and dynamics. Analysis of these experiments is blended with previously reported data from the same cell systems and organized into the present manuscript. The authors argue based on their data that RB status influences DNA and chromosome topology and in particular this effect reorganizes Cohesin complexes and further influences the ability of enhancer-promoter contacts to be made, ultimately altering gene expression patterns.

This was a difficult paper to review from a number of perspectives that prevent me from being enthusiastic about the publication of this study. In its present form it looks like an article from Plos One or Scientific Reports where data is reported for the record, and future investigators may or may not see value in it, but the current take home message is unclear. I've listed the biggest drawbacks to this study below to help the authors improve their work.

1) The authors, for the most part, compare RB wild type status cells with knock out cells. The resulting molecular characteristics observed indicate that alterations take place in RB's absence. It is an overinterpretation to assume that RB has a deliberate regulatory role related to Cohesin positioning and chromatin binding when it is present. The title of the paper includes this error. It is just as likely that the data in this study captures random downstream consequences of RB loss as compared to a deliberate function as the authors hope. The model diagram in figure 5 indicates the authors conclusion of an 'RB/cohesin pathway', but there is no evidence that this pathway exists from data in this paper. Indirect influence of Cohesin by RB status has also been reported by others previously, making this a very modest conceptual advancement.

2) The paper in many places is an unconnected series of observations. Figure 1 is the best example of this as it attempts to link different observations from exogenous non-phosphorylatable RB and RB deficiency. However, the staging of the different RB status cells are starkly different either because of time point choices or manipulation by trypsinization and re-plating. The narrative of the results section that corresponds to this figure states a number of different changes at varying stages of the cell cycle, but the illustrations are more about experimental design than illustrating cell cycle consequences of RB status. The biggest admission from the authors that this figure is incoherent is panel I where they offer brief conclusions as they already expect that readers of their paper won't come to these conclusions on their own from the data presented. The narrative of the results indicates RB gain of function causes downstream alterations in heterochromatin structure, but the microscopy image in figure 1E offers a rather superficial look at gain of RB function in the regulation of heterochromatin and so I struggle to find a way to agree with the conclusions in panel I. In most figures the data displays appear to be mostly a collection of comparisons that show statistically significant differences even though they don't offer a singular conclusion. Instead the more I read on, the more I encountered seemingly random measurements that support the broad conclusion that chromatin structure changes when RB is lost. The figures also don't relate well to one another as there is little reason to expect that figures 1 and 2 lead to a need to understand Cohesin occupancy as a logical progression of the work in figure 3. Many other factors could have been profiled and found to be altered as well.

3) In many places the author's narrative in the results describe changes to chromatin structure and they describe a statistical test and significance level to go with it. However, the data display panel that they cite along with it often shows little evidence of change due to RB status. In figure 1, panels D and H. Panel 2F, panels 3A and B, and 4B and C all contain genotype comparisons that suffer from looking very similar, if not virtually identical. In some cases, very modest changes are illustrated by graphs such as figure 1G. Even if a statistically significant difference does exist in the cases of these displays, what does such a small difference mean? Are these subtle changes really meaningful in biological terms? Highly similar data in control and RB knock out makes the value of this study hard to appreciate.

4) Somewhat related to point 3 and some earlier concerns, the manuscript largely lacks biological consequences to go with the observations of chromatin structure. Subtle changes in chromatin structure may be important biologically, or they could be largely inconsequential. I was surprised to read to the end and see that the authors really don't have key biological relevance for the consequence for Cohesin misregulation by RB loss. Given all of the biological consequences of RB loss that are already published and RB's key role in human cancer, the subtle change in adherence of cells to a culture dish and scratch assay closure are quite minimal functional experiments. Even in the case of these observations, does the data presented really indicate that altered Cohesin loops causes these effects compared to alterations in other aspects of RB function? Some gene categories that they discover in their analysis are interesting. RB loss has been shown to alter properties of advanced cancer cells that are consistent with EMT and RB has also been documented to have a role in transcriptional regulation of myogenesis. The opportunity to connect to more meaningful biological scenarios exists, but at present the manuscript stops short of connecting the chromatin structure details to functions that yield a better understanding of RB.

Some smaller, but also important issues are:
5) The scratch wound experiment either contains an error or it doesn't represent what the authors hope. The Y-axis is a measure of closure of the scratch (presumably through cell migration). However, the control cells display measurements that exceed 100%. Does this mean that control cells migrate in the opposite direction? Without these outlier and counterintuitive data points included, the statistically significant difference with RB knock out would likely be lost. Something is wrong with this display/experiment.

6) The authors offer a comparison with Wapl effects on Cohesin (SMC3) levels. 'Notably, these results resembled the changes observed with cohesin accumulation at TAD boundaries following Wapl depletion (ref 47), albeit at a weaker magnitude.' Without a side-by-side comparison experiment look to look at Wapl depletion, statements like this have little value. This is but one example of many descriptive connections between the observations from their data and the literature that are very much opinion and not relevant to a results section.

7) Most figures contain unreadable text and numbers embedded in them. Sometimes these are labels, sometimes measurements or statistical values. I'm not sure if it is important to be able to read them. Are they already stated in the narrative of the results? Do I need to read them? This deficiency adds to the lack of clarity.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The manuscript by Lee et al homes in on the dynamics of chromatin structure and its role in shaping the expressivity of the genome. Specifically, it addresses the regulation of the interplay between chromatin-bound CTCF and the cohesin complex assigned roles in the partitioning of the genome into chromatin domains. Having previously focused on the inner workings of the tumor repressor protein RB1, the authors perceived that there could be a non-canonical role for this factor going beyond its well-known function of repressing E2F- dependent functions. To deal with the E2F-dependent noise provided by this pivotal function in cell cycle progression, the team resorted to extensive Chip-seq and Micro-C analyses of cells carrying inducible mutant RB1 expression vectors. The key outcome is the documentation that RB1 functions to antagonize the

chromatin-bound CTCF-cohesin complexes to remodel the partitioning of a subset of TAD domains and that phosphorylation of RB1 controls this process. Importantly, it describes a painstaking strategy to uncover that CTCF-cohesin complexes are stabilized in the absence of an RB1 function and that RB1 functions to release the cohesion complex from chromatin-bound CTCF specifically in prometaphase cells. It is of significant interest that chromatin loops tend to get smaller and more numerous in RB1-deficient cells. The authors have adopted heroic efforts to highlight that these outcomes are different from the canonical RB1 function that serves to repress E2F-dependent gene expression. Taken together, the data supports the view that a subset of chromatin domains is rewired in the absence of an RB1 function in a non-canonical manner. I was also intrigued by the discussion that RB1 might control also the role of the condensin complex and that the absence of an RB1 function might generate chromosome instability analogous to its role in the CTCF-cohesin complex. The observations of the manuscript have obvious implications for cancer in instances where the RB1 function is perturbed, genetically or otherwise. The surprising outcome of this reasoning is, however, that the plasticity of the genome function to enable adaptation to changing environments might be compromised.

Technically, the manuscript describes cutting edge approaches that include a balanced consideration of alternative explanations. The experiments appear well executed and with appropriate controls.

To sum up, this is an excellent although dense report, which provides yet another surprising twist to the regulation of the CTCF function. It provides a much needed insight into the dynamics of the formation of chromatin domains that underlie the function of the genome. However, there are a number of loose ends, listed below, that the authors might wish to consider.

• It is not crystal clear to me why the Extended data Figs S5-S7 are treated so summarily. Although I appreciate the space limit of the manuscript format it makes little sense to include this data without explanations.
• I lack an insightful discussion about how the absence of an RB1 function might affect cancer evolution. Although loss of RB1 function occurs late in cancer development as alluded to by the authors, its absence would be expected to freeze/stabilize chromatin interactions underlying the expressivity of the genome running counter to the general view that cancer cells evolve by readily adapting to a changing environment. Even though this is not a topic of the manuscript, its observations have strong implications on this topic. For example, the authors could consider to analyze single cell RNA-seq data to explore the non-canonical function of RB1 even though I realize that the space of the manuscript format limits such options.
• On a similar note, I lack a few sentences discussing the observation that genes encoding factors involved in the EMT process are strongly overrepresented in two of the non-canonical clusters. This is especially relevant as RB1 has been implicated to control this process, which likely underlies the ability of cancer cells to establish metastatic niches.
• In the manuscript it is stated “RB depletion reduced the strength of H3K27ac HiChIP peaks across all three interaction 256 groups despite slightly stronger H3K27ac ChIP-seq signal (Fig. 4c and Extended Data Fig. 15).” I lack a sentence or two explaining this discrepancy.

Rolf Ohlsson

(Remarks on code availability)
see above

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Revised Manuscript NCOMMS-24-71231A: RB loss modulates chromatin organization by regulating cohesion-dependent loops and enhancer-promoter interactions.

Manuscript Summary:
This revised manuscript includes additional experimental data and text/figure revisions, including the title, to strengthen the rigor of the conclusions drawn and the clarity of the presentation. Key additional data buttressing the conclusions include scRNA-seq, STAG1/2 ChIP-seq, MED15 ChIP-seq, proteomics, and quantitative ChIP-PCR. These changes have adequately addressed nearly all the issues we raised on prior review. One experiment was attempted to replicate some of the findings in an additional cell line model, as requested, but this experiment did not yield interpretable results. Given the technical challenges associated with these types of experiments, evidenced by the relative lack of equivalent data in the published literature, I don’t think this should preclude publication of the current manuscript. I support publication of the current version of the manuscript in Nature Communication, perhaps with minor revisions listed below.

In my opinion the work should be published for several key reasons. This is one of the first papers to address the impact of RB on higher order chromatin structure like chromosome looping. A key finding is that RB loss increases the number of cohesin-defined chromosome loops and their insulating activity. Given increased interest in non-canonical functions of RB in cancer, like phenotypic plasticity, the work presented in this manuscript is an early step in trying to elucidate the molecular basis of such non-canonical functions, even though cancer models are not deployed to generate the data. The data does provocatively align with observations in the cancer plasticity field where RB loss induces an EMT as an intermediate plastic state that evolves into lineage variants, suggesting RB’s impact on higher order chromatin structure may be involved. The

data presented is of high quality, especially in light of the difficulty the field has had in generating good RB ChIP-seq data with available antibodies. It has been observed for some time that RB can activate gene expression in addition to its canonical role in gene expression repression. The molecular basis of RB induced gene expression is not well established. This paper provides data to rigorously confirm RB activated gene expression and provides a potential mechanism. Overall, the work presented is important and potentially paradigm shifting.

Minor issues that could be addressed at the editor's or author's discretion:
1) It would be nice to see UMAPs of the scRNA-seq data, perhaps color coded by the transcriptional clusters identified as well as the number of genes/UMI metric.
2) The authors focus the insulator discussion on those separating promoters and enhancers, rightfully given the data they are interpreting. However, insulators also insulate from encroaching heterochromatin. The authors may want to speculate in the discussion how the increased number and size of chromatin loops upon RB loss may affect the organization of heterochromatin.

(Remarks on code availability)
My co-reviewer previously reviewed the code and deemed it appropriate and justified for the data and analyses presented.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
The revised manuscript is an improved version of an already excellent study. Given the space limits the authors have done a good job responding to my concerns.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
Retinoblasma protein is encoded by a major tumor suppressor able to repress cell cycle genes through mechanisms by which RB associates with E2F to counteract its role in gene activation. Mutations in CDKs regulating RB association with E2F have been associated with tumorigenesis due to such E2F regulation, though additional phenotypes often developing in association with RB dysfunction, like sister chromatin cohesion defects, may be due to other reasons than E2F. Here, the authors find a novel role of RB that regulates, on top of E2F, cohesin removal from CTCF insulators. Accordingly, upon RB mutations cohesin subunit binding is stabilized at CTCF sites, and probing long-range interactions support a stronger insulation genome-wide. Moreover, long-range contacts engaging enhancers with promoters are perturbed, while CTCF insulation increases. The authors report additional deregulation of genes, in particular for those with no RB in their promoter. They propose a model whereby by regulating cohesin binding, RB may regulate insulation thereby allowing to activate genes involving enhancer-promoter interactions.

The paper provides lots of interesting data that, altogether, support a model by which RB controls cohesin binding at CTCF sites. Given the major role of cohesin on higher-order chromatin organization, there could be many reasons by which this impedes on gene expression. The proposed model linking the increased insulation with the increasing levels in gene expression is not clearly demonstrated. Although genomic analyses show a tendency for genes being activated to be associated with increased insulation, it is difficult to interpret the data due to the heterogenous nature of the genomic contexts.

Major points

1. In figure 4c, the defects in enhancer-promoter interactions (HiChiP of H3K27ac) should be shown as a function of cohesin accumulation at the insulator site that separate E-P's (as in Fig 4f).

2. Whereas the split between RB+E2F+ from RB-E2F- is probably of use to analyze the data, there may be many reasons for their relative change in RNA expression upon RBkd and the comparison is thus not insightful to demonstrate the mechanism by which RB functions(Fig 4f). Rather, a more direct enhancer-promoter assay with an intervening insulator would allow a more straightforward comparison.

3. The defects in B compartment seen for RBcdk (Figure 1d) should be shown upon RBkd. The real question is whether

gene expression defects (increasing expression levels) could also be the consequence of the defects in B compartments.

4. The impact of RB-kd should also be shown as a function of the cohesin loss, for panels Fig 4a, 4d.

5. Boxplot with p-value should be added to test the significance of the changes in binding profiles (fig3a,3b, 4d). Otherwise, there is a risk of looking at the effect of outliers.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #5

(Remarks to the Author)
The authors have addressed most criticisms. The one remaining item may be addressed as explained in the next paragraph. This would strengthen one of the main conclusion of the paper related to the regulation of E-P by RB.

Former comment: In figure 4c, the defects in enhancer-promoter interactions (HiChiP of H3K27ac) should be shown as a function of cohesin accumulation at the insulator site that separate E-P's (as in Fig 4f).
Former authors'response: In our study, we report that RB loss leads to increased insulation at TAD boundaries and to reduced enhancer-promoter interactions. According to the reviewer's comment, we aimed to quantify the fraction of changes in enhancer-promoter interaction that is attributable to changes in chromatin insulation. Since chromosome conformation capture experiments such as H3K27ac HiChIP did not have the resolution to resolve individual enhancer-promoter interactions, we utilized MED15 ChIP-seq signal as a surrogate measure to quantify enhancer activity (Figure R2).

Reviewer's comment: The authors solely need to reassess E-P interactions as already measured by the APA in Fig 4c. Unlike stated in their answer, there is no need for higher resolution to do so. The APA may be simply redone after splitting E-P into two groups, one defines by E-P with an intervening insulator, versus the other defined by E-P couples not separated by an insulator. Ideally, this can be done for E-P separated by the same range of distances.

