

Polycomb Chromatin Topology Enables Long-Range Enhancer Recruitment during Craniofacial Development

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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)

This study employs a mouse embryonic development model to systematically investigate the dynamic changes of long-range promoter-enhancer interactions and their regulatory roles in gene activation/silencing during cranial neural crest cell (CNCC) migration and subsequent differentiation into four major subpopulations (pharyngeal arch [PA], mandible [Md], maxilla [Mx], and frontonasal prominence [FNP]). The authors selected three critical developmental timepoints (E8.5 [pre-migration], E10.5 and E11.5 [post-migration]) to map promoter-associated chromatin interactions using Promoter Capture Hi-C (PCHI-C), complemented by integrative analysis of ATAC-Seq and multiple ChIP-Seq datasets to characterize the epigenetic states of promoters and their interacting distal accessible elements (DAEs).

Key findings reveal that pre-migratory CNCCs harbor H3K27me3-enriched ultra-long-range chromatin interaction networks, forming PRC-mediated poised promoter-promoter (P-P) and promoter-DAE (P-DAE) architectures reminiscent of embryonic stem cells. During migration and differentiation, this network undergoes tissue-specific dissolution, exhibiting dual regulatory outcomes: promoters escaping the network establish new contacts with non-H3K27me3 DAEs to drive robust transcription, while retained promoters maintain silencing. Using *Ezh2*^{-/-} models, the authors demonstrate PRC2's essential role in maintaining these poised-specific interactions—its loss triggers aberrant short-range contacts and ectopic gene activation, while compromising proper DAE engagement and transcriptional output in differentiated lineages.

Through focused analysis of *Hoxa2* in genetically engineered models and genome-wide validation, the study further uncovers that pre-established Polycomb repressive complex tethering elements (PTEs) spatially facilitate post-differentiation interactions between poised promoters and PTE proximal DAEs (within 50kb from PTE). This work not only elucidates how PRC-mediated 3D genome architecture temporally controls developmental gene expression through dynamic reorganization, but also provides fundamental insights into the mechanistic interplay between chromatin topology and cell fate determination, representing an outstanding study with both conceptual and technical innovation.

While the study presents compelling findings, several analytical aspects require clarification or improvement:

1. Quantification of Network Features:

The network diagrams would benefit from quantitative characterization, such as:

- 1) The number of promoters/DAEs associated with each PcG-activated gene (PcActgenes)
- 2) The genomic distance distribution of these interactions
- 3) Epigenetic state statistics (e.g., H3K27me3/H3K4me3 ratios) for connected elements

Direct comparison of networks across timepoints/cell subtypes is challenging. A summarized visualization (e.g., subnetworks highlighting conserved/dynamic interactions) would facilitate interpretation.

2. Visualization of Differential Interactions:

The differential interaction analysis currently relies on statistical summaries. Including locus-specific examples (e.g., browser tracks of 2-3 representative loci) would strengthen the evidence.

3. Ternary Plot Limitations:

The ternary plot inadequately captures ChIP-seq signal dynamics. A heatmap (clustered by promoter/DAE states across stages) would better illustrate epigenetic heterogeneity.

4. H3K27me3 Signal in Active Promoters:

The violin plots (e.g., Supplementary Figure 4) show residual H3K27me3 at active promoters. The authors should:

- 1) Re-define "active" promoters by excluding those with conflicting marks
- 2) Discuss potential biological implications (e.g., bivalency or technical noise)

5. Correlation Analysis (Figure 4):

The scatterplots poorly convey interaction-expression relationships. Suggested improvements:

- 1) Stratify promoters/DAEs into functional groups (e.g., gained/lost interactions) and plot expression changes as heatmaps/bubble charts
- 2) Extend the analysis to compare pre-migration CNCCs vs. all four subtypes to test generalizability
6. Dynamic Epigenetic Trajectories:
The temporal evolution of chromatin states/interactions could be better visualized using alluvial or sankey diagrams to highlight transition frequencies/percentages.
7. Q3/Q4 Interaction Paradox:
The observed expression differences between Q3/Q4 interactions lack epigenetic explanations. The authors should:
 - 1) Explore additional features (e.g., TF motif enrichment, RNA polymerase occupancy)
 - 2) Hypothesize mechanisms (e.g., cooperative TF binding at specific DAEs)
8. PTE Proximity Hypothesis:
The PTE-mediated DAE engagement model relies heavily on Hoxa2 data. Genome-wide validation requires:
 - 1) Meta-analysis of interaction changes centered on PTEs (e.g., aggregate plots of interaction frequencies within ± 50 kb)
 - 2) Statistical testing for distance-dependent enrichment of new DAEs near PTEs
9. CTCF's Role in PTE Context:
The interplay between CTCF and PTEs remains unclear. Key questions:
 - 1) Are CTCF sites colocalized with PTEs or flanking them?
 - 2) Does CTCF binding correlate with H3K27me3 loss (e.g., in Ezh2-/-)?
 - 3) Are these interactions cohesin-mediated loops or phase-separated compartments?
 Additional analysis of Hoxa2 locus CTCF ChIP data is warranted.
10. HIRE1-Hoxa2 Interactions (Supplementary Figure 16):
The E11.5 PA2-specific HIRE1-Hoxa2 contacts (black arrows) coincide with weak H3K27me3 at HIRE1. This suggests:
 - 1) These interactions may still be PRC2-dependent (lost in Ezh2-/- due to H3K27me3 depletion)
 - 2) Alternatively, PRC2 could indirectly stabilize the architecture
 The authors should clarify whether residual PRC2 activity maintains these contacts despite differentiation.

Reviewer #2

(Remarks to the Author)

I read with great interest the manuscript by Joshi et al. entitled "Polycomb Chromatin Topology Enables Long-Range Enhancer Recruitment during Craniofacial Development". In this manuscript, the authors use a multiomic approach in which they combine Promoter Capture Hi-C (PCHi-C), ChIP-seq, ATAC-seq and RNA-seq to investigate the role of Polycomb in 3D chromatin organization during in vivo craniofacial development. Based on an integrative analysis of the different types of genomic datasets generated, the authors propose that polycomb plays an important regulatory role not only as a classical repressor, but also as chromatin-folding organizer that prepares important developmental genes for future activation. More specifically, using the Hoxa2 locus as an example, they report the presence of polycomb tethering elements that facilitate long-range interactions between developmental genes and distal enhancers.

Overall, this is a very complete and thorough study that adds a 3D genome organization view to previous work from the same lab in which they characterized the chromatin profiles and enhancers landscapes during in vivo craniofacial development. Although the reported findings are clearly relevant in the context of craniofacial biology, conceptually speaking, the role of Polycomb in 3D chromatin organization in general as well as its potential role as a facilitator of long-range enhancer-promoter communication has been previously reported in other cell types (e.g. ESC, neural progenitors) and model organisms (e.g. Drosophila) (Bantignies et al., 2011; Denholtz et al., 2013; Kondo et al., 2014; Schoenfelder et al., 2015; Joshi et al., 2015; Cruz-Molina et al., 2017; Rhodes et al., 2020; Pachano et al., 2021; Denaud et al., 2024). Therefore, the main strength of the manuscript is that it shows that the role of Polycomb in 3D genome organization and as a facilitator of enhancer-promoter contacts, which in vertebrates has been mainly investigated using in vitro cellular systems, seems to be also important in vivo. Moreover, it also adds a slightly different angle to previous studies, as based on the findings for the Hoxa2 locus, the Polycomb tethering elements they describe might not be necessarily closed to the enhancers they assist but up to 50 Kb away. However, I am afraid experimental work in additional loci and/or alternative experimental approaches based on gain-of-function/knock-in (e.g. insert tethering element nearby distal enhancers) strategies would be necessary to make stronger and more general claims about the relevance and mechanism of action of such PcG-tethering elements.

More specifically, I have the following concerns and suggestions:

1. Previous work (e.g. Rhodes et al., 2020) indicates that cohesin-mediated loop extrusion counteracts the long-range interactions between PcG-bound promoters. Therefore, one the increased short-range interactions that PcAct genes display in the absence of EZH2 could be mediated by cohesin, whose loading and extrusion within the affected TADs could be increased due to the overall increase in H3K27ac levels and chromatin accessibility. The authors could perhaps perform ChIP-seq for some cohesin subunits to address this possibility.
2. Similarly, the authors suggest that PcG tethering elements facilitate long-range interactions between PcAct genes and distal enhancers. Do these enhancers have any particular features?. E.g. Are they often located close to CTCF peaks, YY1 peaks or CpG islands that can somehow facilitate the establishment of long-range contacts once these enhancers become active?.
3. Regarding the Hoxa2 locus, I found surprising that the HIRE1 and HIRE2 enhancer clusters seem to be already active (based on H3K27ac) at E8.5 and, moreover, the levels of H3K27ac seem to be similar in PA2 and Md: how do the authors explain that the enhancers only contact the Hoxa2 promoter in PA2 but not in Md?.

4. Upon close inspection, and as the authors briefly mention, there is a putative DAE next to the Pc-Tethering element that displays high H3K4me2 and H3K27ac levels, particularly in PA2. Moreover, this DAE also has a clear CTCF peak. Could this DAE act together with the tethering element in controlling *Hoxa2* expression as previously reported for enhancers associated with orphan CpG islands?. As this DAE displays increased H3K27ac specifically in PA2, I think the authors should consider deleting it alone and together with the tethering element.
5. The authors should perform H3K27ac ChIP-seq experiments in PA2 upon deletion of the Pc-tethering element. Is the activation of the HIRE1 and HIRE2 enhancer clusters affected or is it just their communication with the *Hoxa2* promoter disrupted?. Similarly, are H3K27ac levels affected for the DAE located next to the deleted tethering element?.
6. Do the *Hox2* tethering element as well as those similar polycomb-marked anchors often overlap with orphan CpG islands?. To test this, the authors should use broad definitions of CpG islands based on biochemical rather than purely computational properties (e.g. NMI as defined by Long et al. 2013) as well as evaluate the CG and CpG content of those tethering elements. This would reveal to what extent the proposed tethering elements are genetically and functionally distinct from orphan CpG islands that were previously proposed to act as tethering elements facilitating enhancer-promoter communication (Pachano et al., 2021), as also described in *Drosophila* (Batut et al., 2022).

Reviewer #3

(Remarks to the Author)

Joshi et al. focused on the dynamics of 3D chromatin architecture in the regulation of gene expression in cranial neural crest cells (CNCCs). They investigated how Polycomb proteins repress the expression of specific genes in undifferentiated CNCCs, and how this repression is lifted as CNCCs migrate and differentiate, allowing the formation of enhancer-promoter interactions that activate gene expression. Furthermore, the authors described in detail how the loss of the *Ezh2* gene disrupts both these repressive networks and activating interactions, ultimately impairing proper gene expression.

In this study, the authors employed various genomic datasets including RNA-seq, ChIP-seq, and capture Hi-C (cpHi-C). By classifying different types of chromatin interactions based on epigenetic modification states, they meticulously analyzed the transitions during differentiation and in the *Ezh2* knockout context. In the first part of the paper, they present a classification of promoter-DAE interactions and general trends resulting from *Ezh2* loss. In the latter part, they propose a model in which PcG-dependent long-range loops regulate the long-distance enhancer-promoter interactions and gene activation of *Hoxa2*. This connects the generalized phenomena observed in the first part to concrete, gene-specific outcomes and mechanisms. In particular, the latter part explains how the global changes in chromatin contacts seen in Figure 3a lead to detrimental effects at specific gene loci.

The finding that the *Ezh2* knockout leads to inappropriate activation (de-repression) of genes that should normally remain repressed highlights an important function of Polycomb proteins: beyond merely acting as transcriptional repressors, they also help organize chromatin architecture to physically block unwanted ectopic enhancer interactions. This underscores a crucial role for Polycomb in ensuring gene expression specificity and sheds light on a new functional aspect of Polycomb biology.

In summary, this study is highly novel and certainly of interest to the fields of Polycomb and chromatin biology. While the manuscript is well-structured and generally easy to follow, there are some points where the interpretation of data remains unclear, making the manuscript as it currently stands difficult to accept. The addition of the following data and experiments would likely make the manuscript more acceptable.

The authors have performed a comprehensive and multifaceted analysis of developmental processes in mouse CNCCs, and the biological phenomena they target are highly novel; overall, the study is fundamentally acceptable. However, there are some concerns regarding transparency. Specifically, much of the data are presented in a highly quantitative manner, with relatively few figures such as heatmaps or track views that show data at individual loci or genomic regions. This leaves the reader wishing to see more of the actual raw data.

In addition, although the study features a wealth of informative, detailed analyses, the volume and presentation of the data are rather busy and difficult to follow. In particular, the supplementary figures include excessive data that are not directly central to the main narrative, which may confuse readers. From the perspective of novelty and data robustness, I believe this manuscript would even be acceptable in a higher-tier journal such as *Nature Communications*. However, I suspect that prior reviewers may have issued negative evaluations largely because of how the data are presented and expressed, rather than because of any substantive flaw.

The revisions we have proposed can be broadly summarized in three areas: consistency of data across different cell subtypes; clarifying the timing of chromatin structural changes following *Ezh2* knockout; and further characterization of loop anchor features. We consider these to be reasonable requests that should not require excessive additional effort from the authors. If the authors can adequately address at least the major points raised, I believe the manuscript would be acceptable for this journal, and we submit these comments as our review response.

Major comments

The authors present results from four distinct CNCC subtypes spanning E8.5 to E11.5. However, the datasets shown in the main figures do not appear to be unified across the same cell type, making it difficult to believe that these phenomena are truly generalizable. For example, Figure 2c presents data only from Md, Figure 3 only from PA2, and Figure 4 only from FNP. Are these findings similarly observed across all cell types at E11.5? Would it be possible to consolidate the main figures to show data from a single cell type, and summarize the data from other cell types separately in supplementary figures?

To reveal the dual regulatory mechanisms of Polycomb, the authors use *Ezh2* knockout and explore how the resulting changes in 3D chromatin architecture affect gene expression at later differentiation stages (E10.5 and E11.5 post-migratory CNCCs). However, once PRC2 is knocked out, it would not be surprising for transcriptional changes in various genes to

occur immediately at that time point. This raises concern over whether the observed chromatin architecture changes and phenotypes are truly due to PcG-dependent chromatin organization. To address this, could the authors provide data showing the expression of PcG subunit proteins and H3K27me3 enrichment in each cell type at E8.5, E10.5, and E11.5 after Ezh2 knockout? Clarifying at which stage PcG is completely depleted, and checking for gene expression changes at E8.5 (the time point knockout is initiated), would strengthen the interpretation.

In Figure 6a, even though *Hoxa2* expression emerges normally in Ezh2-deficient cells at E10.5, it decreases at E11.5. Does this suggest that Ezh2 is necessary to maintain the established contacts between HIRE1/2 and the *Hoxa2* promoter? The manuscript appears to imply that contact formation between HIRE1/2 and the *Hoxa2* promoter requires prior contact with the *Hoxa2* promoter-proximal PTE. Is it possible to generate capture promoter Hi-C data at E10.5? Since *Hoxa2* is already expressed at E10.5, does this mean that the contacts between HIRE1/2 and the *Hoxa2* promoter are formed in Ezh2-deficient cells at that stage? Alternatively, another possibility is that at E10.5, Ezh2 is not yet completely depleted. As noted above, data showing the time course of H3K27me3 ChIP-seq depletion and expression of Polycomb subunit proteins are essential. In addition, could the authors provide a ChIP-seq snapshot of the *Hoxa2* locus at E10.5?

In Figure 5, Ezh2 knockout leads to a failure to establish proper enhancer contacts and instead creates interactions with short-range contacts. Regarding these new loop anchors, do they tend to form within specific types of chromatin domains? Are there particular trends associated with epigenetic modifications, CGI content, or binding of CTCF and transcription factors? I am very interested in whether a hierarchy emerges in the placement of these second- and third-choice loop anchors once PcG interactions are lost.

Minor Comments

In Figure 4a, is it possible to show representative loci in Q1–Q4, especially in Q2 and Q3? While the data convincingly suggest general trends, providing concrete examples of specific loci would help the reader better visualize the phenomenon.

It is difficult to understand what Figure 2b is meant to show, and the authors' intended message is not clearly conveyed.

In Figure 5, contacts between the *Hoxa2* promoter and *Hoxa2* PTE remain even in Ezh2-KO cells (E11.5 PA2 Ezh2-KO). What do the authors think these contacts depend on? Could they be maintained by residual PRC1?

Figures 6a and 6b might fit better within Figure 5, which would help readers understand the relationship between gene expression and chromatin interactions as an integrated figure.

Is it possible to provide a simple schematic diagram showing the timing of knockout induction and sample collection in a supplementary figure would make it easier for readers to grasp the actual experimental design.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #1 (Remarks to the Author):

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While the study presents compelling findings, several analytical aspects require clarification or improvement:

1. Quantification of Network Features:

The network diagrams would benefit from quantitative characterization, such as:

1) The number of promoters/DAEs associated with each PcG-activated gene (PcActgenes)

OK. We have generated 2 new Excel tables (Supplementary Tables 4 and 5) that contain this information, namely the number of promoters and DAEs interacting with each PcActTF promoter shown in the networks.

2) The genomic distance distribution of these interactions

OK. The genomic distances between PcActTF gene promoters and their interacting promoters and/or DAEs have been added to the same newly generated Excel tables (Supplementary Tables 4 and 5).

3) Epigenetic state statistics (e.g., H3K27me3/H3K4me3 ratios) for connected elements

OK. Supplementary Tables 4 and 5 provide ChIP-seq signal intensities for H3K27me3, H3K4me3, and H3K27ac at PcActTF gene promoters shown in the networks, as well as at their interacting promoters and distal accessible elements (DAEs).

We thank the authors for providing Supplementary Tables 4 and 5, which contain the requested quantitative information (number of interacting promoters/DAEs, genomic distances, and ChIP seq signal intensities) for the PcActTF gene promoters. While these tables are comprehensive, the data would be more accessible to readers if presented in a graphical format.

Direct comparison of networks across timepoints/cell subtypes is challenging.

A summarized visualization (e.g., subnetworks highlighting conserved/dynamic interactions) would facilitate interpretation. We thank the reviewer for this comment. Indeed, summarizing all promoter–DAE networks across time points and cell subtypes in a single unified representation is not feasible due to the complexity and density of the interactions. However, in line with the reviewer's recommendation, we now present a focused comparative analysis using biologically relevant and representative examples. Specifically, in Fig. 2a and Supplementary Fig. 10, we generated promoter–distal accessible element (P–DAE) interaction networks for selected PcActTF gene promoters in E8.5 pre-migratory progenitors and E10.5 post-migratory cranial neural crest cell (CNCC) subpopulations, in which these promoters either remain Pc-repressed or become transcriptionally active. To facilitate direct visual comparisons, genes and their interacting DAEs are displayed using identical positional coordinates across developmental stages and cell populations. For each selected promoter, we include all DAEs that interact at either stage or cell subpopulation. DAEs that do not interact are shown as unconnected.

This strategy avoids the impracticality of globally summarizing all networks while providing a clear and interpretable view of maintained versus dynamic regulatory contacts across developmental stages allowing direct comparison between pre- and post-migratory stages, and among CNCC subpopulations in which promoters remain Pc-repressed versus those in which the same promoters become active. We believe that this comparison directly addresses the reviewer's request and significantly improves the clarity of the network dynamics.

Of note: Supplementary Fig. 9 in the original version presenting the promoter–promoter (P–P) chromatin interaction networks of PcActTF gene promoters in each E10.5 CNCC subpopulation has been retained in the revised version of the manuscript (kept as Supplementary Fig. 9), with quantitative information for these networks provided in Supplementary Table 4. In contrast, Supplementary Fig. 10 in the original version showing the promoter–distal accessible element (P–DAE) chromatin interaction networks of PcActTF gene promoters in E10.5 CNCC subpopulations has been replaced by figures illustrating P–DAE interaction networks for representative PcActTF genes (Fig. 2a and Supplementary Fig. 10). Quantitative information regarding the P–DAE interaction networks of PcActTF gene promoters across E10.5 CNCC subpopulations is now provided in Supplementary Table 5.