(Remarks on code availability)

Version 3:

Reviewer comments:

Reviewer #5

(Remarks to the Author)
The authors now include an analysis of E-P long-range interactions in RB WT or KD, for E-P separated by a TAD border or not. The interpretation and conclusions are adequate and satisfactory. It also justify why the authors used Med15 ChIP data.

(Remarks on code availability)
A Github linked to the paper is provided with basic functions needed to compute ChIP and Hi-C data, which are clear. A source Matlab code is also provided.

Point-by-point response to the critiques

We sincerely thank the reviewers for their thoughtful and constructive feedback, which has significantly improved the clarity and overall quality of the manuscript, and helped us better highlight the novel aspects of RB's non-canonical activity at chromatin insulators. Our point-by-point responses to the reviewers' comments are provided below:

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

RB is a well-established cell cycle regulator whose canonical mechanism of action is to bind E2F transcription factors at the promoters of cell cycle genes and recruit chromatin regulatory complexes to suppress transcription. RB has been implicated in many other cellular functions beyond the cell cycle and E2F, however, including cellular differentiation and cancer lineage infidelity. The mechanisms underlying such non-canonical functions are largely unknown. This manuscript investigates whether RB influences chromatin looping dynamics and gene expression independent of E2F. This work extends the groups prior Mol. Cell paper that used engineered immortalized cells to perform high quality RB Chip-seq to demonstrate Rb binds enhancers and insulators independent of E2F, as well as promoters in an E2F dependent fashion. The current paper utilizes Micro-C to analyze the same engineered cell system to assess the impact of Rb on higher order chromatin structure. A key finding in this paper is that RB loss prevents normal removal of CTCF sites during mitosis. This results in an elevated number of cohesion-defined chromosome loops with stronger chromatin interactions within them. Another important finding is that RB-bound CTCF sites show increased chromatin accessibility, transcriptional activity, and stronger insulator boundary strength compared to CTCF sites not bound by RB. This suggests a novel transcriptional co-activator mechanism for RB at these regions. Overall, this work represents an important and significant area of inquiry as it has potential to shift paradigms in understanding RB function and the impact of RB deficiency in cancer.

There are some conceptual and technical limitations, however, that should be addressed before publication in Nature Communications. The authors demonstrate RB loss increases the number of chromosome loops and strengthens interactions within those loops. To a first approximation this may be expected to increase the number of enhancer/promoter interactions within a larger number of TADs, increasing the fraction of genes expressed per cell. A standard scRNA-seq analysis could be used to test this. The data would also have the benefit of testing whether RB affects molecular heterogeneity between cells. Molecular heterogeneity could confound interpretation of peak calls in bulk Micro-C, Hi-C, and ChIP-seq data if a larger number of potential genome interactions sites are spread across a larger fraction of the genome. This could explain why the strength of H3K27ac HiChIP peaks is reduced upon RB loss despite a stronger H3K27ac ChIP-seq signal.

Another issue that is not directly addressed is the stability of higher order chromatin structures across cell cycles. This chromatin structure presumably needs to be recreated with some fidelity after every cell division cycle to maintain stable phenotypes. It would be interesting to know if RB loss decreases the fidelity of this process as the findings may be relevant to important non-canonical RB functions like cancer lineage fidelity.

We thank Reviewer 1 for recognizing the significance of our study in advancing the understanding

of the non-canonical, E2F-independent functions of RB. We especially appreciate their acknowledgment that this is the first report to describe a transcriptional co-activator role for RB, representing a paradigm shift in the field.

As the reviewer correctly noted, our analysis of the Micro-C data demonstrated that RB loss increases the number of the cohesin-dependent chromosome loops and strengthens the interactions within TADs. Importantly, our H3K27ac HiChIP revealed that these changes are associated with a reduction in enhancer-promoter interactions. Given that RB loss promotes the gain of cohesin binding and increased insulation at these loci, we interpret the reduction in enhancer-promoter interactions as a consequence of the increased chromatin insulation, as illustrated in the model presented in Figure 5. We agree with the reviewer that a limitation of our approach is that bulk Micro-C, Hi-C, and ChIP-seq data may mask potential cell-to-cell heterogeneity in RB's effects on chromatin organization. To address this limitation, we performed single-cell RNA-seq (scRNA-seq) analysis in RB wild-type and RB knockout cells synchronized in the G1 phase.

The scRNA-seq experiment corroborated non-canonical transcriptional activation by RB, but it did not reveal additional distinct transcriptional profiles at the single-cell level that would support RB's effect on heterogeneity. Principal component analysis showed that all cells clustered together, with a modest separation between RB wild-type and knockout cells, primarily driven, as expected, by differences in RB/E2F target gene expression – cluster 3 (Fig. R1). Notably, the scRNA-seq analysis validated the differential expression of cluster 5 genes, where RB functions as a transcriptional activator during G1 (Fig. R1D). Cluster 5 genes exhibit cohesin gain at insulators upon RB loss, which correlates with reduced expression in RB-deficient cells. In addition, we observed that RB loss not only reduces the expression of cluster 5 genes but also reduces the number of genes expressed per cell upon normalization with UMI (Fig. R1B). This is in line with our H3K27ac HiChIP results which showed that RB loss leads to decreases in enhancer-promoter interactions. We present the scRNA-seq analysis in the new Extended Data Fig. 25 and discuss these findings in the revised manuscript in the paragraph "RB enhances the non-E2F transcriptional program" (page 8, line 39 to page 9, line 5). "To further investigate whether these RB-associated transcriptional changes occur population-wide or are driven by a specific cell population, we performed single-cell RNA-seq (scRNA-seq) experiments in RB^{WT} and RB^{KO} hTERT-RPE1 cells synchronized in the G1 phase via contact inhibition. Principal component analysis revealed that all cells clustered together, with a modest separation between RB^{WT} and RB^{KO} cells, primarily driven, as expected, by differences in RB/E2F target gene expression – cluster 3 (Extended Data Fig. 25). Notably, the scRNA-seq analysis validated the differential expression of cluster 5 genes, where RB functions as a transcriptional activator during G1 (Extended Data Fig. 25). While the scRNA-seq experiment corroborated non-canonical transcriptional activation by RB, it did not reveal additional distinct transcriptional profiles at the single-cell level that would support RB's effect on cellular heterogeneity."

We would also like to acknowledge and thank the reviewer for their insightful comment regarding the stability of higher-order chromatin structures across the cell cycle in RB knockout cells. This is indeed an important and intriguing area of investigation, particularly in the context of understanding RB's role in maintaining cell lineage fidelity. Our data indicate that the altered chromatin distribution of cohesin upon RB loss is rescued by the cell cycle progression since the differential binding of SMC3 at chromatin insulators resolves during the S-phase (Fig. 3B). However, to determine precisely whether these cell cycle-dependent changes in cohesin distribution result in more persistent defects in higher-order chromatin architecture, additional Micro-C profiling at multiple time points following acute RB loss would be required. While this question lies beyond the scope of the current manuscript

and we couldn't answer it within the time frame of this revision process, we agree it represents an important direction for future studies. We now discuss this point in the second paragraph of the revised Discussion (page 10, lines 14-19), stating that "Notably, our data indicate that the altered chromatin distribution of cohesin upon RB loss is resolved during cell cycle progression, as the differential binding of SMC3 at chromatin insulators diminishes in S phase. Future studies employing Micro-C profiling at multiple time points following acute RB loss may answer whether the cell cycle and RB-dependent changes in cohesin distribution result in more persistent defects in higher-order chromatin architecture."

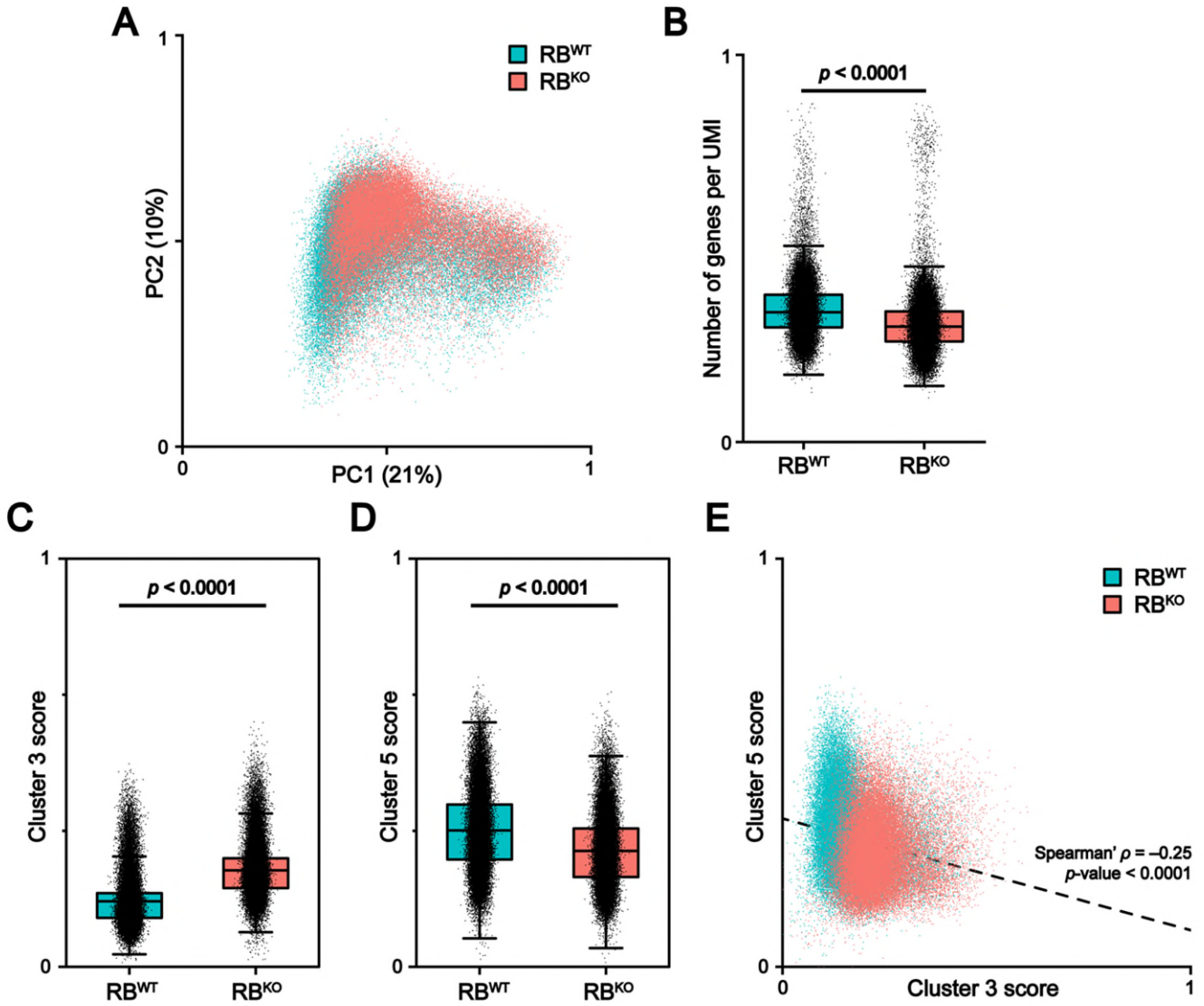

Figure R1. Single cell RNA sequencing of G1-arrested RB^{WT} and RB^{KO} hTERT-RPE1 cells further validates the non-canonical RB transcriptional activation, without detecting distinct transcriptional profiles at the single-cell level A, principal component analysis (PCA) of single-cell transcriptomes from RB^{WT} and RB^{KO} hTERT-RPE1 cells, arrested in G1 by 6 days of contact inhibition. Each dot on the plot represents an individual cell, colored according to its genotype; RB^{WT} (cyan) and RB^{KO} (red). The plot visualizes the principal components (PC1 and PC2) that capture the greatest variance within the dataset. Values on the axes after the principal components indicate explained variance. **B**, quantification of unique gene counts per cell in G1 arrested RB^{WT} and RB^{KO} hTERT-RPE1 cells reveals that RB^{KO} cells exhibit fewer number of genes per cell compared to RB^{WT}. Gene counts were calculated by Unique Molecular Identifiers (UMI) and normalized to account for differences in sequencing depth. Box plot shows the distribution of the number of unique genes detected per cell in RB^{WT} (cyan) and RB^{KO} (red) hTERT-RPE1 cells. Horizontal lines indicate mean values. Statistical significance was determined by a two-tailed Student's *t*-test,

$p<0.0001$****. **C** and **D**, average expression levels of cluster 3 and cluster 5 genes in G1 arrested RB^{WT} and RB^{KO} hTERT-RPE1 cells reveal that upon RB loss, cluster 3 genes get upregulated while cluster 5 genes get downregulated. Box plots showing the average normalized expression levels of genes belonging to cluster 3 (C) or cluster 5 (D) in RB^{WT} (cyan) and RB^{KO} (red) cells. Median is indicated by the horizontal lines. Statistical significance was determined by a two-tailed Student's *t*-test, $p<0.0001$****. **E**, average expression levels of cluster 3 and cluster 5 genes reveal modest transcriptional shifts in RB^{KO} G1 arrested hTERT-RPE1 cells. Scatter plot visualizes the single-cell relationship between the average expression scores for genes within cluster 3 (x axis) and cluster 5 (y axis). Each dot represents an individual cell, colored by genotype; pink for RB^{WT} and maroon for RB^{KO}. A modest, but statistically significant negative Spearman correlation (ρ= -0.25, $p<0.0001$****) is observed between clusters 3 and 5 scores across total cells population, indicated by the dashed trend line. The plot annotates the inverse transcriptional regulation of these two gene sets (cluster 3 and cluster 5) at the single-cell level.

Additional Strengths:

1) The generation and analysis of data is robust and well controlled, with exceptions noted below. The experimental approaches and methodology used are appropriate.

2) The computational approaches for all omics analyses were sound and justified based on the methodology and hypotheses. Gold-standard chromatin conformation pipelines such as 'Juicer/JuiceBox', 'cooltools', 'HiCCUPS' were described appropriately for reproducible analyses.

3) Complementary RB knockout and RB activation models are employed in a non-transformed, genetically stable cell line so RB-dependent effects can be cleanly identified.

4) Use of Micro-Capture technology, which provides nucleotide-resolution and higher sensitivity for chromatin interactions, offered a distinct advantage in detecting the subtle (but significant) genome-wide RB1-dependent chromatin organization effects while minimizing digestion bias which can sometimes hinder interpretations when other chromatin conformation assays are used. The use of Hi-ChIP (H3K27ac) is also appropriate for investigating RB's impact on enhancer- promoter interactions.

We thank the reviewer for highlighting the additional strengths of our study.

Additional Weaknesses:

1) It would be nice to see the distribution of mapped sequencing reads across the genome to get a sense of how the MicroC, HiC, and ChIP signals are distributed in the presence and absence of RB.

In Fig. R2, we present the distribution of mapped sequencing reads from Micro-C and ChIP-seq experiments across chromosome 11 and the representative genomic region in chromosome 11 described in the main Fig. 3h. Overall, we did not observe significant differences in sequencing coverage across experiments. However, in the absence of RB, we observed specific enrichment of cohesin at insulators at regions of differential chromatin loops, supporting our observation that RB loss increases insulator activity.

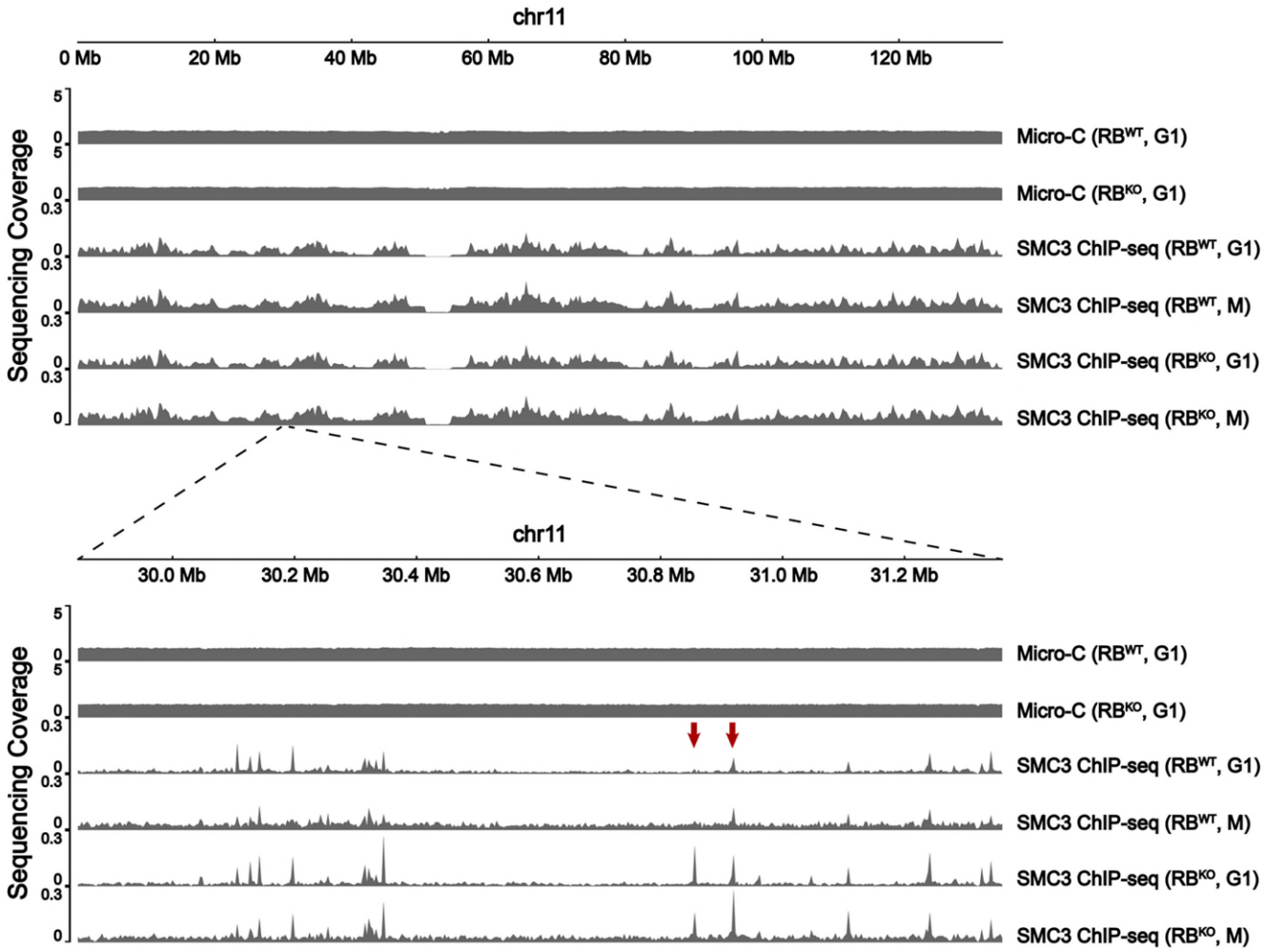

Figure R2. Overview of sequencing coverage for Micro-C and SMC3 ChIP-seq experiments across chromosome 11. Genome browser tracks display the normalized sequencing coverage (Counts Per Million, CPM) for various experiments (as indicated) across a broad region of human chromosome 11. Red arrows indicate the regions where significant changes in chromatin loop formation are observed upon RB loss (Micro-C contact maps are not shown here). Overall sequencing coverage is comparable across experimental conditions (upper panel). However, SMC3 Chip-seq tracks reveal differential enrichment patterns at specific genomic loci (lower panel, red arrows).

2) The authors claim that RB promotes removal of cohesion, a significant finding, but no complementary or orthogonal experiments were performed to confirm this.

We thank the reviewer for the suggestion to add complementary and orthogonal experiments. In the revised manuscript, we performed additional experiments to corroborate RB's role in cohesin distribution, chromatin organization, and transcriptional regulation. More specifically:

1. While the redistribution of SMC3, a core component of the cohesin complex, suggested cohesin alterations upon RB loss, additional data on other cohesin subunits would strengthen our conclusions. In the revised manuscript, we have included ChIP-seq data for two additional proteins essential for cohesin function, STAG1 and STAG2. In Figure R3, we present the SMC3, STAG1, and STAG2 ChIP-seq tracks at the representative genomic region in chromosome 11 described in Fig. 3h. We observed stronger STAG1 signals compared to STAG2, in line with previous reports that STAG2 is primarily associated with sister chromatin cohesion. Notably, we observed redistribution of STAG1 upon RB loss at regions of differential chromatin loop

formation, supporting the model that RB loss leads to increased cohesin binding at insulators. This redistribution was observed genome-wide across insulators (Fig. R3B). The new STAG1 ChIP-seq data are shown in the revised Figure 3 h and i.

2. To confirm that the observed RB-mediated transcriptional regulation is due to the enrichment of cohesin at insulators and not the differences in cohesin expression, we performed proteomics experiment and confirmed that protein levels of the cohesin complex components SMC3, SMC1A, PDS5B, PDS5A, RAD21, WAPL, STAG1, and STAG2 were comparable between RB^{WT} and RB^{KO} cells (Extended Data Fig. 11).
3. We also performed complementary SMC3 ChIP-quantitative PCR experiment to confirm the increased SMC3 binding upon RB loss at the locus of differential chromatin loop formation described in Fig. 3h.

We discuss these new data in the revised text on page 7, lines 11-14: “Inspection of individual regions in chromosomes 9 and 11 revealed visually striking gains in chromatin contacts and showed that the formation of differential loops in RBKO is driven by an increase in cohesin signal at insulators, including SMC3 and STAG1 (Figs. 3h and i). Additional SMC3 ChIP-quantitative PCR further confirmed the increase of SMC3 binding at chromosome 9 locus following RB loss”, and page 5, lines 22-31: “To rule out the possibility that the observed increase is an indirect effect of RB loss, we compared the proteomes of RB wild-type and RB knockout cells and found no significant differences in the protein levels of SMC3 or other core components of the cohesin complex (Extended Data Fig. 11).”

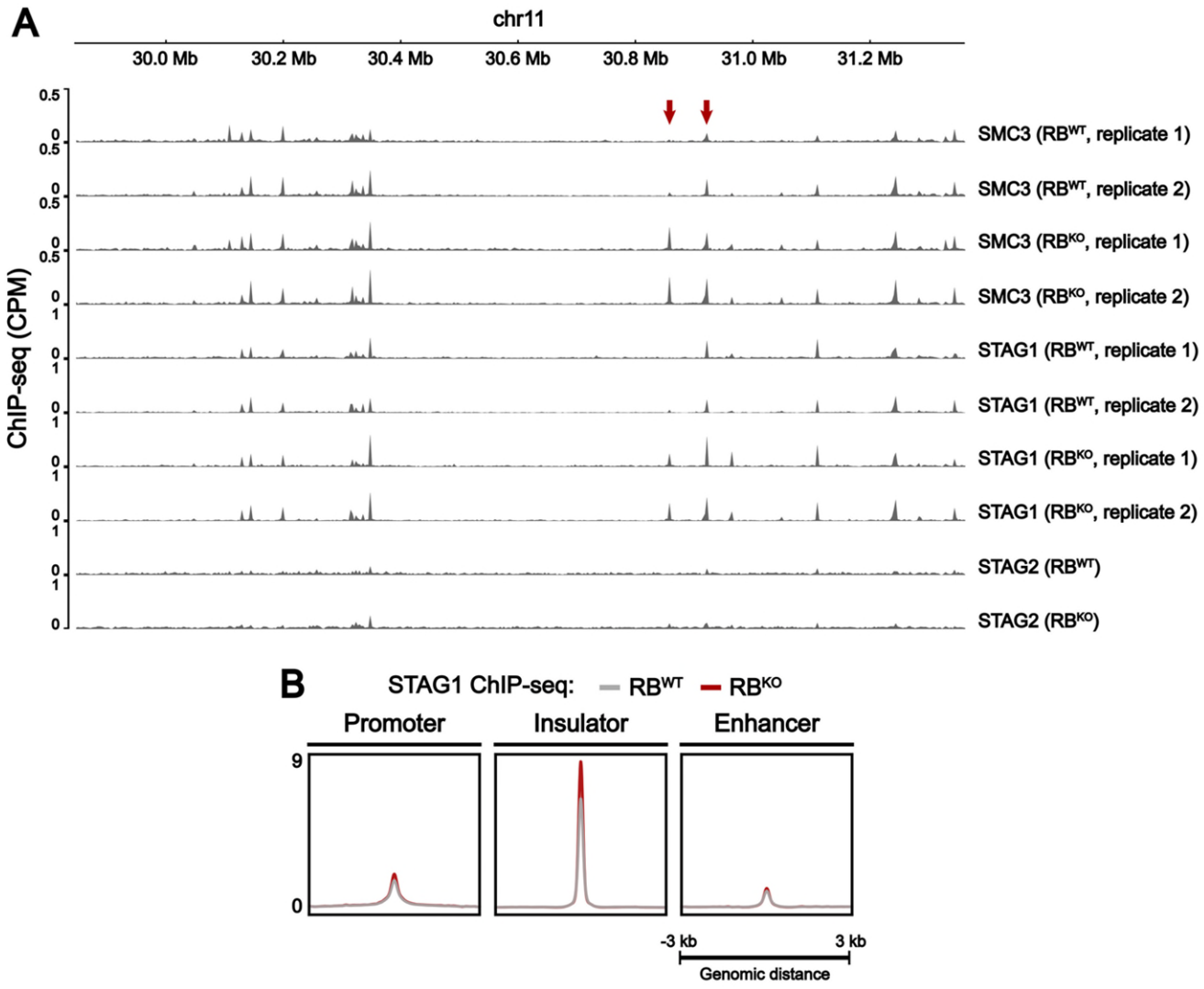

Figure R3. SMC3, STAG1 and STAG2 ChIP-seq reveal redistribution of STAG1 upon RB loss. A, STAG1 and STAG2 ChIP-seq tracks across region of differential chromatin loop formation in chr11. Genome browser tracks display the normalized sequencing coverage (Counts Per Million, CPM) for various experiments (as indicated) across a broad region of human chromosome 11. Red arrows indicate regions where significant changes in chromatin loop formation are observed upon RB loss (Micro-C contact maps, not show here). Both SMC3 and STAG1 exhibit redistribution upon RB loss in distinct genomic loci (red arrows), indicating that RB loss enriches the cohesin complex at insulators. **B**, Aggregate plots showing the average STAG1 ChIP-seq signal across indicated genomic features (Promoter, Insulator, Enhancer) in G1 arrested RB^{WT} (grey) and RB^{KO} (maroon) hTERT-RPE1 cells. The x-axis represents genomic distance centered at the respective feature and the y-axis represents normalized ChIP-seq signal. The plots reveal a distinct increase of STAG1 enrichment at insulator regions upon RB loss.

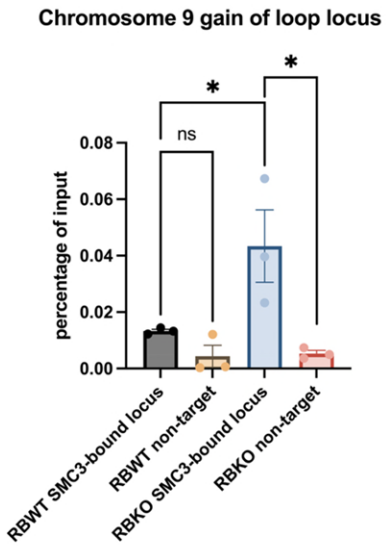

Figure R4. ChIP-qPCR confirms differential SMC3 recruitment to locus of chromatin loop gain at chromosome 9. ChIP-qPCR experiment was performed at the site of differential chromatin loop formation in chromosome 9 identified in Fig. 3i. Primer pairs targeting the SMC3-bound locus was used to probe SMC3 binding at the locus, while primer pairs targeting the non-targeting locus 500 bp away from the SMC3-bound locus were utilized as the negative control. ChIP-qPCR signal for SMC3 increased upon RB loss, corroborating our ChIP-seq and Micro-C results (not shown here). Statistical significance was determined by one-way ANOVA, $p<0.05$*.

4. In addition, we performed orthogonal assays to corroborate reduced enhancer-promoter interaction upon RB loss. To a first approximation, reduced enhancer-promoter interactions observed in H3K27ac HiChIP experiment should lead to reduced mediator complex recruitment to enhancers and promoters. We therefore performed MED15 ChIP-seq on RB^{WT} and RB^{KO} hTERT-RPE1 cells and confirmed reduced MED15 signal specifically at promoters and enhancers (Figure R5). The new MED15 ChIP-seq data are shown in the revised Figure 4d.

We discuss these new data in the revised text, page 7, lines 33-34: "As an orthogonal approach, we performed MED15 ChIP-seq to assess mediator recruitment and observed reduced MED15 occupancy at enhancers and promoters following RB loss (Fig. 4d)."