We thank the authors for their thoughtful explanation and the revised presentation of the network comparisons.

2. Visualization of Differential Interactions:

The differential interaction analysis currently relies on statistical summaries.

Including locus-specific examples (e.g., browser tracks of 2-3 representative loci) would strengthen the evidence.

We thank the reviewer for this valuable suggestion. As noted above, the new subnetwork analysis (presented in Fig. 2a and Supplementary Fig. 10) introduced in response to comment 1 already provides locus-specific visualizations of interaction dynamics, and therefore directly addresses the reviewer's request for more explicit examples of differential interactions across stages and CNCC subpopulations. In addition, we now include genome browser views of *Hand2* and *Lhx8* (Supplementary Fig. 17) showing interaction and ChIP-seq profiles in E8.5 WT pre-migratory progenitors and E11.5 WT mandibular CNCCs, where these genes reach their highest expression levels. These views highlight changes in promoter–DAE contacts together with the corresponding chromatin state transitions, providing a locus-specific illustration of differential interaction patterns.

We thank the authors for addressing this point thoroughly.

3. Ternary Plot Limitations:

The ternary plot inadequately captures ChIP-seq signal dynamics. A heatmap (clustered by promoter/DAE states across stages) would better illustrate epigenetic heterogeneity.

We thank the reviewer for this comment and appreciate the opportunity to clarify the purpose of the ternary plots. The aim of these plots was not to display the epigenetic heterogeneity at the level of individual elements, nor to track gene-by-gene dynamics across CNCC subpopulations. For this level of resolution, we now provide dedicated Excel tables reporting the quantitative ChIP-seq signal for each histone mark at PcActTF gene promoters and their interacting promoters and DAEs (Supplementary tables 4 and 5).

Rather, the ternary plots were intended to provide a global, interaction-centered, visualization of the epigenetic states of promoters and DAEs that interact with PcAct gene promoters in distinct cellular contexts; namely in E8.5 pre-migratory progenitors and in post-migratory CNCCs, in which PcAct promoters either remain Pc-repressed or have switched to an active state. This analysis reveals that PcAct promoters that remain Pc-repressed in post-migratory CNCCs preferentially interact with Pc-marked DAEs, as was the case in E8.5 progenitors, whereas promoters that become transcriptionally active tend to contact active DAEs.

Such interaction-level and group-level comparisons that provide a visualization of the chromatin interaction dynamics associated with PcAct genes cannot be meaningfully captured by a conventional heatmap, which is intrinsically element-centric rather than interaction-centric.

However, to improve clarity and readability, we have implemented several modifications:

- we revised the main text, figure legends, and Methods to clarify the interpretation of the ternary plots.
- The ternary plots are now positioned below the corresponding subnetworks in Fig. 2, to more clearly link locus-specific representations with the global interaction-level view.
- The *Hoxa2* locus, presented as an illustrative example in the subnetwork analysis (Fig. 2a), is now highlighted directly within the ternary plots (outlined (black), Fig. 2b-d), thereby explicitly connecting the locus-specific example with the global representation.
- We now use a darker orange color for promoters, enhancing visual discrimination between promoters and DAEs.

Nonetheless, in consideration of the reviewer's suggestion, we generated an additional heatmap comparing pre-migratory and post-migratory WT CNCCs. This heatmap includes unique PcAct gene promoters and DAEs and displays, for each stage, whether the element engages in interactions, along with its epigenetic state. While this visualization is helpful for examining individual promoter/DAE states, it does not fully capture the interaction-based mechanisms emphasized in the paper. For this reason, the heatmap is provided to address the reviewer's request but has not been included in the manuscript (Fig.1_Reviewer 1.1).

Taken together, the revised text, improved ternary plots, and additional heatmap clarify both element- level epigenetic information and global interaction-level patterns, thereby addressing the reviewer's concerns while preserving the mechanistic framework central to our study.

We acknowledge the generation of the additional heatmap (Fig.1_Reviewer 1.1) comparing pre- and post-migratory states. While the heatmap is element-centric and does not directly capture interaction dynamics, it provides complementary information on individual promoter and DAE states. We suggest including this heatmap as a supplementary figure (e.g., Supplementary Fig. X) to make it accessible to readers who may wish to examine element-level epigenetic states across stages. We noticed that the color key for promoters and DAEs in this heatmap appears to be reversed; please ensure this is corrected upon incorporation.

H3K27me3 Signal in Active Promoters:

The violin plots (e.g., Supplementary Figure 4) show residual H3K27me3 at active promoters. The authors should:

- 1) Re-define "active" promoters by excluding those with conflicting marks
- 2) Discuss potential biological implications (e.g., bivalency or technical noise)

We thank the reviewer for these valuable comments, providing us with the opportunity to explain and discuss these important points. Below we clarify why redefining "active" promoters would not be appropriate for our *in vivo* system and further explain the biological basis of the residual H3K27me3 signal at active genes.

1) Redefining "active" promoters.

Redefining "active" promoters by excluding those that retain residual H3K27me3 would not be appropriate in the context of our *in vivo* CNCC system. Within each CNCC subpopulation, gene expression exhibits a spatially restricted pattern rather than uniform expression across all CNCCs within a given facial prominence. Because bulk ChIP-seq captures this biological heterogeneity, promoters that are active only in subsets of CNCCs often show both H3K27ac enrichment and residual H3K27me3 signal.

Excluding these promoters would eliminate important craniofacial regulators that are active in specific CNCC subsets—such as *Pitx1*, *Lhx8*, *Alx4*, and *Hand2*—but not across the entire population. Our classification strategy was specifically designed to account for this *in vivo* heterogeneity. This approach captures distinct epigenetic states across cells within each CNCC subpopulation, thereby preserving critical promoters and their associated DAEs in our analysis.

2) Biological implications of residual H3K27me3.

Given the above considerations, the residual H3K27me3 signal is not technical noise but instead reflects genuine biological heterogeneity in epigenetic state and gene expression patterns within CNCC subpopulations (see above). It likely represents partial Polycomb retention at genes that are not uniformly expressed across all CNCCs within a given subpopulation. This observation aligns with the region- specific patterns of gene expression and developmental regulation seen *in vivo*.

Detailed descriptions of the methodology and rationale supporting our classification framework are presented in the Methods

section and depicted in the Supplementary Fig. 2.

We thank the authors for their detailed and thoughtful explanation regarding the residual H3K27me3 signal observed in active promoters. With these explanations, we consider this point fully addressed.

4. Correlation Analysis (Figure 4):

The scatterplots poorly convey interaction-expression relationships. Suggested improvements:

- 1) Stratify promoters/DAEs into functional groups (e.g., gained/lost interactions) and plot expression changes as heatmaps/bubble charts
 - 2) Extend the analysis to compare pre-migration CNCCs vs. all four subtypes to test generalizability
- We thank the reviewer for these suggestions, giving us the opportunity to further clarify the purpose of the analysis shown in Fig. 5 (former Fig. 4). The scatterplot was not meant to display a direct correlation between interaction counts and gene expression in wild-type CNCCs. Instead, it was designed to illustrate how promoter-DAE interactions, target gene expression, and the epigenetic states of both promoters and interacting DAEs change in a coordinated manner when comparing WT and Ezh2cKO CNCC subpopulations.

In the revised version, we have implemented several modifications and clarifications to improve readability:

- In Fig. 5a, we have added a schematic illustrating the changes in P-DAE interactions and corresponding changes in gene expression (expression down or up) in Ezh2cKO vs. WT, representing the four scenarios (Q1-Q4) shown in the scatter plots in Fig. 5b.
 - At the top of each scatter plot in Fig. 5b, we included a schematic highlighting (in black) the epigenetic state of each target promoter (left) or DAE (right) in WT. In each quadrant (Q1-Q4) each dot is an interaction. Dot positions are identical across the left and right Q1-Q4 scatter plots, reflecting the same interactions. The schematics presented at the top of each scatter plot help visualizing how the color-coded epigenetic state of promoters or DAEs in WT correlate with gain or loss of interactions and expression changes in Ezh2cKO CNCCs.
 - We have clarified that although each single P-DAE interaction (single dots) is unique and assigned to a single quadrant (Q1-Q4), a single target gene promoter can participate in multiple interactions in two quadrants, namely Q1 + Q2 or Q3 + Q4, depending on its expression changes in Ezh2cKO CNCCs.
 - The Venn diagrams, already included in the first version of the manuscript, show the percentage and number of unique promoters showing interactions in both Q3 (gained) and Q4 (lost) (Fig. 5e) or in both Q2 (gained) and Q1 (lost) (Fig 5f) in Ezh2cKO CNCCs, from the scatter plots shown in Fig. 5b.
- Schematics in Fig. 5f and 5e illustrate the representative behavior of Q3/Q4 and Q1/Q2 shared promoters, namely: i) representative Q1/Q2 shared promoters correspond to Pc-marked promoters in WT that are up-regulated in Ezh2cKO CNCCs, and display simultaneous loss of interactions with Pc-marked DAEs and gain of new interactions with ectopic DAEs (Fig. 5f); and, ii) representative Q4/Q3 shared promoters correspond to active promoters in WT that are down-regulated in Ezh2cKO, and display simultaneous loss of interactions with active DAEs and gain of new interactions with ectopic DAEs (Fig. 5e).

Regarding the stratification of promoters/DAEs into functional groups, double-stratification bar plots were already included in the original manuscript and have been retained in the revised version (Fig. 5c,d and Supplementary Fig. 15a,b,d,e,g,h). In this analysis, unique promoters and DAEs were respectively stratified according to their assigned quadrant and epigenetic state. Stratifying promoters/DAEs into functional groups based solely on gained versus lost interactions would not effectively capture differences in expression patterns, as many promoters simultaneously exhibit both gained and lost interactions. However, in consideration of the reviewer's request, we additionally generated a heatmap summarizing the information shown in the scatter plots of Fig 5b. This heatmap was produced specifically for the reviewer (Fig.2_Reviewer 1.3) and is not included in the main manuscript, as it may not provide additional information beyond that already conveyed by the scatter plots.