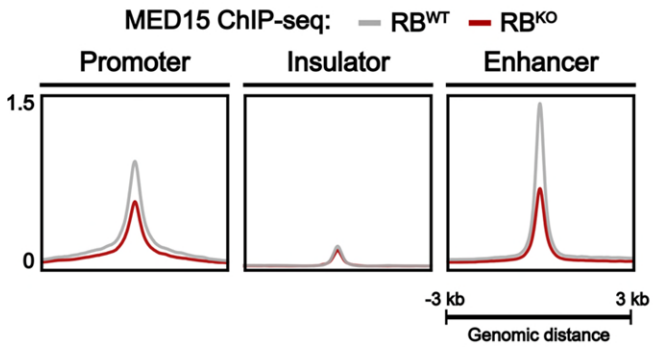

Figure R5. MED15 recruitment to promoters and enhancers is reduced upon RB loss. Aggregate plots showing the average MED15 ChIP-seq signal across indicated genomic features (Promoter, Insulator, Enhancer) in G1 arrested RB^{WT} (grey) and RB^{KO} (red) hTERT-RPE1 cells. The x-axis represents genomic distance centered at the respective feature and the y-axis represents normalized ChIP-seq signal. The plots reveal a distinct decrease of MED15 enrichment at promoter and enhancer regions upon RB loss, in line with H3K27ac HiChIP data (Fig. 4b).

3) The mechanisms by which RB removes cohesion or co-occupies SMC3 sites independent of E2F is unknown, but the authors could speculate in the discussion section. Potential interactions with Wapl could also be discussed,

We have expanded our discussion to include this point on page 11, lines 3-5, noting that "Although RB and SMC3 co-occupy the same chromatin loci, the presence of RB is associated with reduced cohesin binding. This supports the hypothesis that RB may promote cohesin removal at TAD boundaries during prophase by facilitating the recruitment of Wapl."

4) The figures are difficult to interpret easily given their size and resolution. For example, it was hard to discern the subtle differences in TAD boundaries between RB KO and RB WT (Figure 1H and 2F).

We have increased the size and resolution of Figures 1h and 2f to more clearly highlight the differences described in the text.

5) The descriptions provided in the figure legends are cursory, making it difficult to interpret figures without frequent consultation of the main text.

We have revised the figure legends to make them more descriptive.

6) The deltaCDK RB activation experiments do not control for cell cycle phase distribution differences as far as I can tell. Pre and post Dox induction time points are compared, but only the Dox induced cell will undergo cell cycle arrest. This could be controlled by comparing confluent, G1 arrested Dox induced and Dox uninduced cells.

The goal of this experiment was to compare cells treated with DOX for 24 hours versus 72 hours, both of which are arrested in G1. The 24-hour treatment induces a reversible G1 arrest, whereas the 72-hour treatment leads to a non-reversible G1 arrest associated with chromatin dispersion. We agree with the reviewer that differences observed between pre- and post-DOX induction time points may result from RBΔCDK-induced cell cycle arrest. We have revised the text on page 3, lines 33-35, to clarify this point, stating that "Although changes occurred between 0 h and 24 h or 72 h of RBΔCDK expression may be associated with G1-arrest, most compartmental changes occurred between 24 h and 72 h of RBΔCDK expression, coinciding with the irreversible cell cycle exit, as previously described."

7) While the engineered cell model is a strength, it is the only model employed so the generality of the findings are unclear. Whether findings are applicable in the more clinically relevant context of RB deficiency in cancer is unclear. Targeted experiments to validate some key observations in other cell lines would be helpful.

We appreciate the reviewer's comment that the role of RB in regulating chromatin conformation would have greater clinical relevance if examined specifically in the context of RB-deficient cancer cells. However, this study represents the first demonstration of a novel role for RB in higher-order chromatin organization, and extending these findings broadly across cancer models is beyond the scope of the current manuscript.

To strengthen our conclusions and validate key observations, we have included the following new data in the revised manuscript:

i) SMC3 ChIP-seq in RB wild-type and RB knockout cells synchronized in prometaphase using inhibition of Eg5 kinesin activity, as an alternative to nocodazole-induced arrest (Fig. R6). Notably, SMC3 accumulation at chromatin insulators upon RB loss is observed regardless of the synchronization method or RB knockout clones used. These new data have been shown in the revised Extended Data Fig. 12.

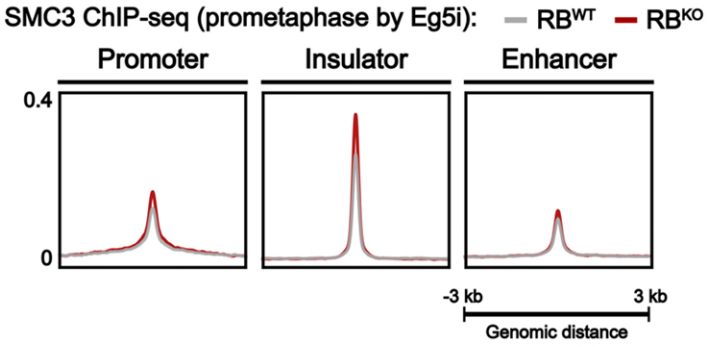

Figure R6. Prometaphase arrest by Eg5 inhibition reveals SMC3 redistribution toward insulators upon RB loss. Aggregate plots showing the average SMC3 ChIP-seq signal across indicated genomic features (Promoter, Insulator, Enhancer) in prometaphase arrested RB^{WT} (grey) and RB^{KO} (red) hTERT-RPE1 cells. Prometaphase arrest was performed by Eg5 inhibition. The x-axis represents genomic distance centered at the respective feature and the y-axis represents normalized ChIP-seq signal. The plots reveal a distinct increase of SMC3 enrichment at insulator regions upon RB loss, similar to the results obtained by nocodazole-induced prometaphase arrest (Fig. 3b).

ii) MED15 ChIP-seq in RB wild-type and RB knockout cells synchronized in G1 (Fig. R5). The observed reduction in mediator complex binding at promoters and enhancers upon RB loss supports the enhancer-promoter interaction defects identified by H3K27ac HiChIP.

We discuss these new data in the revised text at page 6, lines 2-5: "SMC3 ChIP-seq in RB^{WT} and RB^{KO} hTERT-RPE1 cells synchronized in prometaphase using inhibition of Eg5 kinesin activity, as an alternative to nocodazole-induced arrest, similarly revealed cohesin enrichment at insulators following RB loss (Extended Data Fig. 12)", and page 7, lines 33-35: "As an orthogonal approach, we performed MED15 ChIP-seq to assess mediator recruitment and observed reduced MED15 occupancy at enhancers and promoters following RB loss (Fig. 4d)."

To extend our analysis to additional normal immortalized cell lines, we generated genetic knockouts of RB in hTERT-BJ1 fibroblasts. However, we were unable to obtain high-quality ChIP-seq or CUT&RUN data, largely due to the limited number of mitotically arrested cells. In Figure R7, we present a CUT&RUN experiment we performed in RB^{WT} and RB^{KO} hTERT-BJ1 cells following prometaphase arrest via Eg5 inhibition.

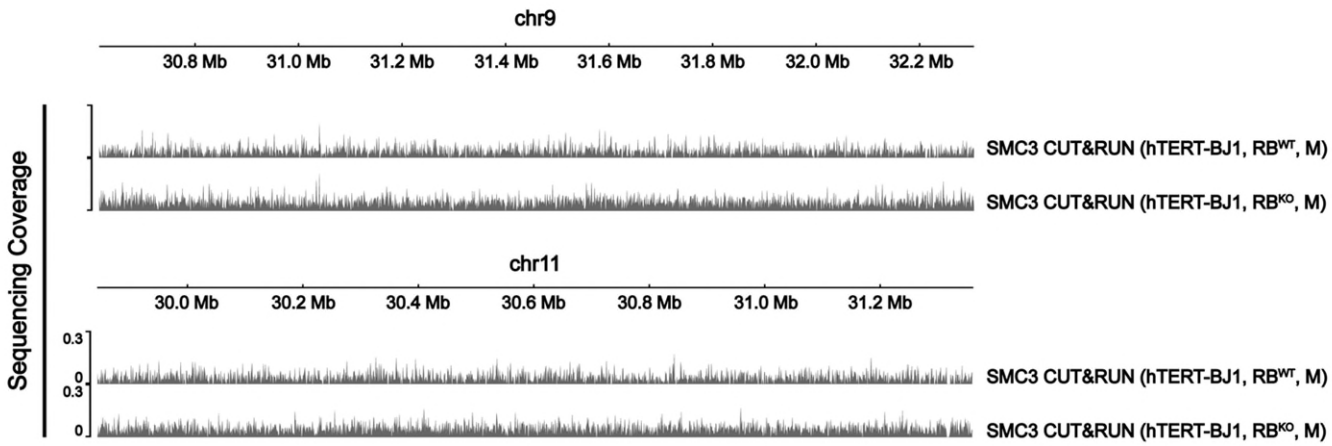

Figure R7. SMC3 CUT&RUN experiment in hTERT-BJ1 cells yielded very low signal-to-noise ratio. Genome browser tracks display the normalized sequencing coverage (Counts Per Million, CPM) for mitotically arrested RB^{WT} and RB^{KO} hTERT-BJ1 cells across a broad region of human chromosome 9 (top) and 11 (bottom). Both experiments yielded very low signal-to-noise ratio, due to a small fraction of cells arrested in prometaphase.

8) The manuscript would benefit from a clearer and more detailed graphical abstract which depicts the RB-dependent effects on TAD boundaries, enhancer-promoter interactions, and gene expression. The current graphical abstract does not clearly convey the high-level chromatin organizational changes that the paper is demonstrating.

We have revised Figure 5 to illustrate the RB-dependent effects on chromatin insulation and E/P interactions.

9) The gene set enrichment and functional analysis of EMT needs clarification. The PI focuses on 181 genes showing an increase in SMC3 binding in the absence of RB. These genes are enriched for EMT genes and the PI provides functional evidence that RB loss causes EMT properties. However, the authors also conclude that RB depletion significantly decreases expression of the "cohesion gain" gene set. These conclusions seem at odds with one another.

We apologize for not explaining this more clearly in the original text. While the gene set is enriched for EMT-related genes, the majority are involved in maintaining epithelial characteristics, including genes associated with extracellular matrix organization and cell adhesion. We have revised the text on page 8, line 12, to clarify this distinction: "…genes regulating epithelial-mesenchymal transition by maintaining epithelial characteristics…"

Reviewer #1 (Remarks on code availability):

My co-reviewer reviewed the code and felt it appropriate based on standards in the field. The paper makes use of publicly available code and thus is generally available to the community.

We thank the co-reviewer for taking the time to review the code used for our data analysis and its availability.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is

part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

In this manuscript, Lee et al. investigate Retinoblastoma protein (RB) dependent effects on chromatin and chromosome organization. They utilize comparisons between control and knock out cells as well as an exogenous expression system to induce a non-phosphorylatable version of RB. They use chromosome conformation capture based methods to investigate chromosome organization in the different genotypes of cells under varying conditions related to RB phosphorylation or cell cycle position. The also undertake SMC3 ChIP-seq and H3K27ac HiChIP experiments to further elucidate RB status effects on chromosome organization and dynamics. Analysis of these experiments is blended with previously reported data from the same cell systems and organized into the present manuscript. The authors argue based on their data that RB status influences DNA and chromosome topology and in particular this effect reorganizes Cohesin complexes and further influences the ability of enhancer-promoter contacts to be made, ultimately altering gene expression patterns.

This was a difficult paper to review from a number of perspectives that prevent me from being enthusiastic about the publication of this study. In its present form it looks like an article from Plos One or Scientific Reports where data is reported for the record, and future investigators may or may not see value in it, but the current takefsc home message is unclear. I've listed the biggest drawbacks to this study below to help the authors improve their work.

We thank the reviewer for their time and effort in reviewing our manuscript. We appreciate the specific concerns raised and have carefully considered each of them, as discussed below. However, we respectfully disagree with the comment that our manuscript presents data without drawing significant or novel conclusions. This study provides the first evidence that RB and cohesin co-occupy specific genomic regions and that RB loss leads to enrichment of cohesin at insulators. Our Micro-C data, representing the first such dataset generated in RB-deficient cells, demonstrate that RB-dependent regulation of cohesin alters chromatin insulation, disrupts enhancer-promoter interactions, and reveals a previously unrecognized mechanism by which RB acts as a transcriptional activator. Given the mechanistic insights provided and the novelty of the findings, we believe that this manuscript will be of significant interest to the scientific community.

1) The authors, for the most part, compare RB wild type status cells with knock out cells. The resulting molecular characteristics observed indicate that alterations take place in RB's absence. It is an overinterpretation to assume that RB has a deliberate regulatory role related to Cohesin positioning and chromatin binding when it is present. The title of the paper includes this error. It is just as likely that the data in this study captures random downstream consequences of RB loss as compared to a deliberate function as the authors hope. The model diagram in figure 5 indicates the authors conclusion of an 'RB/cohesin pathway', but there is no evidence that this pathway exists from data in this paper. Indirect influence of Cohesin by RB status has also been reported by others previously, making this a very modest conceptual advancement.

While our study provides for the first time a comprehensive analysis of the chromatin architecture of

RB wild-type and RB knockout cells, we agree with the reviewer that these models cannot sufficiently distinguish direct from indirect regulatory effects. We have performed several new orthogonal assays to further strengthen our conclusions, moderated the language throughout the manuscript and the title, and revised Figure 5 to incorporate the reviewer's suggestions. Specifically, in the revised figure we removed the section on the "RB/cohesin pathway" and focused on the RB-dependent changes in TADs, enhancer-promoter interactions, and gene expression, observations that are directly supported by our experiments. We believe that the revised schematic provides a more thorough and scientifically accurate interpretation of our findings and proposed model. We also revised the title of the manuscript according to the reviewer's suggestion. The revised version of the manuscript now highlights the effect of RB loss: "**RB loss modulates chromatin organization by regulating cohesin-dependent loops and enhancer-promoter interactions**". In addition, we performed the following orthogonal assays to corroborate our observations:

i) **ChIP-seq experiments on other cohesin subunits (STAG1 and STAG2)**. We show, in the revised manuscript, that most SMC3 binding sites are co-occupyied by STAG1 and that the effect of RB loss on SMC3 is also observed in STAG1 (Fig. 3h-i and Fig. R3). RB loss leads to redistribution of both SMC3 and STAG1 towards insulators, supporting the interpretation that RB loss alters the chromatin localization of the cohesin complex.

ii) **SMC3 ChIP-seq experiments after different prometaphase arrest protocols (Eg5 inhibition)**. While we previously showed that RB loss has the strongest effect on insulators during prometaphase, the generalizability of this observation was limited as only the nocodazole-induced arrest was tested. In the revised manuscript, we utilize Eg5 inhibitor as an alternative and orthogonal method of arresting RB wild-type and RB knockout cells in prometaphase. SMC3 ChIP-seq experiments after Eg5 inhibitor-induced prometaphase arrest show that the effect of RB loss is the strongest in prometaphase cells, regardless of the synchronization method, and that RB loss leads to significant enrichment of cohesin towards insulators (Extended Data Fig. 12).

iii) **MED15 ChIP-seq experiments to validate enhancer-promoter interaction.** While our H3K27ac HiChIP data suggested that RB loss led to reduced enhancer-promoter interaction, which correlated with our Micro-C, ChIP-seq, and RNA-seq experiments, the limitation of this experiment has been the lack of orthogonal assays. As a first approximation, reduced enhancer-promoter interaction should lead to reduced mediator complex enrichment in enhancers and promoters. To test this, we performed MED15 ChIP-seq experiments and found that RB loss indeed leads to reduced mediator occupancy at enhancers and promoters (Fig. 4d). Together with the H3K27ac HiChIP results (Fig. 4a-c), these data support the interpretation that RB loss leads to reduced enhancer-promoter interactions.

iv) **SMC3 ChIP-qPCR on sites of differential chromatin contact.** For the region of differential chromatin contact presented in Fig. 3i, we further performed SMC3 ChIP-qPCR experiments and validated the SMC3 enrichment upon RB loss.

v) **Proteomics analysis of cohesin subunits upon RB loss.** We performed proteomics analysis in G1-arrested RB wild-type and RB knockout cells in biological quadruplicates and found that none of the cohesin comlex components, including SMC3, SMC1A, PDS5B, PDS5A, RAD21, WAPL, STAG1, and STAG2, exhibited statistically significant changes upon RB loss (Extended Data Fig. 11). This new analysis confirm that RB does not significantly alter the expression or stability of cohesin subunits, supporting the conclusion that RB directly influences cohesin association with chromatin. This data, combined with our observation that the effect of RB loss on cohesin is specific to insulators and is the strongest in the mitotic phase, highlights the specificity of this deregulatory mechanism and argues against a

nonspecific effect. We have revised the text on page 5, lines 22-32, to reflect and clarify these points: "To rule out the possibility that the observed increase is an indirect effect of RB loss, we compared the proteomes of RB^{WT} and RB^{KO} cells and found no significant differences in the protein levels of SMC3 or other core components of the cohesin complex (Extended Data Fig. 11). To exclude the possibility that the observed changes resulted from normalization artifacts, we utilized spike-in DNA from *Escherichia coli* as additional control and confirmed the increase in SMC3 signal at insulators (Fig. 3a). Notably, we did not observe analogous increases in SMC3 signal at promoters (+2.8% upon RB loss; n = 4) or enhancers (-3.4% upon RB loss; n = 4), further supporting the specificity of RB's regulatory role at insulator sites."

2) The paper in many places is an unconnected series of observations. Figure 1 is the best example of this as it attempts to link different observations from exogenous non- phosphorylatable RB and RB deficiency. However, the staging of the different RB status cells are starkly different either because of time point choices or manipulation by trypsinization and re- plating. The narrative of the results section that corresponds to this figure states a number of different changes at varying stages of the cell cycle, but the illustrations are more about experimental design than illustrating cell cycle consequences of RB status. The biggest admission from the authors that this figure is incoherent is panel I where they offer brief conclusions as they already expect that readers of their paper won't come to these conclusions on their own from the data presented. The narrative of the results indicates RB gain of function causes downstream alterations in heterochromatin structure, but the microscopy image in figure 1E offers a rather superficial look at gain of RB function in the regulation of heterochromatin and so I struggle to find a way to agree with the conclusions in panel I. In most figures the data displays appear to be mostly a collection of comparisons that show statistically significant differences even though they don't offer a singular conclusion. Instead the more I read on, the more I encountered seemingly random measurements that support the broad conclusion that chromatin structure changes when RB is lost. The figures also don't relate well to one another as there is little reason to expect that figures 1 and 2 lead to a need to understand Cohesin occupancy as a logical progression of the work in figure 3. Many other factors could have been profiled and found to be altered as well.

The overarching goal of Fig. 1 was to compare the effects of RB loss versus gain of non-phosphorylated RB (RBΔCDK) in the same cell type, focusing specifically on chromatin structural changes across distinct cell cycle stages. These models were chosen to provide complementary insight: RB knockout cells represent a chronic RB-deficient state, while RBΔCDK expression allows for temporally controlled RB activation which, depending on the activation time, can cause G1 arrest or trigger cell cycle exit. While the experimental systems differ in setup, we used cell cycle synchronization to mitigate confounding variables. We now clarify these details in the revised text (page 3, lines 2-6) to help readers more easily follow the logic and biological relevance of each comparison: "We conducted two complementary sets of experiments in human retinal pigment epithelial cells immortalized by telomerase reverse transcriptase (hTERT1-RPE1) (Figs. 1a and b) to compare the effects of (1) chronic RB deficient state with (2) conditional gain of constitutively active RB. Cell cycle synchronization was used to minimize potential confounding variables between the two experimental systems."

Regarding the microscopy image in Figure 1e, this panel was intended as a validation step to demonstrate that our observations on RBΔCDK-induced chromatin changes are consistent with previously published findings of heterochromatin dispersion after 72 hours of RBΔCDK expression (Krishnan et al. 2022). We have clarified this point in the revised text (page 3, line 37): "…concordant with the previously described heterochromatin dispersion phenotype (Fig. 1e).

With respect to Figure 1i, we acknowledge the reviewer's concern and agree that clearer integration of the data is warranted. To improve the narrative flow, we have rewritten this section (page 4, lines 12-15) to guide the reader more explicitly through the results and their interpretation: "In summary, we observe that RB exerts broad effects on chromatin organization (Fig. 1i), with RBΔCDK expression promoting primarily heterochromatin spreading and dispersion (Figs. 1d–e), and RB loss in G1-arrested cells altering cohesin-dependent loop number, loop length, and TAD boundary strength (Figs. 1g–h)."

Regarding the progression to cohesin occupancy in Figure 3, we respectfully note that this direction was motivated by two key observations: (1) our Micro-C analysis in Figure 1 revealed increased chromatin loop formation and stronger TAD boundaries upon RB loss, features typically associated with cohesin activity, and (2) our ChIP-seq data in Figure 2 showed extensive co-localization between chromatin-bound RB and SMC3. Together, these findings naturally led us to investigate whether RB regulates cohesin localization directly. We now emphasize this connection more explicitly in the revised text (page 5, lines 15-17): "The extensive co-localization of chromatin-bound RB and SMC3, along with the observed impact of RB loss on cohesin-dependent loop formation and TAD boundary strength, prompted us to investigate RB's role in cohesin localization."

While we acknowledge that many chromatin-associated factors could have been explored, we prioritized cohesin due to its direct involvement in the architectural features highlighted by our Micro-C data. Moreover, our previous studies have shown that RB depletion does not alter CTCF association with chromatin (Sanidas et al. 2022), further supporting our focus on cohesin as a specific mediator of RB's effect on chromatin architecture.

3) In many places the author's narrative in the results describe changes to chromatin structure and they describe a statistical test and significance level to go with it. However, the data display panel that they cite along with it often shows little evidence of change due to RB status. In figure 1, panels D and H. Panel 2F, panels 3A and B, and 4B and C all contain genotype comparisons that suffer from looking very similar, if not virtually identical. In some cases, very modest changes are illustrated by graphs such as figure 1G. Even if a statistically significant difference does exist in the cases of these displays, what does such a small difference mean? Are these subtle changes really meaningful in biological terms? Highly similar data in control and RB knock out makes the value of this study hard to appreciate.

The chromatin-associated changes presented in the figures mentioned by the reviewer reflect aggregate signals across the genome. Because these represent averaged differences over thousands of loci, changes may appear modest but are statistically significant and biologically consistent. Importantly, when we focused on subsets of loci most strongly regulated by RB, such as the top-ranked insulators in Fig. 3g, or selected genomic domains (Figs. 3h–i), the differences were considerably stronger. In the revised manuscript, we observe that RB loss leads to a strong effect in H3K27ac HiChIP as well as in MED15 ChIP-seq (Fig 4a-d). Through these experiments, we significantly strengthened our argument that RB loss leads to reduced enhancer-promoter interactions. We also observed strong enrichment of cohesin at insulators upon RB loss in prometaphase cells (Fig. 3b, +56% across n=21,498 insulators), and we reproduced these findings when we tested different knockout clones as well as using different prometaphase arrest methods (Extended Data Fig. 12). Subtle alterations in enhancer-promoter proximity or domain insulation can significantly influence transcriptional output (e.g., Xiao JY et al., 2021, PMID: 34240703). This principle is reflected in our data as well. In Figure 1g, for example, while the change in loop length appears modest, it reflects a consistent trend across the genome that correlates with increased loop formation (Fig. 1f) and changes in TAD boundary strength (Fig. 1h), both of which are characteristic

of cohesin-mediated chromatin organization. We also observe that in prometaphase cells, where we observe the strongest differences upon RB loss (+56% across n=21,498 insulators), the differential enrichment of cohesin at these loci is reproduced, further corroborating our interpretation. Similarly, in Figure 4, panels B and C, although the overall changes in contact enrichment between enhancers and promoters may appear modest, they were consistently observed across biological replicates. In the revised manuscript, these changes are further supported by corresponding reductions in Mediator complex enrichment following RB loss at the same loci (Figure 4D), highlighting the functional relevance of these architectural shifts.

4) Somewhat related to point 3 and some earlier concerns, the manuscript largely lacks biological consequences to go with the observations of chromatin structure. Subtle changes in chromatin structure may be important biologically, or they could be largely inconsequential. I was surprised to read to the end and see that the authors really don't have key biological relevance for the consequence for Cohesin misregulation by RB loss. Given all of the biological consequences of RB loss that are already published and RB's key role in human cancer, the subtle change in adherence of cells to a culture dish and scratch assay closure are quite minimal functional experiments. Even in the case of these observations, does the data presented really indicate that altered Cohesin loops causes these effects compared to alterations in other aspects of RB function? Some gene categories that they discover in their analysis are interesting. RB loss has been shown to alter properties of advanced cancer cells that are consistent with EMT and RB has also been documented to have a role in transcriptional regulation of myogenesis. The opportunity to connect to more meaningful biological scenarios exists, but at present the manuscript stops short of connecting the chromatin structure details to functions that yield a better understanding of RB.

We agree that connecting chromatin structure changes to downstream biological consequences is a critical next step in understanding the broader significance of RB-mediated regulation. While the primary focus of our study was on uncovering a novel role for RB in modulating higher-order chromatin organization through cohesin redistribution, we recognize that the functional implications of these structural changes, particularly in disease-relevant contexts, require further investigation.

Importantly, our transcriptomic analyses revealed that the genes most strongly regulated by RB and cohesin are involved in processes such as EMT and extracellular matrix organization. In this initial study, we chose to explore two phenotypes: 1) cell adhesion and 2) wound-healing, as proof-of-concept readouts for these signatures. However, we acknowledge that these phenotypes are modest and do not fully capture the breadth of RB's biological impact. We now clarify this in the revised text (page 8, lines 21-24) by adding the phrase: "While these phenotypes do not represent comprehensive functional validation of RB-regulated chromatin architecture, these cell cycle-independent effects of RB may be a good starting point for connecting architectural chromatin changes to cellular behavior of RB-deficient cells."

Some smaller, but also important issues are:

5) The scratch wound experiment either contains an error or it doesn't represent what the authors hope. The Y-axis is a measure of closure of the scratch (presumably through cell migration). However, the control cells display measurements that exceed 100%. Does this mean that control cells migrate in the opposite direction? Without these outlier and counterintuitive data points included, the statistically significant difference with RB knock out would likely be lost. Something is wrong with this display/experiment.

The scores in this graph represent changes relative to the average initial wound length in control cells at time 0 (n = 8). Because the initial wound sizes varied across samples, some wound measurements

at 48 hours, particularly in RB^{WT} cells, fall above 100%, reflecting cases where the wound length remained greater than the average baseline. To prevent any confusion, we recalculated the change in the wound length relative to time = 0 for each individual experiment. As a result, all values now fall below 100%. We have clarified this point in the revised Figure 4i, the revised extended data Figure 22, and the corresponding figure legends.

6) The authors offer a comparison with Wapl effects on Cohesin (SMC3) levels. 'Notably, these results resembled the changes observed with cohesin accumulation at TAD boundaries following Wapl depletion (ref 47), albeit at a weaker magnitude.' Without a side-by-side comparison experiment look to look at Wapl depletion, statements like this have little value. This is but one example of many descriptive connections between the observations from their data and the literature that are very much opinion and not relevant to a results section.

We are grateful to the reviewer for this suggestion. Our intention was not to imply equivalence between RB loss and WAPL depletion but rather to provide contextual insight by noting that the pattern of cohesin accumulation at TAD boundaries upon RB loss qualitatively resembles previously published findings in the WAPL depletion setting (Ref. 47). To avoid potential overinterpretation, we have revised the relevant sentence and moved it from the Results to the Discussion section (page 10, lines 13-14), where it is now presented as a contextual comparison rather than part of the primary data interpretation: "The changes in cohesin occupancy upon RB loss qualitatively resemble the accumulation of cohesin at TAD boundaries reported in WAPL-depleted cells[47], albeit at a weaker magnitude."

7) Most figures contain unreadable text and numbers embedded in them. Sometimes these are labels, sometimes measurements or statistical values. I'm not sure if it is important to be able to read them. Are they already stated in the narrative of the results? Do I need to read them? This deficiency adds to the lack of clarity.

We acknowledge that in the original submission, some figure panels contained embedded text, labels, or statistical values that were difficult to read due to font size or resolution. To improve readability, we have now provided high-resolution figures. In addition, we have ensured that all key numerical values and statistical results are explicitly described in the corresponding figure legends and/or the main text so that the information remains accessible even if not visible directly in the figure panels. We hope these revisions significantly improve the clarity and interpretability of the figures.