To further address the request of the reviewer to stratify promoters/DAEs based on interaction dynamics in WT between pre-migratory and post-migratory stages and relate these changes to gene expression, we had already performed a stage-matched comparison of chromatin contacts (Fig. 3b). However, rather than directly comparing pre-migratory progenitors to each individual post-migratory CNCC subtype, we focused on PcAct promoters that in post-migratory CNCCs either remain Pc-repressed (Fig. 3b, left) or have switched to an active state (Fig. 3b, right). For both categories, we assessed enrichment of acquired, inherited, and lost P-DAE interactions according to the E11.5 epigenetic state (active or Pc-marked) of the DAEs. We visualized these dynamics in a stage-resolved heatmap with p-values corresponding to the overdispersion-adjusted χ^2 test of independence between interaction types and DAE categories. Furthermore, this analysis has now been completed by the Sankey diagram performed to answer point 6 below. Lastly, our subnetwork analysis (Fig. 2a and Supplementary Fig. 10), performed in response to point 1, also provides a direct visualization of interaction rewiring between pre-migratory and post-migratory stages. In this analysis, that includes all four CNCC subpopulations, pre- and post-migratory interactions are contrasted using identical coordinates.

We thank the authors for providing the heatmap (Fig.2_Reviewer 1.3). This visualization is much clearer than the scatterplots alone, and we recommend including it as a supplementary figure. Importantly, the heatmap reveals an intriguing pattern: in Q2 (upregulated genes), the most strongly expressed genes—many of which are bivalent in WT—do not show the strongest gains in promoter-DAE interactions. This suggests that bivalent genes can be robustly activated upon PRC2 loss without extensive enhancer rewiring, possibly reflecting their poised chromatin architecture. We suggest that the authors to briefly discuss its biological implications (e.g., bivalent promoters may be primed for activation without requiring new enhancer contacts).

5. Dynamic Epigenetic Trajectories:

The temporal evolution of chromatin states/interactions could be better visualized using alluvial or sankey diagrams to highlight transition frequencies/percentages.

We thank the reviewer for this suggestion. To address this point, we generated a simplified Sankey diagram focusing on PcAct genes that have switched to an active state in E11.5 WT post-migratory CNCCs, illustrating both the epigenetic and interaction dynamics of their associated DAEs from E8.5 to E11.5. This analysis summarizes how distal accessible elements connected to active PcAct genes at E11.5 change their chromatin and interaction states from E8.5 to E11.5. The resulting figure has been added as

Supplementary Fig. 7e, along with a detailed description in the Methods section explaining how the diagram was constructed.

We thank the authors for generating the Sankey diagram (now Supplementary Fig. 7e), which effectively visualizes the epigenetic and interaction dynamics of DAEs associated with activated PcAct genes from E8.5 to E11.5. This added panel significantly improves the presentation of temporal transitions and addresses our concern.

6. Q3/Q4 Interaction Paradox:

The observed expression differences between Q3/Q4 interactions lack epigenetic explanations. The authors should:

- 1) Explore additional features (e.g., TF motif enrichment, RNA polymerase occupancy)
- 2) Hypothesize mechanisms (e.g., cooperative TF binding at specific DAEs)

We thank the reviewer for these comments. We first would like to clarify that both the Q3 and Q4 groups of interactions show reduced expression of their target genes in Ezh2cKO CNCCs. Distinct expression behavior (expression down or expression up) of individual target genes is indeed observed for Q1/Q2 and Q3/Q4 interactions in Ezh2cKO. We have now schematically visualised these distinct transcriptional behaviors and interaction patterns in Ezh2cKO CNCCs by the diagram added in Fig. 5a (see above, point 5), summarizing the four scenarios shown in the Q1-Q4 scatter plots in Fig. 5b. The reviewer may be specifically referring to the apparent paradox between Q2 and Q3, which are both associated with gain of proximal ectopic loops in Ezh2cKO CNCCs. Although both Q2 and Q3 correspond to increased promoter-DAE interactions in Ezh2cKO CNCCs, Q2 genes display increased expression whereas Q3 genes show decreased expression. To address the reviewer's question about potential additional features of DAEs explaining the differences in Q2 vs. Q3 and Q3 vs. Q4, we performed a motif enrichment analysis using the R package monaLisa. Briefly, peaks (DAEs) shared between the quadrants to be compared were removed prior to analysis. Motif enrichment was then assessed using `monaLisa::calcBinnedMotifEnrR()` with default parameters, and the top 10-20 motifs were visualized using `monaLisa::plotMotifHeatmaps()`. An adjusted p-value threshold of $-\log_{10}(\text{padj}) > 1.3$ was considered significant. Transcription factor (TF) binding motif analysis comparing exclusive DAEs in Q3 vs. Q4 and in Q2 vs. Q3 did not reveal any specific TF motif enrichment that could account for the differences between Q3 and Q4 and between Q2 and Q3. The corresponding heatmaps are provided for the reviewer's reference (Fig.3_Reviewer 1.7; In the Q3 vs. Q4 comparison, the apparent statistical significance of Nr2e3 in the heatmap is attributable to its correspondence to a HindIII restriction fragment, the enzyme used in promoter Capture Hi-C).

As noted above, both Q2 and Q3 interactions correspond to proximal ectopic loops gained in Ezh2cKO CNCCs. Following the reviewer's comments, we further characterized these ectopic loops and found that DAEs involved in ectopic interactions are more enriched for CpG islands (CGIs; NMI (Long et al., 201353) and CTCF binding sites than DAEs not involved in such interactions (now in Fig. 4g,h). This suggests that intrinsic sequence features and TAD-level architecture may contribute to the formation of these ectopic loops.

We thank the authors for performing the motif enrichment analysis and for clarifying the features of ectopic loops gained in Ezh2cKO CNCCs. While the absence of specific TF motifs that distinguish Q2 vs. Q3 or Q3 vs. Q4 is noted, the finding that DAEs involved in ectopic interactions are enriched for CpG islands and CTCF binding sites (new Fig. 4g,h) is valuable and provides insight into the structural basis of these loops. This remains an interesting but unresolved observation that would benefit from further discussion and analysis.

7. PTE Proximity Hypothesis:

The PTE-mediated DAE engagement model relies heavily on Hoxa2 data. Genome-wide validation requires:

- 1) Meta-analysis of interaction changes centered on PTEs (e.g., aggregate plots of interaction frequencies within $\pm 50\text{kb}$)
- 2) Statistical testing for distance-dependent enrichment of new DAEs near PTEs

We thank the reviewer for this comment. To address the reviewer's request, and within the technical constraints inherent to PCHi-C data, we attempted a genome-wide identification of putative PTEs using the same analytical framework applied to the Hoxa2 locus.

The guiding principle of this framework is to identify Polycomb domains present at the pre-migratory stage (E8.5) that are spatially associated with Q4 active DAEs that loose/reduce contacts with their active target promoters in E11.5 Ezh2cKO post-migratory CNCCs. Conceptually, these early Polycomb domains are hypothesized to facilitate the recruitment of these enhancers to their target H3K27me3-positive promoters, once the latter are activated. Detailed methodological procedures are provided in the Methods section. Using this framework, we identified 174 Polycomb domains that meet our operational criteria for putative PTEs. We additionally found that PTEs are enriched for orphan CpG islands (oCGIs) (see Reviewer 2, point 6) and CTCF binding sites (see below, point 9). The genomic coordinates of these candidate PTEs, their interacting H3K27me3-positive promoters at E8.5, and distances to the nearest E11.5 active Q4 DAE are provided in Supplementary Table 7.

We would like to emphasize, however, that this approach should not be interpreted as a definitive genome-wide annotation of PTEs. The identification of bona fide PTEs is intrinsically challenging and likely to be highly locus-specific, as their definition depends on precise regulatory and chromatin-context features that are difficult to generalize across the genome. Our objective here was therefore not to establish a catalogue of PTEs, but rather to extend, in a systematic manner, the

conceptual and analytical framework developed at the *Hoxa2* locus to explore whether similar regulatory configurations could be detected elsewhere. Consistent with this regulatory logic, and in addition to the *Hoxa2* PTE, we identified further PTE candidates, including regions located near putative long-range enhancers of *Hand2* and *Lhx8* (Supplementary Fig. 17; Supplementary Table 7). Notably, these loci also exhibited reduced interaction frequencies in *Ezh2*cKO CNCCs (Supplementary Fig. 17), supporting the notion that analogous Polycomb-associated regulatory configurations may operate beyond the *Hoxa2* locus.

We thank the authors for their efforts to extend the PTE analysis genome-wide and for providing Supplementary Table 7 with candidate PTE coordinates. While these tables are comprehensive, the data would be more accessible to readers if presented in a graphical format.

The identification of 174 putative PTEs using a defined operational framework, along with the examples of *Hand2* and *Lhx8* (Supplementary Fig. 17), suggests that the regulatory logic observed at *Hoxa2* may indeed apply more broadly.

8. CTCF's Role in PTE Context:

The interplay between CTCF and PTEs remains unclear. Key questions:

- 1) Are CTCF sites colocalized with PTEs or flanking them?
- 2) Does CTCF binding correlate with H3K27me3 loss (e.g., in *Ezh2*^{-/-})?
- 3) Are these interactions cohesin-mediated loops or phase-separated compartments?
- 4) Additional analysis of *Hoxa2* locus CTCF ChIP data is warranted.

We thank the reviewer for these important points. We have addressed each of the questions as follows:

1) Are CTCF sites colocalized with PTEs or flanking them?

To assess whether CTCF binding sites are associated with PTEs, we performed CTCF ChIP-seq on E11.5 WT prominences (merged FNP, Mx, Md, and PA2). We then conducted a genome-wide enrichment analysis of CTCF ChIP-seq peaks within PTE coordinates. Using permutation testing (1,000 iterations) with E8.5 WT Polycomb domains as the background universe, we observed a significant enrichment of CTCF peak overlap within PTEs compared with a random Polycomb domain set ($p < 0.05$) (Fig. 5n).

We further show that PTEs are significantly enriched for orphan CpG islands (Fig. 5m) (see responses to point 8 and Reviewer 2, point 6).

Moreover, through targeted sub-deletions at the *Hoxa2*PTE (now referred to as *Hoxa2*PTE-42kb and *Hoxa2*PTE-36kb; see response to Reviewer 2, point 4) we show that Pc, CTCF, and CGIs cooperate to position the Polycomb-marked *Hoxa2* promoter in spatial proximity to its long-range enhancers, thus enabling efficient enhancer-promoter communication in PA2 CNCCs.

Altogether, our findings suggest that distal Polycomb-marked anchors in pre-migratory progenitors integrate architectural and sequence-based regulatory features that cooperate with Pc to function as essential structural elements enabling the later recruitment of long-range active enhancers required for proper gene regulation in post-migratory CNCCs.

2) Does CTCF binding correlate with H3K27me3 loss (e.g., in *Ezh2*^{-/-})?

To assess changes in CTCF binding upon *Ezh2* deletion, we performed CTCF ChIP-seq on E11.5 prominences (merged FNP, Mx, Md, and PA2) in both WT and *Ezh2*cKO conditions.

Comparison of CTCF occupancy between WT and *Ezh2*cKO reveals largely conserved global binding, with localized increases preferentially occurring at regions marked by H3K27me3 in WT (Supplementary Fig. 14h). These observations indicate that loss of Polycomb activity does not globally rewire CTCF binding but instead leads to local modulation of CTCF occupancy at Polycomb-associated regions, consistent with a model in which Polycomb constrains the functional use of pre-existing CTCF-associated architectural features rather than preventing CTCF binding per se.

3) Are these interactions cohesin-mediated loops or phase-separated compartments?

While promoter-capture Hi-C data do not allow us to directly distinguish between cohesin-mediated loop extrusion and compartment-level interactions, several observations argue against a purely compartment-driven mechanism. First, ectopic interactions are predominantly proximal and intra-TAD, rather than reflecting long-range compartmental shifts. Second, the enrichment of CTCF at PTEs and ectopic DAEs, together with localized changes in CTCF occupancy in *Ezh2*cKO CNCCs, indicates that these interactions occur within a pre-existing architectural framework. We therefore propose that CTCF provides a stable structural context, while Polycomb-dependent mechanisms confer specificity to promoter-DAE engagement; loss of Polycomb activity disrupts this specificity, leading to miswired interactions within an otherwise CTCF-constrained chromatin environment.

4) Additional analysis of *Hoxa2* locus CTCF ChIP data is warranted.

We have now added the CTCF binding profiles for E10.5 WT Md, E10.5 WT PA2 and E11.5 WT FNP/Mx/Md/PA2 at the *Hoxa2* locus and at its interacting long-range enhancers on the genome browser views shown in the Fig. 6a, 7a,b; Supplementary Fig. 18a, 19a, 20b,d.

We thank the authors for the comprehensive response and the new data addressing the role of CTCF in the PTE context. The additional CTCF ChIP-seq experiments in both WT and *Ezh2*cKO conditions, together with the enrichment analyses and the updated genome browser views at the *Hoxa2* locus, substantially strengthen the manuscript.

9. HIRE1-*Hoxa2* Interactions (Supplementary Figure 16):

The E11.5 PA2-specific HIRE1-*Hoxa2* contacts (black arrows) coincide with weak H3K27me3 at HIRE1. This suggests:

- 1) These interactions may still be PRC2-dependent (lost in *Ezh2*^{-/-} due to H3K27me3 depletion)
- 2) Alternatively, PRC2 could indirectly stabilize the architecture

The authors should clarify whether residual PRC2 activity maintains these contacts despite differentiation.

We thank the reviewer for this comment that provides us with the opportunity to discuss this important points. Below, the individual responses to each point.

1) These interactions may still be PRC2-dependent (lost in *Ezh2*^{-/-} due to H3K27me3 depletion)

We detect only very low H3K27me3 levels at HIRE1 in E11.5 WT PA2 CNCCs, which are comparable to input controls and therefore consistent with background signal. To directly address whether residual PRC2 activity at E11.5 contributes to maintaining HIRE1–Hoxa2 interactions, we performed H3K27me3 ChIP–seq in E11.5 PA2 from *Hoxa2*PTE-36kdel/del embryos and repeated the experiment in WT controls for direct comparison (Supplementary Fig. 19c). In both conditions, the H3K27me3 signal at HIRE1 remained at background levels.

Importantly, in *Hoxa2*PTE-36kdel/del embryos, *Hoxa2*–HIRE1 interactions are strongly reduced, phenocopying the *Ezh2*cKO condition, despite intact PRC2 machinery. This demonstrates that the loss of interactions cannot be attributed to depletion of H3K27me3 at E11.5. Furthermore, in addition to the qPCR analysis performed at E10.5 in PA2 of *Hoxa2*PTE-36kdel/del embryos, we carried out qPCR at earlier developmental stages, specifically in PA2 at E9.5 and in the heads of E8.5 embryos (down to r5). We observed reduced *Hoxa2* transcript levels at E9.5 and already at E8.5 (Fig. 7c), i.e. the stage at which HIRE1 is required for *Hoxa2* induction in the neural tube and CNCC progenitors (Kessler et al., 2023). Together, these findings argue against a model in which PRC2 directly maintains HIRE1–Hoxa2 contacts at the post-migratory stage. Instead, our data support a developmental model in which Polycomb-dependent chromatin architecture established at the pre-migratory stage (E8.5) primes or constrains the regulatory landscape, thereby enabling subsequent enhancer–promoter engagement upon activation. In this context, PRC2 is required for the early establishment of regulatory topology rather than for the direct maintenance of active enhancer–promoter contacts at E11.5.

2) Alternatively, PRC2 could indirectly stabilize the architecture

Our chromatin interaction data indicate that in PA2 CNCCs, where *Hoxa2* is selectively activated, contacts shift away from the *Hoxa2*PTE-36kb region toward HIRE1/HIRE2 and the adjacent H3K27ac-marked enhancer RE5.5 (Fig. 6a; Supplementary Fig. 19a). Importantly, at this stage, the *Hoxa2*PTE-36kb region no longer directly contacts the *Hoxa2* promoter.

Although the *Hoxa2*PTE-36kb region remains engaged in Polycomb–Polycomb interactions with neighboring repressed *Hoxa* cluster genes (Supplementary Fig. 19b), these Pc–Pc contacts are spatially separated from the active HIRE1–Hoxa2 loop. Thus, the active enhancer–promoter configuration observed in PA2 CNCCs does not appear to be maintained through direct PRC2-mediated interactions at this stage.

While we cannot formally exclude a minor indirect architectural contribution of surrounding Pc domains, this mechanism is unlikely to represent a general feature of PRC2-dependent PcAct genes. Notably, additional loci that show reduced interactions in *Ezh2*cKO embryos (e.g., *Hand2* and *Lhx8*) do not reside within large Polycomb domains, arguing against a universal indirect stabilization mechanism mediated by persistent Pc–Pc contacts.

Together, these observations support a model in which PRC2 is required for establishing regulatory topology at the pre-migratory stage, but not for directly maintaining active enhancer–promoter contacts after *Hoxa2* activation.

We thank the authors for this thorough response. The additional experiments, particularly H3K27me3 ChIP–seq in PTE mutants and qPCR at early stages, provide compelling evidence that PRC2 is required for establishing regulatory architecture at the pre-migratory stage rather than maintaining active loops after differentiation. We suggest briefly highlighting this developmental priming model in the Discussion.

Reviewer #2

(Remarks to the Author)

I would like to thank the authors for their efforts in addressing my previous concerns, including the generation of new transgenic animals with additional deletions, as well as new ChIP-seq experiments. Overall, I believe the authors have successfully addressed all of my main concerns. Therefore, I congratulate them on their interesting and comprehensive work, which I recommend to be published in Nature Communications.

Reviewer #3

(Remarks to the Author)

I would like to thank the authors for their thorough response and for addressing the points raised in the previous review. The additional experiments and clarifications provided in the revised manuscript have successfully addressed my concerns. Specifically, the new data regarding the timing and hierarchy of PcG-mediated 3D chromatin architecture in higher-order genome structure formation provides the necessary evidence to support the authors' claims. I believe the manuscript has been significantly improved and is now suitable for publication.

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POINT-BY-POINT RESPONSE TO REVIEWERS' COMMENTS NCOMMS-25-41946-T

We are grateful that all three reviewers recognized the significance and novelty of our study. Reviewer #1 described it as “an **outstanding study** with both conceptual and technical innovation”; Reviewer #2 highlighted its completeness and the **importance of uncovering the role** of Polycomb in 3D genome organization and enhancer–promoter interactions *in vivo*; and Reviewer #3 emphasized its **high novelty and relevance** to the fields of Polycomb and chromatin biology. We sincerely thank the reviewers for their constructive feedback, which has helped us strengthen the manuscript through additional experiments, data analyses, figure revisions, and revisions to the text. Please find below a detailed account of the changes and experimental additions to the current revision of the manuscript. We do hope that the reviewers will be fully satisfied by these revisions.

Reviewer #1 (Remarks to the Author):

This study employs a mouse embryonic development model to systematically investigate the dynamic changes of long-range promoter-enhancer interactions and their regulatory roles in gene activation/silencing during cranial neural crest cell (CNCC) migration and subsequent differentiation into four major subpopulations (pharyngeal arch [PA], mandible [Md], maxilla [Mx], and frontonasal prominence [FNP]). The authors selected three critical developmental timepoints (E8.5 [pre-migration], E10.5 and E11.5 [post-migration]) to map promoter-associated chromatin interactions using Promoter Capture Hi-C (PCHi-C), complemented by integrative analysis of ATAC-Seq and multiple ChIP-Seq datasets to characterize the epigenetic states of promoters and their interacting distal accessible elements (DAEs).