Reviewer #4 (Remarks to the Author):

The manuscript by Lee et al homes in on the dynamics of chromatin structure and its role in shaping the expressivity of the genome. Specifically, it addresses the regulation of the interplay between chromatin-bound CTCF and the cohesin complex assigned roles in the partitioning of the genome into chromatin domains. Having previously focused on the inner workings of the tumor repressor protein RB1, the authors perceived that there could be a non-canonical role for this factor going beyond its well-known function of repressing E2F- dependent functions. To deal with the E2F-dependent noise provided by this pivotal function in cell cycle progression, the team resorted to extensive Chip-seq and Micro-C analyses of cells carrying inducible mutant RB1 expression vectors. The key outcome is the documentation that RB1 functions to antagonize the chromatin-bound CTCF-cohesin complexes to remodel the partitioning of a subset of TAD domains and that phosphorylation of RB1 controls this process. Importantly, it describes a painstaking strategy to

uncover that CTCF-cohesin complexes are stabilized in the absence of an RB1 function and that RB1 functions to release the cohesion complex from chromatin-bound CTCF specifically in prometaphase cells. It is of significant interest that chromatin loops tend to get smaller and more numerous in RB1-deficient cells. The authors have adopted heroic efforts to highlight that these outcomes are different from the canonical RB1 function that serves to repress E2F-dependent gene expression. Taken together, the data supports the view that a subset of chromatin domains is rewired in the absence of an RB1 function in a non-canonical manner. I was also intrigued by the discussion that RB1 might control also the role of the condensin complex and that the absence of an RB1 function might generate chromosome instability analogous to its role in the CTCF-cohesin complex. The observations of the manuscript have obvious implications for cancer in instances where the RB1 function is perturbed, genetically or otherwise. The surprising outcome of this reasoning is, however, that the plasticity of the genome function to enable adaptation to changing environments might be compromised.

Technically, the manuscript describes cutting edge approaches that include a balanced consideration of alternative explanations. The experiments appear well executed and with appropriate controls.

To sum up, this is an excellent although dense report, which provides yet another surprising twist to the regulation of the CTCF function. It provides a much needed insight into the dynamics of the formation of chromatin domains that underlie the function of the genome. However, there are a number of loose ends, listed below, that the authors might wish to consider.

We thank the reviewer for highlighting the significance of our manuscript in advancing the understanding of how chromatin-bound CTCF–cohesin complexes and a subset of TAD domains are regulated by the cell cycle machinery, and how this regulation may be linked to the non-canonical, E2F-independent transcriptional activity of RB.

- It is not crystal clear to me why the Extended data Figs S5-S7 are treated so summarily. Although I appreciate the space limit of the manuscript format it makes little sense to include this data without explanations.

We thank the reviewer for pointing this out. The reason we did not include a discussion of the analysis presented in Extended Data Figs. 5-7 in the main text was solely due to word count limitations. This analysis was designed to estimate the probability of observing specific chromatin architectures based on our Micro-C data. It confirmed the high quality of our dataset, as we were able to reconstruct three-dimensional chromatin organization with confidence. Notably, the analysis also revealed potential differences in the spatial arrangement of acrocentric chromosomes in RB knockout cells. To address this, we have added the following text to the main text on page 4, lines 5-9: “Furthermore, three-dimensional reconstruction of the chromatin architecture via a cell population-based algorithm revealed that RB loss weakens the spatial clustering of acrocentric chromosomes harboring nucleolus organizer regions (NORs). Specifically, the average distance between centromeres of acrocentric chromosomes grew from ~1.81 µm in RBWT to ~2.52 µm in RBKO (Extended Data Figs. 5–7).”

- I lack an insightful discussion about how the absence of an RB1 function might affect cancer evolution. Although loss of RB1 function occurs late in cancer development as alluded to by the authors, its absence would be expected to freeze/stabilize chromatin interactions underlying the expressivity of the genome running counter to the general view that cancer cells evolve by

readily adapting to a changing environment. Even though this is not a topic of the manuscript, its observations have strong implications on this topic. For example, the authors could consider to analyze single cell RNA-seq data to explore the non-canonical function of RB1 even though I realize that the space of the manuscript format limits such options.

We agree with the reviewer that this is an important and timely topic for discussion. Recent studies have demonstrated that 3D chromatin reorganization is associated with neuroendocrine transformation in castration-resistant prostate cancer (Lu X. et al., 2024, PMID: 39677680), a process that is also linked to RB1 inactivation (Ku et al. and Mu et al., 2017). To explore whether chromatin reorganization following RB loss contributes to cell-to-cell heterogeneity or shifts in lineage specificity, we performed single-cell RNA-seq (scRNA-seq) analysis in RB wild-type and RB knockout cells synchronized in the G1 phase. While this analysis confirmed the transcriptional differences in RB-deregulated genes reported in Figure 4, it did not reveal distinct subpopulations or transcriptional heterogeneity at the single-cell level (Fig. R1; Extended Data Fig. 25; see also response to Reviewer 1). We acknowledge, however, that in genetically altered cancer models or under therapeutic pressure, we cannot rule out the effect of RB loss on cell-to-cell heterogeneity. We now discuss this point in the revised manuscript on page 10, lines 28-35: "Recent findings showing that 3D chromatin reorganization is linked to neuroendocrine transformation in castration-resistant prostate cancer[64] suggest that RB's role in regulating chromatin insulators may contribute to cell-to-cell heterogeneity and drive tumor progression. Although our scRNA-seq analysis in RPE1 RB^{WT} and RB^{KO} cells did not reveal such heterogeneity, we acknowledge that in genetically altered cancer models or under therapeutic pressure, these effects could become more pronounced. We appreciate that RB's involvement in chromatin architecture and its impact on enhancer-promoter interactions opens new avenues for exploring its role in the activation of lineage-specific programs seen in lung and prostate cancers following RB loss[14,15,17-19]."

- On a similar note, I lack a few sentences discussing the observation that genes encoding factors involved in the EMT process are strongly overrepresented in two of the non-canonical clusters. This is especially relevant as RB1 has been implicated to control this process, which likely underlies the ability of cancer cells to establish metastatic niches.

We thank the reviewer for pointing this out. To address this, we have added the following statement to the Discussion on page 10, lines 35-38: "In addition, the newly identified role of RB in regulating enhancer-promoter interactions and activating genes involved in maintaining epithelial cell identity offers a novel mechanistic perspective on how RB loss may facilitate the ability of cancer cells to establish metastatic niches[65,66]."

- In the manuscript it is stated "RB depletion reduced the strength of H3K27ac HiChIP peaks across all three interaction 256 groups despite slightly stronger H3K27ac ChIP-seq signal (Fig.4c and Extended Data Fig. 15)." I lack a sentence or two explaining this discrepancy.

We rephrased this sentence to clarify the point on page 7, lines 30-32, as follows: "Although *RB1* knockout cells display slightly increased H3K27ac ChIP-seq signal compared to *RB1* wild-type RPE1 cells, consistent with transcriptional de-repression[23], RB depletion reduced the strength of H3K27ac HiChIP peaks across all three interaction groups (Fig. 4c and Extended Data Fig. 15)."

Point-by-point response to the critiques

We sincerely thank the reviewers for their thoughtful and constructive feedback, which has significantly improved the clarity and overall quality of the manuscript. Our point-by-point responses to the reviewers' comments are provided below:

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Revised Manuscript: RB loss modulates chromatin organization by regulating cohesion-dependent loops and enhancer-promoter interactions.

Manuscript Summary:
This revised manuscript includes additional experimental data and text/figure revisions, including the title, to strengthen the rigor of the conclusions drawn and the clarity of the presentation. Key additional data buttressing the conclusions include scRNA-seq, STAG1/2 ChIP-seq, MED15 ChIP-seq, proteomics, and quantitative ChIP-PCR. These changes have adequately addressed nearly all the issues we raised on prior review. One experiment was attempted to replicate some of the findings in an additional cell line model, as requested, but this experiment did not yield interpretable results. Given the technical challenges associated with these types of experiments, evidenced by the relative lack of equivalent data in the published literature, I don't think this should preclude publication of the current manuscript. I support publication of the current version of the manuscript in Nature Communication, perhaps with minor revisions listed below.

In my opinion the work should be published for several key reasons. This is one of the first papers to address the impact of RB on higher order chromatin structure like chromosome looping. A key finding is that RB loss increases the number of cohesin-defined chromosome loops and their insulating activity. Given increased interest in non-canonical functions of RB in cancer, like phenotypic plasticity, the work presented in this manuscript is an early step in trying to elucidate the molecular basis of such non-canonical functions, even though cancer models are not deployed to generate the data. The data does provocatively align with observations in the cancer plasticity field where RB loss induces an EMT as an intermediate plastic state that evolves into lineage variants, suggesting RB's impact on higher order chromatin structure may be involved. The data presented is of high quality, especially in light of the difficulty the field has had in generating good RB ChIP-seq data with available antibodies. It has been observed for some time that RB can activate gene expression in addition to its canonical role in gene expression repression. The molecular basis of RB induced gene expression is not well established. This paper provides data to rigorously confirm RB activated gene expression and provides a potential mechanism. Overall, the work presented is important and potentially paradigm shifting.

We are grateful to the reviewer for the comments and suggestions.

Minor issues that could be addressed at the editor's or author's discretion:
1) It would be nice to see UMAPs of the scRNA-seq data, perhaps color coded by the transcriptional clusters identified as well as the number of genes/UMI metric.

In the revised manuscript, we have added Extended Data Fig. 27, which presents UMAP visualizations of the scRNA-seq dataset in RB^{WT} and RB^{KO} hTERT-RPE1 cells. To identify whether the expression of each transcriptional cluster is enhanced or repressed by RB, we have color-coded each dots based on the genotype (RB^{WT} vs RB^{KO}) and based on the average expression of genes in each transcriptional cluster (Figure R1). Overall, our analysis reveals clearer separation of RB^{WT} and RB^{KO} hTERT-RPE1 cells and confirms that cluster 5 genes, which is linked to non-canonical transcriptional activation by RB, are preferentially expressed in RB^{WT} cells. In contrast, cluster 3 genes, which correspond to the canonical RB/E2F-pathway target genes, are preferentially expressed in RB^{KO} cells.

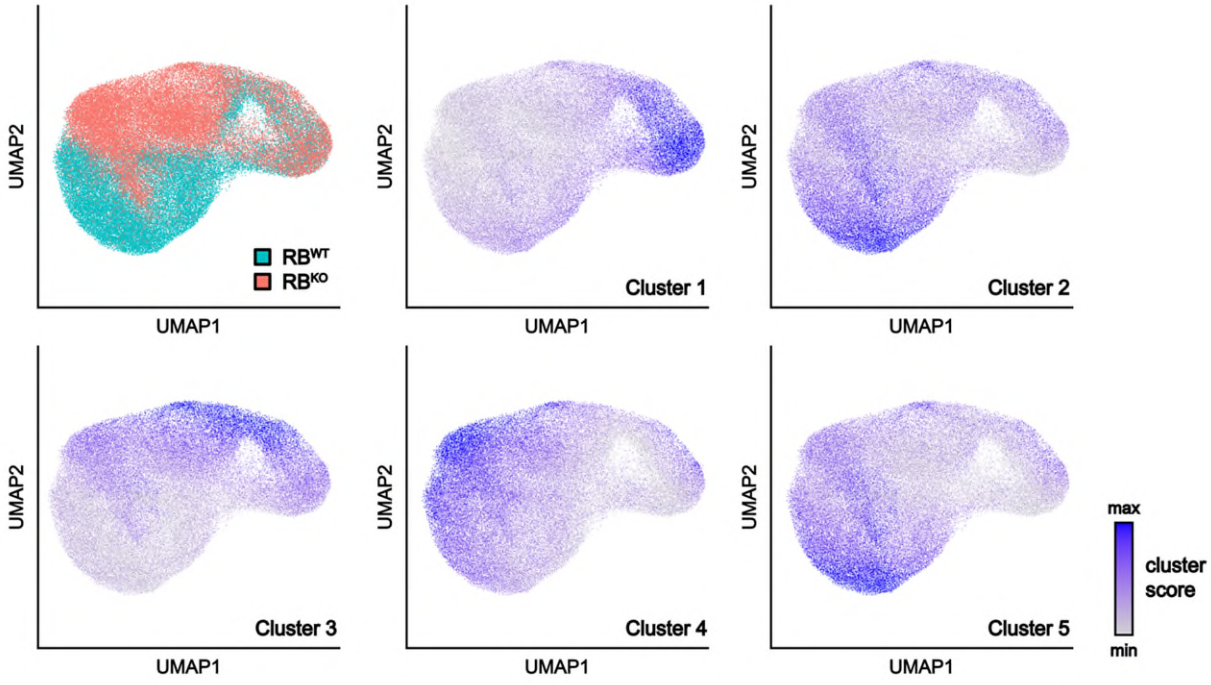

Figure R1. UMAP visualization of single-cell RNA sequencing of G1-arrested RB^{WT} and RB^{KO} hTERT-RPE1 cells corroborates differential transcriptional regulation by RB.
Uniform Manifold Approximation and Projection (UMAP) visualization of single-cell transcriptomes from RB^{WT} and RB^{KO} hTERT-RPE1 cells, arrested in G1 by 6 days of contact inhibition. Each dot on the plot represents an individual cell. Top left panel is colored according to its genotype: RB^{WT} (cyan) and RB^{KO} (magenta). Average expression levels of clusters 1–5 genes in G1 arrested RB^{WT} and RB^{KO} hTERT-RPE1 cells reveal that cluster 2 and cluster 5 gene expression are enhanced in RB^{WT} hTERT-RPE1 cells while cluster 3 gene expression is repressed in RB^{WT} hTERT-RPE1 cells. The plot further corroborates the inverse transcriptional regulation of cluster 3 and cluster 5 gene sets at the single-cell level.

2) The authors focus the insulator discussion on those separating promoters and enhancers, rightfully given the data they are interpreting. However, insulators also insulate from encroaching heterochromatin. The authors may want to speculate in the discussion how the increased number and size of chromatin loops upon RB loss may affect the organization of heterochromatin.

Our chromosome conformation capture data reveal a clear distinction between the effects of transiently active RB in the G1-phase and constitutively active $RB^{\Delta CDK}$ expression on higher-order chromatin organization. In G1, RB modulates cohesin-dependent loop formation in a transient manner that is restored upon S-phase entry. In contrast, sustained $RB^{\Delta CDK}$ expression induces persistent alterations in

nuclear compartments accompanied by heterochromatin dispersion, likely representing an irreversible process. These findings suggest that the changes in TAD boundary insulation observed upon RB depletion are unlikely to drive compartmental reorganization. This conclusion is consistent with prior work showing that acute cohesin degradation abolishes loop domains but does not disrupt nuclear compartmentalization (PMID: 28985562). Instead, the heterochromatin alterations triggered by constitutive $RB^{\Delta CDK}$ activity appear independent of cohesin-mediated looping and may arise from the formation of far-*cis* contacts between nuclear compartments (Fig. 1D) and likely involve $RB^{\Delta CDK}$-mediated recruitment of heterochromatin factors, including HP1, SUV39H1, and DNMT1 (PMID: 11484059, 17245754).