Key findings reveal that pre-migratory CNCCs harbor H3K27me3-enriched ultra-long-range chromatin interaction networks, forming PRC-mediated poised promoter-promoter (P-P) and promoter-DAE (P-DAE) architectures reminiscent of embryonic stem cells. During migration and differentiation, this network undergoes tissue-specific dissolution, exhibiting dual regulatory outcomes: promoters escaping the network establish new contacts with non-H3K27me3 DAEs to drive robust transcription, while retained promoters maintain silencing. Using *Ezh2*^{-/-} models, the authors demonstrate PRC2's essential role in maintaining these poised-specific interactions—its loss triggers aberrant short-range contacts and ectopic gene activation, while compromising proper DAE engagement and transcriptional output in differentiated lineages.

Through focused analysis of *Hoxa2* in genetically engineered models and genome-wide validation, the study further uncovers that pre-established Polycomb repressive complex tethering elements (PTEs) spatially facilitate post-differentiation interactions between poised promoters and PTE proximal DAEs (within 50kb from PTE). This work not only elucidates how PRC-mediated 3D genome architecture temporally controls developmental gene expression through dynamic reorganization but also provides fundamental insights into the mechanistic interplay between chromatin topology and cell fate determination, representing an outstanding study with both conceptual and technical innovation. While the study presents compelling findings, several analytical aspects require clarification or improvement:

1. Quantification of Network Features:

The network diagrams would benefit from quantitative characterization, such as:

1) The number of promoters/DAEs associated with each PcG-activated gene (PcActgenes)

OK. We have generated 2 new Excel tables (Supplementary Tables 4 and 5) that contain this information, namely the number of promoters and DAEs interacting with each PcActTF promoter shown in the networks.

2) The genomic distance distribution of these interactions

OK. The genomic distances between PcActTF gene promoters and their interacting promoters and/or DAEs have been added to the same newly generated Excel tables (Supplementary Tables 4 and 5).

3) Epigenetic state statistics (e.g., H3K27me3/H3K4me3 ratios) for connected elements

OK. Supplementary Tables 4 and 5 provide ChIP-seq signal intensities for H3K27me3, H3K4me3, and H3K27ac at PcActTF gene promoters shown in the networks, as well as at their interacting promoters and distal accessible elements (DAEs).

Direct comparison of networks across timepoints/cell subtypes is challenging.

A summarized visualization (e.g., subnetworks highlighting conserved/dynamic interactions) would facilitate interpretation.

We thank the reviewer for this comment. Indeed, summarizing all promoter–DAE networks across time points and cell subtypes in a single unified representation is not feasible due to the complexity and density of the interactions. However, in line with the reviewer’s recommendation, we now present a focused comparative analysis using biologically relevant and representative examples. Specifically, in Fig. 2a and Supplementary Fig. 10, we generated promoter–distal accessible element (P–DAE) interaction networks for selected PcActTF gene promoters in E8.5 pre-migratory progenitors and E10.5 post-migratory cranial neural crest cell (CNCC) subpopulations, in which these promoters either remain Pc-repressed or become transcriptionally active. To facilitate direct visual comparisons, genes and their interacting DAEs are displayed using identical positional coordinates across developmental stages and cell populations. For each selected promoter, we include all DAEs that interact at either stage or cell subpopulation. DAEs that do not interact are shown as unconnected.

This strategy avoids the impracticality of globally summarizing all networks while providing a clear and interpretable view of maintained versus dynamic regulatory contacts across developmental stages allowing direct comparison between pre- and post-migratory stages, and among CNCC subpopulations in which promoters remain Pc-repressed versus those in which the same promoters become active. We believe that this comparison directly addresses the reviewer’s request and significantly improves the clarity of the network dynamics.

Of note: Supplementary Fig. 9 in the original version presenting the promoter–promoter (P–P) chromatin interaction networks of PcActTF gene promoters in each E10.5 CNCC subpopulation has been retained in the revised version of the manuscript (kept as Supplementary Fig. 9), with quantitative information for these networks provided in Supplementary Table 4.

In contrast, Supplementary Fig. 10 in the original version showing the promoter–distal accessible element (P–DAE) chromatin interaction networks of PcActTF gene promoters in E10.5 CNCC subpopulations has been replaced by figures illustrating P–DAE interaction networks for representative PcActTF genes (Fig. 2a and Supplementary Fig. 10). Quantitative information regarding the P–DAE interaction networks of PcActTF gene promoters across E10.5 CNCC subpopulations is now provided in Supplementary Table 5.

2. Visualization of Differential Interactions:

The differential interaction analysis currently relies on statistical summaries.

Including locus-specific examples (e.g., browser tracks of 2-3 representative loci) would strengthen the evidence.

We thank the reviewer for this valuable suggestion. As noted above, the new subnetwork analysis (presented in Fig. 2a and Supplementary Fig. 10) introduced in response to comment 1 already provides locus-specific visualizations of interaction dynamics, and therefore directly addresses the reviewer's request for more explicit examples of differential interactions across stages and CNCC subpopulations. In addition, we now include genome browser views of *Hand2* and *Lhx8* (Supplementary Fig. 17) showing interaction and ChIP-seq profiles in E8.5 WT pre-migratory progenitors and E11.5 WT mandibular CNCCs, where these genes reach their highest expression levels. These views highlight changes in promoter-DAE contacts together with the corresponding chromatin state transitions, providing a locus-specific illustration of differential interaction patterns.

3. Ternary Plot Limitations:

The ternary plot inadequately captures ChIP-seq signal dynamics. A heatmap (clustered by promoter/DAE states across stages) would better illustrate epigenetic heterogeneity.

We thank the reviewer for this comment and appreciate the opportunity to clarify the purpose of the ternary plots. The aim of these plots was not to display the epigenetic heterogeneity at the level of individual elements, nor to track gene-by-gene dynamics across CNCC subpopulations. For this level of resolution, we now provide dedicated Excel tables reporting the quantitative ChIP-seq signal for each histone mark at PcActTF gene promoters and their interacting promoters and DAEs (Supplementary tables 4 and 5).

Rather, the ternary plots were intended to provide a global, interaction-centered, visualization of the epigenetic states of promoters and DAEs that interact with PcAct gene promoters in distinct cellular contexts; namely in E8.5 pre-migratory progenitors and in post-migratory CNCCs, in which PcAct promoters either remain Pc-repressed or have switched to an active state. This analysis reveals that PcAct promoters that remain Pc-repressed in post-migratory CNCCs preferentially interact with Pc-marked DAEs, as was the case in E8.5 progenitors, whereas promoters that become transcriptionally active tend to contact active DAEs.

Such interaction-level and group-level comparisons that provide a visualization of the chromatin interaction dynamics associated with PcAct genes cannot be meaningfully captured by a conventional heatmap, which is intrinsically element-centric rather than interaction-centric.

However, to improve clarity and readability, we have implemented several modifications:

- we revised the main text, figure legends, and Methods to clarify the interpretation of the ternary plots.
- The ternary plots are now positioned below the corresponding subnetworks in Fig. 2, to more clearly link locus-specific representations with the global interaction-level view.
- The *Hoxa2* locus, presented as an illustrative example in the subnetwork analysis (Fig. 2a), is now highlighted directly within the ternary plots (outlined (black), Fig. 2b-d), thereby explicitly connecting the locus-specific example with the global representation.
- We now use a darker orange color for promoters, enhancing visual discrimination between promoters and DAEs.

Nonetheless, in consideration of the reviewer's suggestion, we generated an additional heatmap comparing pre-migratory and post-migratory WT CNCCs. This heatmap includes unique PcAct gene promoters and DAEs and displays, for each stage, whether the element engages in interactions, along with its epigenetic state. While this visualization is helpful for examining individual promoter/DAE states, it does not fully capture the interaction-based mechanisms emphasized in the paper. For this reason, the heatmap is provided to address the reviewer's request but has not been included in the manuscript (Fig.1_Reviewer 1.1).

Taken together, the revised text, improved ternary plots, and additional heatmap clarify both element-level epigenetic information and global interaction-level patterns, thereby addressing the reviewer's concerns while preserving the mechanistic framework central to our study.

4. H3K27me3 Signal in Active Promoters:

The violin plots (e.g., Supplementary Figure 4) show residual H3K27me3 at active promoters. The authors should:

- 1) Re-define "active" promoters by excluding those with conflicting marks
- 2) Discuss potential biological implications (e.g., bivalency or technical noise)

We thank the reviewer for these valuable comments, providing us with the opportunity to explain and discuss these important points. Below we clarify why redefining "active" promoters would not be appropriate for our *in vivo* system and further explain the biological basis of the residual H3K27me3 signal at active genes.

1) Redefining "active" promoters.

Redefining "active" promoters by excluding those that retain residual H3K27me3 would not be appropriate in the context of our *in vivo* CNCC system. Within each CNCC subpopulation, gene expression exhibits a spatially restricted pattern rather than uniform expression across all CNCCs within a given facial prominence. Because bulk ChIP-seq captures this biological heterogeneity, promoters that are active only in subsets of CNCCs often show both H3K27ac enrichment and residual H3K27me3 signal. Excluding these promoters would eliminate important craniofacial regulators that are active in specific CNCC subsets—such as *Pitx1*, *Lhx8*, *Alx4*, and *Hand2*—but not across the entire population. Our classification strategy was specifically designed to account for this *in vivo* heterogeneity. This approach captures distinct epigenetic states across cells within each CNCC subpopulation, thereby preserving critical promoters and their associated DAEs in our analysis.

2) Biological implications of residual H3K27me3.

Given the above considerations, the residual H3K27me3 signal is not technical noise but instead reflects genuine biological heterogeneity in epigenetic state and gene expression patterns within CNCC subpopulations (see above). It likely represents partial Polycomb retention at genes that are not uniformly expressed across all CNCCs within a given subpopulation. This observation aligns with the region-specific patterns of gene expression and developmental regulation seen *in vivo*.

Detailed descriptions of the methodology and rationale supporting our classification framework are presented in the *Methods* section and depicted in the Supplementary Fig. 2.

5. Correlation Analysis (Figure 4):

The scatterplots poorly convey interaction-expression relationships. Suggested improvements:

- 1) Stratify promoters/DAEs into functional groups (e.g., gained/lost interactions) and plot expression changes as heatmaps/bubble charts
- 2) Extend the analysis to compare pre-migration CNCCs vs. all four subtypes to test generalizability

We thank the reviewer for these suggestions, giving us the opportunity to further clarify the purpose of the analysis shown in Fig. 5 (former Fig. 4). The scatterplot was not meant to display a direct correlation between interaction counts and gene expression in wild-type CNCCs. Instead, it was designed to illustrate how promoter–DAE interactions, target gene expression, and the epigenetic states of both promoters and interacting DAEs change in a coordinated manner when comparing WT and *Ezh2cKO* CNCC subpopulations.

In the revised version, we have implemented several modifications and clarifications to improve readability:

- In Fig. 5a, we have added a schematic illustrating the changes in P-DAE interactions and corresponding changes in gene expression (expression down or up) in *Ezh2cKO* vs. WT, representing the four scenarios (Q1-Q4) shown in the scatter plots in Fig. 5b.
- At the top of each scatter plot in Fig. 5b, we included a schematic highlighting (in black) the epigenetic state of each target promoter (left) or DAE (right) in WT. In each quadrant (Q1-Q4) each dot is an

interaction. Dot positions are identical across the left and right Q1-Q4 scatter plots, reflecting the same interactions. The schematics presented at the top of each scatter plot help visualizing how the color-coded epigenetic state of promoters or DAEs in WT correlate with gain or loss of interactions and expression changes in *Ezh2cKO* CNCCs.

- We have clarified that although each single P-DAE interaction (single dots) is unique and assigned to a single quadrant (Q1-Q4), a single target gene promoter can participate in multiple interactions in two quadrants, namely Q1 + Q2 or Q3 + Q4, depending on its expression changes in *Ezh2cKO* CNCCs.
- The Venn diagrams, already included in the first version of the manuscript, show the percentage and number of unique promoters showing interactions in both Q3 (gained) and Q4 (lost) (Fig. 5e) or in both Q2 (gained) and Q1 (lost) (Fig 5f) in *Ezh2cKO* CNCCs, from the scatter plots shown in Fig. 5b. Schematics in Fig. 5f and 5e illustrate the representative behavior of Q3/Q4 and Q1/Q2 shared promoters, namely: i) representative Q1/Q2 shared promoters correspond to Pc-marked promoters in WT that are up-regulated in *Ezh2cKO* CNCCs, and display simultaneous loss of interactions with Pc-marked DAEs and gain of new interactions with ectopic DAEs (Fig. 5f); and, ii) representative Q4/Q3 shared promoters correspond to active promoters in WT that are down-regulated in *Ezh2cKO*, and display simultaneous loss of interactions with active DAEs and gain of new interactions with ectopic DAEs (Fig. 5e).

Regarding the stratification of promoters/DAEs into functional groups, double-stratification bar plots were already included in the original manuscript and have been retained in the revised version (Fig. 5c,d and Supplementary Fig. 15a,b,d,e,g,h). In this analysis, unique promoters and DAEs were respectively stratified according to their assigned quadrant and epigenetic state. Stratifying promoters/DAEs into functional groups based solely on gained versus lost interactions would not effectively capture differences in expression patterns, as many promoters simultaneously exhibit both gained and lost interactions. However, in consideration of the reviewer's request, we additionally generated a heatmap summarizing the information shown in the scatter plots of Fig 5b. This heatmap was produced specifically for the reviewer (Fig.2_Reviewer 1.3) and is not included in the main manuscript, as it may not provide additional information beyond that already conveyed by the scatter plots.

To further address the request of the reviewer to stratify promoters/DAEs based on interaction dynamics in WT between pre-migratory and post-migratory stages and relate these changes to gene expression, we had already performed a stage-matched comparison of chromatin contacts (Fig. 3b). However, rather than directly comparing pre-migratory progenitors to each individual post-migratory CNCC subtype, we focused on PcAct promoters that in post-migratory CNCCs either remain Pc-repressed (Fig. 3b, left) or have switched to an active state (Fig. 3b, right). For both categories, we assessed enrichment of acquired, inherited, and lost P-DAE interactions according to the E11.5 epigenetic state (active or Pc-marked) of the DAEs. We visualized these dynamics in a stage-resolved heatmap with p-values corresponding to the overdispersion-adjusted χ^2 test of independence between interaction types and DAE categories. Furthermore, this analysis has now been completed by the Sankey diagram performed to answer point 6 below. Lastly, our subnetwork analysis (Fig. 2a and Supplementary Fig. 10), performed in response to point 1, also provides a direct visualization of interaction rewiring between pre-migratory and post-migratory stages. In this analysis, that includes all four CNCC subpopulations, pre- and post-migratory interactions are contrasted using identical coordinates.

6. Dynamic Epigenetic Trajectories:

The temporal evolution of chromatin states/interactions could be better visualized using alluvial or sankey diagrams to highlight transition frequencies/percentages.

We thank the reviewer for this suggestion. To address this point, we generated a simplified Sankey diagram focusing on PcAct genes that have switched to an active state in E11.5 WT post-migratory CNCCs, illustrating both the epigenetic and interaction dynamics of their associated DAEs from E8.5 to E11.5. This analysis summarizes how distal accessible elements connected to active PcAct genes at E11.5 change their chromatin and interaction states from E8.5 to E11.5. The resulting figure has been added as

Supplementary Fig. 7e, along with a detailed description in the Methods section explaining how the diagram was constructed.

7. Q3/Q4 Interaction Paradox:

The observed expression differences between Q3/Q4 interactions lack epigenetic explanations. The authors should:

- 1) *Explore additional features (e.g., TF motif enrichment, RNA polymerase occupancy)*
- 2) *Hypothesize mechanisms (e.g., cooperative TF binding at specific DAEs)*

We thank the reviewer for these comments. We first would like to clarify that both the Q3 and Q4 groups of interactions show reduced expression of their target genes in *Ezh2cKO* CNCCs. Distinct expression behavior (expression down or expression up) of individual target genes is indeed observed for Q1/Q2 and Q3/Q4 interactions in *Ezh2cKO*. We have now schematically visualised these distinct transcriptional behaviors and interaction patterns in *Ezh2cKO* CNCCs by the diagram added in Fig. 5a (see above, point 5), summarizing the four scenarios shown in the Q1-Q4 scatter plots in Fig. 5b. The reviewer may be specifically referring to the apparent paradox between Q2 and Q3, which are both associated with gain of proximal ectopic loops in *Ezh2cKO* CNCCs. Although both Q2 and Q3 correspond to increased promoter-DAE interactions in *Ezh2cKO* CNCCs, Q2 genes display increased expression whereas Q3 genes show decreased expression. To address the reviewer's question about potential additional features of DAEs explaining the differences in Q2 vs. Q3 and Q3 vs. Q4, we performed a motif enrichment analysis using the R package *monaLisa*. Briefly, peaks (DAEs) shared between the quadrants to be compared were removed prior to analysis. Motif enrichment was then assessed using `monaLisa::calcBinnedMotifEnrR()` with default parameters, and the top 10-20 motifs were visualized using `monaLisa::plotMotifHeatmaps()`. An adjusted *p*-value threshold of $-\log_{10}(p_{adj}) > 1.3$ was considered significant. Transcription factor (TF) binding motif analysis comparing exclusive DAEs in Q3 vs. Q4 and in Q2 vs. Q3 did not reveal any specific TF motif enrichment that could account for the differences between Q3 and Q4 and between Q2 and Q3. The corresponding heatmaps are provided for the reviewer's reference (Fig.3_Reviewer 1.7; In the Q3 vs. Q4 comparison, the apparent statistical significance of *Nr2e3* in the heatmap is attributable to its correspondence to a HindIII restriction fragment, the enzyme used in promoter Capture Hi-C).

As noted above, both Q2 and Q3 interactions correspond to proximal ectopic loops gained in *Ezh2cKO* CNCCs. Following the reviewer's comments, we further characterized these ectopic loops and found that DAEs involved in ectopic interactions are more enriched for CpG islands (CGIs; NMI (Long *et al.*, 2013⁵³) and CTCF binding sites than DAEs not involved in such interactions (now in Fig. 4g,h). This suggests that intrinsic sequence features and TAD-level architecture may contribute to the formation of these ectopic loops.

8. PTE Proximity Hypothesis:

The PTE-mediated DAE engagement model relies heavily on Hoxa2 data. Genome-wide validation requires:

- 1) *Meta-analysis of interaction changes centered on PTEs (e.g., aggregate plots of interaction frequencies within $\pm 50\text{kb}$)*
- 2) *Statistical testing for distance-dependent enrichment of new DAEs near PTEs*

We thank the reviewer for this comment. To address the reviewer's request, and within the technical constraints inherent to PCHi-C data, we attempted a genome-wide identification of putative PTEs using the same analytical framework applied to the *Hoxa2* locus.

The guiding principle of this framework is to identify Polycomb domains present at the pre-migratory stage (E8.5) that are spatially associated with Q4 active DAEs that loose/reduce contacts with their active target promoters in E11.5 *Ezh2cKO* post-migratory CNCCs. Conceptually, these early Polycomb domains are hypothesized to facilitate the recruitment of these enhancers to their target H3K27me3-positive promoters, once the latter are activated. Detailed methodological procedures are provided in the Methods section. Using this framework, we identified 174 Polycomb domains that meet our operational criteria for

putative PTEs. We additionally found that PTEs are enriched for orphan CpG islands (oCGIs) (see Reviewer 2, point 6) and CTCF binding sites (see below, point 9). The genomic coordinates of these candidate PTEs, their interacting H3K27me3-positive promoters at E8.5, and distances to the nearest E11.5 active Q4 DAE are provided in Supplementary Table 7.