We discuss this observation in the revised discussion page 10, lines 19-28: "In our chromosome conformation capture experiments, RB loss led to increased insulation at TAD boundaries and decreased enhancer-promoter interactions without significantly altering the composition or interactions within nuclear compartments. This observation is consistent with previous work showing that acute cohesin degradation abolishes loop domains but does not disrupt nuclear compartmentalization[63]. Furthermore, RB-mediated changes in cohesin-dependent loop formation observed in Micro-C experiments were transient and restored upon S-phase entry, suggesting that it is not likely to be related to RB's role in epigenetic alterations associated with heterochromatin dynamics[64,65]. Thus, we interpret that the deregulation of gene expression upon RB loss observed in our cell line model is therefore less likely to be linked to changes in heterochromatin dynamics and the position effects they may exert."

Reviewer #1 (Remarks on code availability):

My co-reviewer previously reviewed the code and deemed it appropriate and justified for the data and analyses presented.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are grateful to the reviewer for their comments throughout the entire revision process. Their comments have helped us significantly improve our manuscript.

Reviewer #4 (Remarks to the Author):

The revised manuscript is an improved version of an already excellent study. Given the space limits the authors have done a good job responding to my concerns.

We are delighted to hear that the reviewer is positive about our study and the revisions we have introduced according to their suggestions. We are grateful to the reviewer for their comments, which have helped us significantly improve our study.

Reviewer #5 (Remarks to the Author):

Retinoblasma protein is encoded by a major tumor suppressor able to repress cell cycle genes through mechanisms by which RB associates with E2F to counteract its role in gene activation. Mutations in CDKs regulating RB association with E2F have been associated with tumorigenesis due to such E2F regulation, though additional phenotypes often developing in association with RB dysfunction, like sister chromatin cohesion defects, may be due to other reasons than E2F. Here, the authors find a novel role of RB that regulates, on top of E2F, cohesin removal from CTCF insulators. Accordingly, upon RB mutations cohesin subunit binding is stabilized at CTCF sites, and probing long-range interactions support a stronger insulation genome-wide. Moreover, long-range contacts engaging enhancers with promoters are perturbed, while CTCF insulation increases. The authors report additional deregulation of genes, in particular for those with no RB in their promoter. They propose a model whereby by regulating cohesin binding, RB may regulate insulation thereby allowing to activate genes involving enhancer-promoter interactions.

The paper provides lots of interesting data that, altogether, support a model by which RB controls cohesin binding at CTCF sites. Given the major role of cohesin on higher-order chromatin organization, there could be many reasons by which this impedes on gene expression. The proposed model linking the increased insulation with the increasing levels in gene expression is not clearly demonstrated. Although genomic analyses show a tendency for genes being activated to be associated with increased insulation, it is difficult to interpret the data due to the heterogenous nature of the genomic contexts.

We are grateful to the reviewer for their comments and suggestions. In the revised manuscript, we have included analyses that address the points raised by the reviewer and have edited the text according to the reviewer's suggestions.

Major points

1. In figure 4c, the defects in enhancer-promoter interactions (HiChiP of H3K27ac) should be shown as a function of cohesin accumulation at the insulator site that separate E-P's (as in Fig 4f).

In our study, we report that RB loss leads to increased insulation at TAD boundaries and to reduced enhancer-promoter interactions. According to the reviewer's comment, we aimed to quantify the fraction of changes in enhancer-promoter interaction that is attributable to changes in chromatin insulation. Since chromosome conformation capture experiments such as H3K27ac HiChIP did not have the resolution to resolve individual enhancer-promoter interactions, we utilized MED15 ChIP-seq signal as a surrogate measure to quantify enhancer activity (Figure R2). MED15 ChIP-seq is an orthogonal experiment we performed to corroborate reduced enhancer-promoter interactions upon RB loss. We observed that in RB^{WT} hTERT-RPE1 cells, TAD boundary exerts a distance-dependent effect on the enhancer activity. In contrast, in RB^{KO} hTERT-RPE1 cells, the distance-dependent effect is abolished. This observation is consistent with RB-mediated repression of insulation at TAD boundaries and increased enhancer-promoter interactions associated with wild-type RB. Notably, the TAD boundary-dependent effects on MED15 recruitment to enhancers did not fully explain the difference between RB^{WT} and RB^{KO} hTERT-RPE1 cells, suggesting that there may be effects of RB loss that are independent of its role in regulating insulation at TAD boundaries. One potential mechanism whereby RB may directly regulate enhancer-promoter interactions would be through the direct interaction of RB with AP-1 transcription factors at enhancers (Sanidas *et al*. 2022). Indeed, we observed that MED15 peaks that are depleted upon RB loss are enriched with AP-1 and TEAD transcription factor binding motifs (Figure R3 and Extended Data Fig. 19). In light of the report that RB loss is associated with reduced YAP/TAZ-TEAD activity in cancers (PMID: 34270926) and our

previous observation that dominant negative c-Fos prevents RB association with enhancers, we interpret that RB loss may have at least two different mechanisms leading to reduced enhancer-promoter interactions. We have revised the main text of the manuscript to include alternative mechanisms: page 7, lines 35-38: “Motif enrichment analysis of enhancers with decreased MED15 binding upon RB loss revealed enrichment of AP-1 and TEAD transcription factors motifs (Extended Data Fig. 19). RB has been reported to associate with these enhancer regions[23,56], suggesting that RB may regulate enhancer activity through multiple, potentially distinct mechanisms.” We have also revised the schematic presented in Figure 5 to more clearly present our model for non-canonical transcriptional regulation associated with RB.

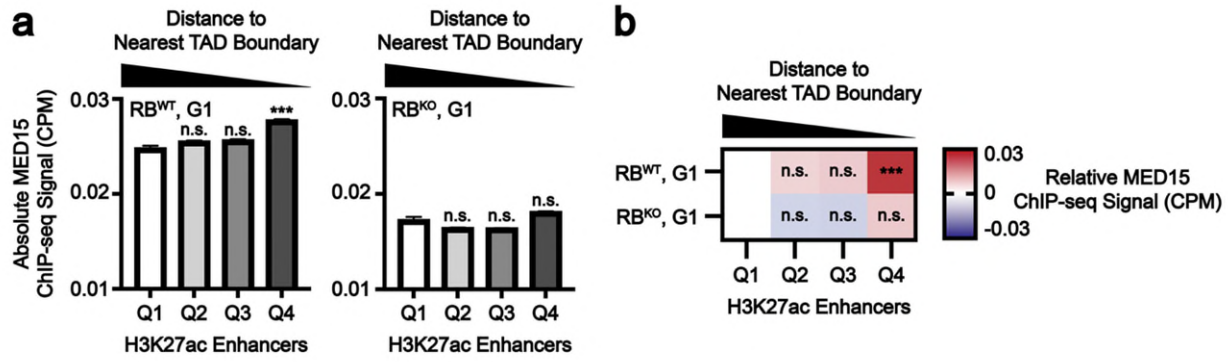

Figure R2. TAD boundary exerts an RB- and distance-dependent effect on MED15 recruitment to enhancers.

(**a**) Absolute MED15 ChIP-seq signals averaged across enhancers marked with H3K27ac modifications. Enhancers were categorized into quartiles based on their distance to nearest TAD boundary. Average MED15 ChIP-seq signal is presented as counts per million (CPM). Left panel presents MED15 ChIP-seq signals in RB^{WT} hTERT-RPE1 cells arrested in G1 phase, while right panel present MED15 ChIP-seq signals in RB^{KO} hTERT-RPE1 cells arrested in G1 phase. TAD boundary exerted a distance-dependent effect on MED15 recruitment to enhancers in RB^{WT} hTERT-RPE1 cells. This distance-dependent effect was abolished in RB^{KO} hTERT-RPE1 cells. (**b**) Heatmap of relative MED15 ChIP-seq signal compared to the enhancer quartile that is most distal from TAD boundaries. Notably, the TAD boundary-dependent effects on MED15 recruitment to enhancers did not fully explain the difference between RB^{WT} and RB^{KO} hTERT-RPE1 cells, suggesting that there may be effects of RB loss that are independent of its role in regulating insulation at TAD boundaries. Two-tailed Student’s *t*-test was performed to assess statistical significance, and Bonferroni correct was performed for multiple hypotheses correction. **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001.

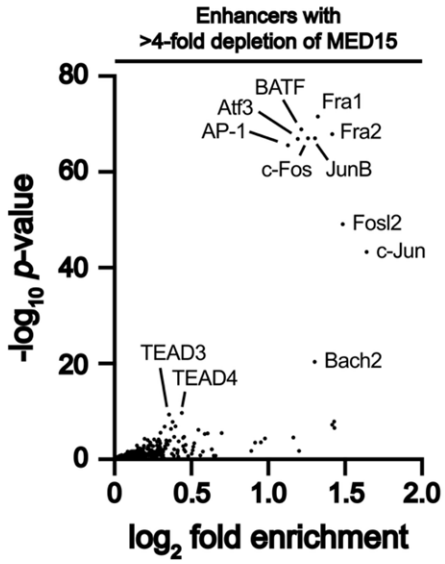

Figure R3. MED15 peaks in enhancers depleted upon RB loss are enriched with AP-1 and TEAD binding motifs.
Motif enrichment analysis of enhancers with more than 4-fold depletion of MED15 revealed enrichment of DNA bindings motifs for AP-1 and TEAD transcription factors.

2. Whereas the split between RB+E2F+ from RB-E2F- is probably of use to analyze the data, there may be many reasons for their relative change in RNA expression upon RBkd and the comparison is thus not insightful to demonstrate the mechanism by which RB functions(Fig 4f). Rather, a more direct enhancer-promoter assay with an intervening insulator would allow a more straightforward comparison.

This is an excellent experiment and we are grateful to the reviewer for the suggestion. However, it would require the generation of genetically engineered cells suitable for the reporter assay, and this could not be completed within the provided timeframe for the revision.

3. The defects in B compartment seen for RBcdk (Figure 1d) should be shown upon RBkd. The real question is whether gene expression defects (increasing expression levels) could also be the consequence of the defects in B compartments.

The analysis of the change in nuclear compartments upon $RB^{\Delta CDK}$ expression and upon RB loss is shown in Figure R4. $RB^{\Delta CDK}$ expression led to dramatic spreading of compartment B as well as reduced interaction within compartment B, consistent with heterochromatin dispersion phenotype that has been microscopically observed (Krishnan *et al*. 2022). Notably, the observed change in compartment B occurred between 24 h and 72 h of $RB^{\Delta CDK}$ expression, coinciding with cell cycle exit. In contrast, we did not observe analogous changes in compartment B upon RB loss (Figure R4).

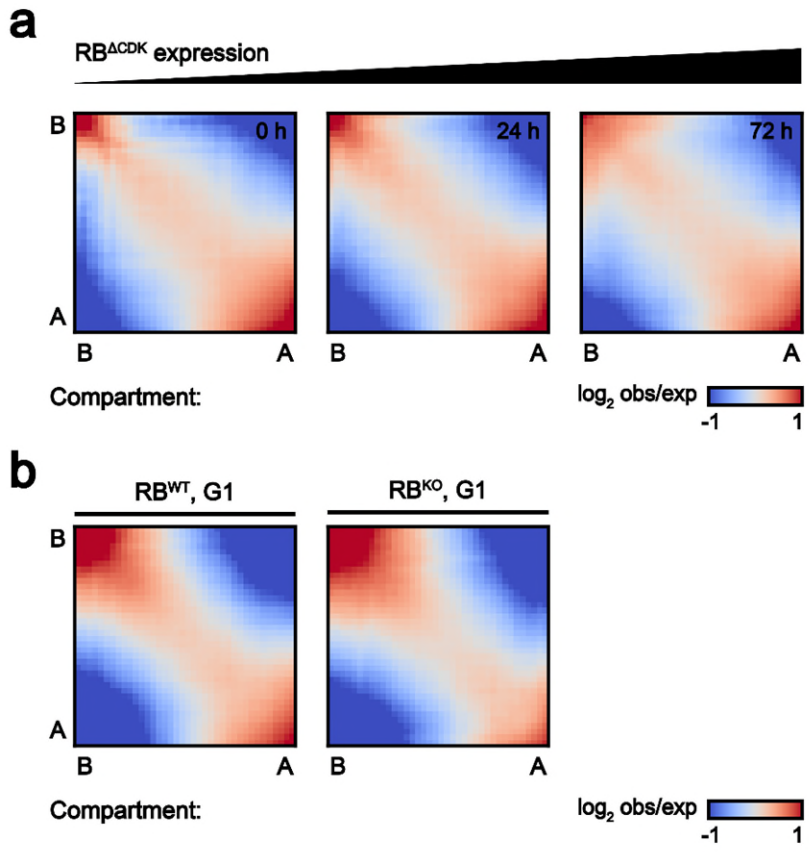

Figure R4. $RB^{\Delta CDK}$ expression but not RB loss induces compartment B spreading and dispersion.
(**a** and **b**) Saddle plot indicating the distribution and the interaction strength between different compartments (A and B). (a) The progression from left to right panels shows changes in compartmentalization over time (0 h, 24 h, 72 h), upon $RB^{\Delta CDK}$ expression. Between 24 h and 72 h of $RB^{\Delta CDK}$ expression, spreading of compartment B as well as reduced interaction within compartment B are observed, consistent with heterochromatin dispersion phenotype observed upon cell cycle exit. (b) RB^{WT} and RB^{KO} hTERT-RPE1 cells exhibited similar nuclear compartmentalization. No evidence of heterochromatin dispersion was observed upon RB loss.

Our chromosome conformation capture data reveal a clear distinction between the effects of transiently active RB in the G1-phase and constitutively active $RB^{\Delta CDK}$ expression on higher-order chromatin organization. In G1, RB modulates cohesin-dependent loop formation in a transient manner that is restored upon S-phase entry. In contrast, sustained $RB^{\Delta CDK}$ expression induces persistent alterations in nuclear compartments accompanied by heterochromatin dispersion, likely representing an irreversible process. These findings suggest that the changes in TAD boundary insulation observed upon RB depletion are unlikely to drive compartmental reorganization. This conclusion is consistent with prior work showing that acute cohesin degradation abolishes loop domains but does not disrupt nuclear compartmentalization (PMID: 28985562). Instead, the heterochromatin alterations triggered by constitutive $RB^{\Delta CDK}$ activity appear independent of cohesin-mediated looping and may arise from the formation of far-*cis* contacts between nuclear compartments (Fig. 1D) and likely involve $RB^{\Delta CDK}$-mediated recruitment of heterochromatin factors, including HP1, SUV39H1, and DNMT1 (PMID: 11484059, 17245754).

We discuss this observation in the revised discussion page 10, lines 19-28: "In our chromosome conformation capture experiments, RB loss led to increased insulation at TAD boundaries and decreased enhancer-promoter interactions without significantly altering the composition or interactions within nuclear compartments. This observation is consistent with previous work showing that acute cohesin degradation abolishes loop domains but does not disrupt nuclear compartmentalization[63]. Furthermore, RB-mediated changes in cohesin-dependent loop formation observed in Micro-C experiments were transient and restored upon S-phase entry, suggesting that it is not likely to be related to RB's role in epigenetic alterations associated with heterochromatin dynamics[64,65]. Thus, we interpret that the deregulation of gene expression upon RB loss observed in our cell line model is therefore less likely to be linked to changes in heterochromatin dynamics and the position effects they may exert."

4. The impact of RB-kd should also be shown as a function of the cohesin loss, for panels Fig 4a, 4d.

We are grateful to the reviewer's suggestions. This comment is linked to the reviewer's 1st major point, and we have addressed the points in our reply therein. In short, we have added extra analyses linking the impact of RB loss on enhancer-promoter interactions to TAD boundaries. TAD boundary exerted an RB- and distance-dependent effect on MED15 recruitment to enhancers, consistent with increased insulation at TAD boundaries and reduced enhancer-promoter interactions observed upon RB loss.

5. Boxplot with p-value should be added to test the significance of the changes in binding profiles (fig3a,3b, 4d). Otherwise, there is a risk of looking at the effect of outliers.

We are very grateful to the reviewer's suggestions to include box plots. In the revised manuscript, we have included box plots as Extended Data Figures for all the main Figures suggested by the reviewer (Figure R5, Extended Data Figs. 10e, 10f, and 19a). Overall, our analyses show that the presented changes in binding profiles are not driven by the effect of outliers and that these changes are statistically significant.

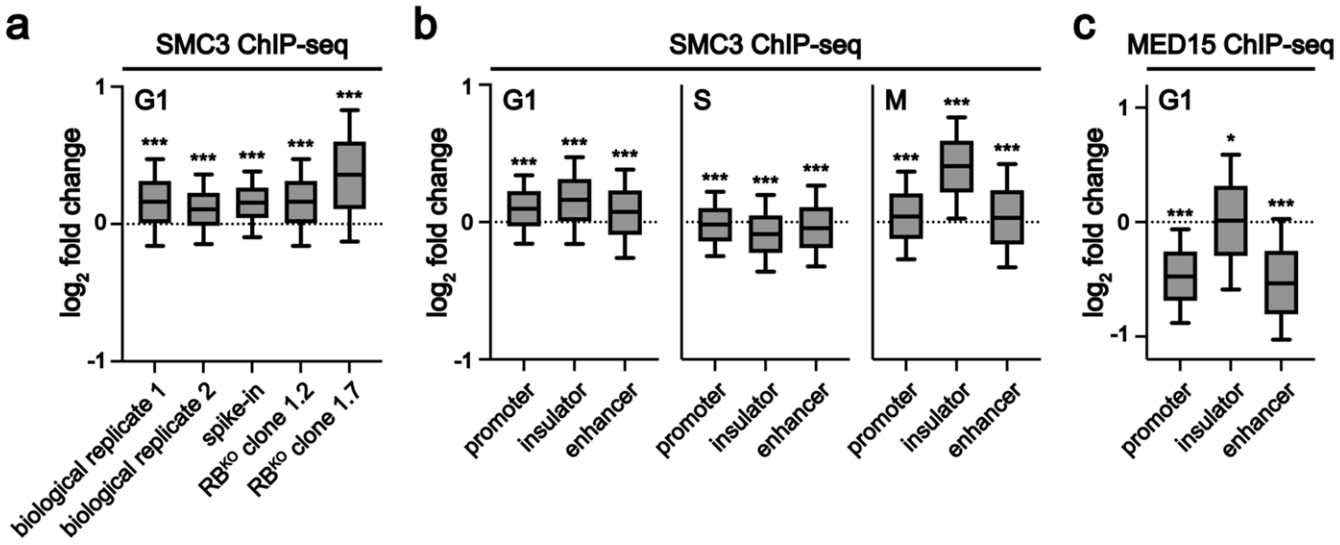

Figure R5. Box plots for SMC3 and MED15 ChIP-seq signals show that the observed differences are not driven by the effect of outliers and are statistically significant.
(**a** and **b**) Box plot of log2 fold change of SMC3 ChIP-seq signals at SMC3 peaks upon RB loss across experiments. SMC3 ChIP-seq signals at insulators in G1 phase (a) and across *cis*-regulatory elements in G1, S, and prometaphase (b) are shown. The data confirms that the observed increase in SMC3 ChIP-seq signal at insulators in G1 and prometaphase cells occurs genome-wide and is not driven by outliers. (**c**) Box plot of log2 fold change of MED15 ChIP-seq signals across *cis*-regulatory elements in hTERT-RPE1 cells. RB loss led to statistically significant depletion of MED15 at promoters and enhancers. One-sample Student's *t*-test was utilized to assess statistical significance and Bonferroni correction was utilized for multiple hypotheses correction. **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001.

Point-by-point response to the critiques

REVIEWER COMMENTS

Reviewer #5 (Remarks to the Author):

The authors have addressed most criticisms. The one remaining item may be addressed as explained in the next paragraph. This would strengthen one of the main conclusion of the paper related to the regulation of E-P by RB.

Former comment: In figure 4c, the defects in enhancer-promoter interactions (HiChiP of H3K27ac) should be shown as a function of cohesin accumulation at the insulator site that separate E-P's (as in Fig 4f).

Former authors' response: In our study, we report that RB loss leads to increased insulation at TAD boundaries and to reduced enhancer-promoter interactions. According to the reviewer's comment, we aimed to quantify the fraction of changes in enhancer-promoter interaction that is attributable to changes in chromatin insulation. Since chromosome conformation capture experiments such as H3K27ac HiChIP did not have the resolution to resolve individual enhancer-promoter interactions, we utilized MED15 ChIP-seq signal as a surrogate measure to quantify enhancer activity (Figure R2).

Reviewer's comment: The authors solely need to reassess E-P interactions as already measured by the APA in Fig 4c. Unlike stated in their answer, there is no need for higher resolution to do so. The APA may be simply redone after splitting E-P into two groups, one defines by E-P with an intervening insulator, versus the other defined by E-P couples not separated by an insulator. Ideally, this can be done for E-P separated by the same range of distances.

We sincerely thank the Reviewer for this comment, as the suggested analysis enhances the interpretation of the role of RB on E-P interactions. In the revised manuscript, we now compare Aggregate Peak Analyses (APA) of enhancer-promoter (E-P) interactions that cross a TAD boundary (inter-TAD) with those that do not (intra-TAD) (Figure R1). To control distance-dependent effects on APA, we randomly pooled a subset of E-P interactions with identical distance distributions (Figure R1b). This led to n=165 E-P interactions for each group.

We then generated aggregate peaks for each group using JuicerTools's APA function at 25 kb resolution. As expected, intra-TAD E-P interactions were stronger and more well-defined, while inter-TAD E-P interactions were weaker and less distinct in both RB^{WT} and RB^{KO} hTERT-RPE1 cells (Figure R1a). Comparing E-P interactions between RB^{WT} and RB^{KO} cells showed that both intra- and inter-TAD interactions become weaker upon RB loss, with the RB-dependent effect appearing more pronounced when E-P interactions cross a TAD boundary.

Due to the clustered nature of enhancers over several tens or hundreds of kilobases, E-P interactions in HiChIP exhibited wider peaks compared to cohesin-dependent loops, especially when E-P interactions crossed a TAD boundary. This led quantification using APA P2LL (Peak to Lower Left) score, which relies on the single pixel on the center, a suboptimal approach for our analysis. Therefore, to better quantify the RB-dependent effects on E-P interaction strength, we also used MED15 ChIP-seq signal as a surrogate measure of enhancer activity and compared it against the distance from a nearest insulator site. MED15 ChIP-seq is an orthogonal experiment we performed to corroborate reduced E-P interactions observed upon RB loss (Figure 4d). Further

analysis of these data demonstrated that in RB^{WT} cells, TAD boundaries impose a distance-dependent effect on enhancer activity, whereas in RB^{KO} cells this distance-dependent effect is abolished (Figure R1c and d). These findings are consistent with RB-mediated repression of insulation at TAD boundaries and increased E-P interactions in the presence of wild-type RB, corroborating observations from Aggregate Peak Analyses (APA) of H3K27ac HiChIP experiments.

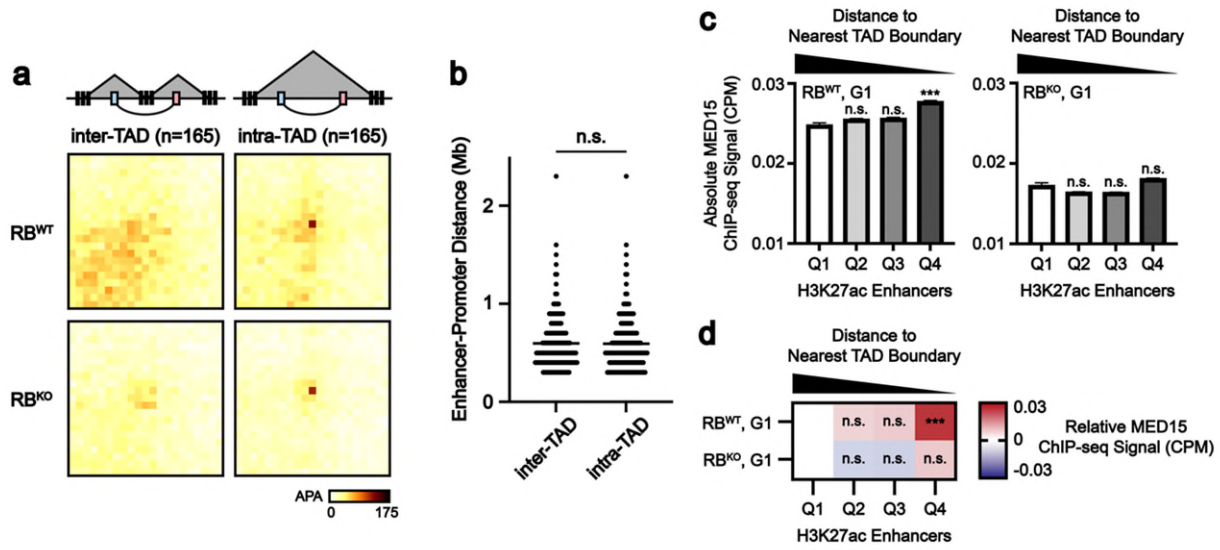

Figure R1. **RB-dependent effects on enhancer-promoter interaction.**
(**a**) Aggregate Peak Analyses (APA) of H3K27ac HiChIP experiment for inter-TAD and intra-TAD enhancer-promoter interactions controlled for enhancer-promoter distance (n=165 each). Intra-TAD enhancer-promoter interactions were stronger and more well-defined, while inter-TAD enhancer-promoter interactions were weaker and less distinct in both RB^{WT} and RB^{KO} hTERT-RPE1 cells. While both intra- and inter-TAD interactions become weaker upon RB loss, RB-dependent effect was more pronounced when enhancer-promoter interactions cross a TAD boundary. (**b**) Comparison of enhancer-promoter distance between inter-TAD and intra-TAD enhancer-promoter interaction sets controlled for enhancer-promoter distance. The distribution of enhancer-promoter distance was highly similar in these sets (*p*-value=1.0, two-tailed Student's *t*-test). (**c**) Absolute MED15 ChIP-seq signals averaged across enhancers marked with H3K27ac modifications. Enhancers were categorized into quartiles based on their distance to nearest TAD boundary. Average MED15 ChIP-seq signal is presented as counts per million (CPM). Left panel presents MED15 ChIP-seq signals in RB^{WT} hTERT-RPE1 cells arrested in G1 phase, while right panel presents MED15 ChIP-seq signals in RB^{KO} hTERT-RPE1 cells arrested in G1 phase. TAD boundary exerted a distance-dependent effect on MED15 recruitment to enhancers in RB^{WT} hTERT-RPE1 cells. This distance-dependent effect was abolished in RB^{KO} hTERT-RPE1 cells. (**d**) Heatmap of relative MED15 ChIP-seq signal compared to the enhancer quartile that is most distal from TAD boundaries. Notably, the TAD boundary-dependent effects on MED15 recruitment to enhancers did not fully explain the difference between RB^{WT} and RB^{KO} hTERT-RPE1 cells, suggesting that there may be effects of RB loss that are independent of its role in regulating insulation at TAD boundaries. Two-tailed Student's *t*-test was performed to assess statistical significance, and Bonferroni correct was performed for multiple hypotheses correction. **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001.

These new analyses are now included in the Extended Data Figure 20 and discussed in the main text of the manuscript page 7, line 35 to page 8, line 10: “To further investigate whether the role of RB in regulating enhancer-promoter interactions associated with its activity in repressing insulation at TAD boundaries, we compared interactions between enhancer-promoter pairs that cross a TAD boundary (inter-TAD) with those that do not (intra-TAD). To control distance-dependent effects, we selected a subset of enhancer-promoter pairs matched for distance (Extended Data Fig. 20). As expected, intra-TAD enhancer-promoter interactions were stronger and more well-defined, while inter-TAD interactions were weaker and less distinct in both RB^{WT} and RB^{KO} hTERT-RPE1 cells (Extended Data Fig. 20). Comparing RB^{WT} and RB^{KO} cells revealed that both intra- and inter-TAD interactions weaken upon RB loss, with the RB-dependent effect appearing more pronounced when the enhancer-promoter pair spans a TAD boundary. Consistent with this, MED15 ChIP-seq analysis demonstrated that in RB^{WT} cells, TAD boundaries impose a distance-dependent effect on MED15 ChIP-seq signal and enhancer activity, whereas this pattern is lost in RB^{KO} cells (Extended Data Figs. 20c and d). Together, these findings suggest that RB-mediated repression of insulation at TAD boundaries facilitates increased enhancer-promoter interactions in the presence of wild-type RB, although additional mechanisms may also contribute. Motif enrichment analysis of enhancers with decreased MED15 binding upon RB loss revealed enrichment of AP-1 and TEAD transcription factors motifs (Extended Data Fig. 19). Given that RB has been reported to associate with these enhancer regions[23,56], these results raise the possibility that RB regulates enhancer activity through additional mechanistically distinct pathways.”