We would like to emphasize, however, that this approach should not be interpreted as a definitive genome-wide annotation of PTEs. The identification of bona fide PTEs is intrinsically challenging and likely to be highly locus-specific, as their definition depends on precise regulatory and chromatin-context features that are difficult to generalize across the genome. Our objective here was therefore not to establish a catalogue of PTEs, but rather to extend, in a systematic manner, the conceptual and analytical framework developed at the *Hoxa2* locus to explore whether similar regulatory configurations could be detected elsewhere. Consistent with this regulatory logic, and in addition to the *Hoxa2* PTE, we identified further PTE candidates, including regions located near putative long-range enhancers of *Hand2* and *Lhx8* (Supplementary Fig. 17; Supplementary Table 7). Notably, these loci also exhibited reduced interaction frequencies in *Ezh2cKO* CNCCs (Supplementary Fig. 17), supporting the notion that analogous Polycomb-associated regulatory configurations may operate beyond the *Hoxa2* locus.

9. CTCF's Role in PTE Context:

The interplay between CTCF and PTEs remains unclear. Key questions:

- 1) *Are CTCF sites colocalized with PTEs or flanking them?*
- 2) *Does CTCF binding correlate with H3K27me3 loss (e.g., in *Ezh2*^{-/-})?*
- 3) *Are these interactions cohesin-mediated loops or phase-separated compartments?*
- 4) *Additional analysis of *Hoxa2* locus CTCF ChIP data is warranted.*

We thank the reviewer for these important points. We have addressed each of the questions as follows:

1) *Are CTCF sites colocalized with PTEs or flanking them?*

To assess whether CTCF binding sites are associated with PTEs, we performed CTCF ChIP-seq on E11.5 WT prominences (merged FNP, Mx, Md, and PA2). We then conducted a genome-wide enrichment analysis of CTCF ChIP-seq peaks within PTE coordinates. Using permutation testing (1,000 iterations) with E8.5 WT Polycomb domains as the background universe, we observed a significant enrichment of CTCF peak overlap within PTEs compared with a random Polycomb domain set ($p < 0.05$) (Fig. 5n). We further show that PTEs are significantly enriched for orphan CpG islands (Fig. 5m) (see responses to point 8 and Reviewer 2, point 6).

Moreover, through targeted sub-deletions at the *Hoxa2*PTE (now referred to as *Hoxa2*PTE-42kb and *Hoxa2*PTE-36kb; see response to Reviewer 2, point 4) we show that Pc, CTCF, and CGIs cooperate to position the Polycomb-marked *Hoxa2* promoter in spatial proximity to its long-range enhancers, thus enabling efficient enhancer-promoter communication in PA2 CNCCs.

Altogether, our findings suggest that distal Polycomb-marked anchors in pre-migratory progenitors integrate architectural and sequence-based regulatory features that cooperate with Pc to function as essential structural elements enabling the later recruitment of long-range active enhancers required for proper gene regulation in post-migratory CNCCs.

2) *Does CTCF binding correlate with H3K27me3 loss (e.g., in *Ezh2*^{-/-})?*

To assess changes in CTCF binding upon *Ezh2* deletion, we performed CTCF ChIP-seq on E11.5 prominences (merged FNP, Mx, Md, and PA2) in both WT and *Ezh2cKO* conditions. Comparison of CTCF occupancy between WT and *Ezh2cKO* reveals largely conserved global binding, with localized increases preferentially occurring at regions marked by H3K27me3 in WT (Supplementary Fig. 14h). These observations indicate that loss of Polycomb activity does not globally rewire CTCF binding but instead leads to local modulation of CTCF occupancy at Polycomb-associated regions, consistent with a model in which Polycomb constrains the functional use of pre-existing CTCF-associated architectural features rather than preventing CTCF binding per se.

3) *Are these interactions cohesin-mediated loops or phase-separated compartments?*

While promoter-capture Hi-C data do not allow us to directly distinguish between cohesin-mediated loop extrusion and compartment-level interactions, several observations argue against a purely compartment-driven mechanism. First, ectopic interactions are predominantly proximal and intra-TAD, rather than reflecting long-range compartmental shifts. Second, the enrichment of CTCF at PTEs and ectopic DAEs, together with localized changes in CTCF occupancy in *Ezh2cKO* CNCCs, indicates that these interactions occur within a pre-existing architectural framework. We therefore propose that CTCF provides a stable structural context, while Polycomb-dependent mechanisms confer specificity to promoter–DAE engagement; loss of Polycomb activity disrupts this specificity, leading to miswired interactions within an otherwise CTCF-constrained chromatin environment.

4) *Additional analysis of Hoxa2 locus CTCF ChIP data is warranted.*

We have now added the CTCF binding profiles for E10.5 WT Md, E10.5 WT PA2 and E11.5 WT FNP/Mx/Md/PA2 at the *Hoxa2* locus and at its interacting long-range enhancers on the genome browser views shown in the Fig. 6a, 7a,b; Supplementary Fig. 18a, 19a, 20b,d.

10. *HIRE1-Hoxa2 Interactions (Supplementary Figure 16):*

The E11.5 PA2-specific HIRE1-Hoxa2 contacts (black arrows) coincide with weak H3K27me3 at HIRE1. This suggests:

- 1) *These interactions may still be PRC2-dependent (lost in Ezh2-/- due to H3K27me3 depletion)*
- 2) *Alternatively, PRC2 could indirectly stabilize the architecture*

The authors should clarify whether residual PRC2 activity maintains these contacts despite differentiation.

We thank the reviewer for this comment that provides us with the opportunity to discuss this important points. Below, the individual responses to each point.

1) *These interactions may still be PRC2-dependent (lost in Ezh2-/- due to H3K27me3 depletion)*

We detect only very low H3K27me3 levels at HIRE1 in E11.5 WT PA2 CNCCs, which are comparable to input controls and therefore consistent with background signal. To directly address whether residual PRC2 activity at E11.5 contributes to maintaining HIRE1–*Hoxa2* interactions, we performed H3K27me3 ChIP–seq in E11.5 PA2 from *Hoxa2*PTE-36kb^{del/del} embryos and repeated the experiment in WT controls for direct comparison (Supplementary Fig. 19c). In both conditions, the H3K27me3 signal at HIRE1 remained at background levels.

Importantly, in *Hoxa2*PTE-36kb^{del/del} embryos, *Hoxa2*–HIRE1 interactions are strongly reduced, phenocopying the *Ezh2cKO* condition, despite intact PRC2 machinery. This demonstrates that the loss of interactions cannot be attributed to depletion of H3K27me3 at E11.5. Furthermore, in addition to the qPCR analysis performed at E10.5 in PA2 of *Hoxa2*PTE-36kb^{del/del} embryos, we carried out qPCR at earlier developmental stages, specifically in PA2 at E9.5 and in the heads of E8.5 embryos (down to r5). We observed reduced *Hoxa2* transcript levels at E9.5 and already at E8.5 (Fig. 7c), i.e. the stage at which HIRE1 is required for *Hoxa2* induction in the neural tube and CNCC progenitors (Kessler et al., 2023). Together, these findings argue against a model in which PRC2 directly maintains HIRE1–*Hoxa2* contacts at the post-migratory stage. Instead, our data support a developmental model in which Polycomb-dependent chromatin architecture established at the pre-migratory stage (E8.5) primes or constrains the regulatory landscape, thereby enabling subsequent enhancer–promoter engagement upon activation. In this context, PRC2 is required for the early establishment of regulatory topology rather than for the direct maintenance of active enhancer–promoter contacts at E11.5.

2) *Alternatively, PRC2 could indirectly stabilize the architecture*

Our chromatin interaction data indicate that in PA2 CNCCs, where *Hoxa2* is selectively activated, contacts shift away from the *Hoxa2*PTE-36kb region toward HIRE1/HIRE2 and the adjacent H3K27ac-

marked enhancer RE5.5 (Fig. 6a; Supplementary Fig. 19a). Importantly, at this stage, the *Hoxa2*PTE-36kb region no longer directly contacts the *Hoxa2* promoter.

Although the *Hoxa2*PTE-36kb region remains engaged in Polycomb–Polycomb interactions with neighboring repressed *Hoxa* cluster genes (Supplementary Fig. 19b), these Pc–Pc contacts are spatially separated from the active HIRE1–*Hoxa2* loop. Thus, the active enhancer–promoter configuration observed in PA2 CNCCs does not appear to be maintained through direct PRC2-mediated interactions at this stage.

While we cannot formally exclude a minor indirect architectural contribution of surrounding Pc domains, this mechanism is unlikely to represent a general feature of PRC2-dependent PcAct genes. Notably, additional loci that show reduced interactions in *Ezh2cKO* embryos (e.g., *Hand2* and *Lhx8*) do not reside within large Polycomb domains, arguing against a universal indirect stabilization mechanism mediated by persistent Pc–Pc contacts.

Together, these observations support a model in which PRC2 is required for establishing regulatory topology at the pre-migratory stage, but not for directly maintaining active enhancer–promoter contacts after *Hoxa2* activation.

Reviewer #2 (Remarks to the Author):

I read with great interest the manuscript by Joshi et al. entitled “Polycomb Chromatin Topology Enables Long-Range Enhancer Recruitment during Craniofacial Development”. In this manuscript, the authors use a multiomic approach in which they combine Promoter Capture Hi-C (PCHi-C), ChIP-seq, ATAC-seq and RNA-seq to investigate the role of Polycomb in 3D chromatin organization during in vivo craniofacial development. Based on an integrative analysis of the different types of genomic datasets generated, the authors propose that polycomb plays an important regulatory role not only as a classical repressor, but also as chromatin-folding organizer that prepares important developmental genes for future activation. More specifically, using the *Hoxa2* locus as an example, they report the presence of polycomb tethering elements that facilitate long-range interactions between developmental genes and distal enhancers.

Overall, this is a very complete and thorough study that adds a 3D genome organization view to previous work from the same lab in which they characterized the chromatin profiles and enhancers landscapes during in vivo craniofacial development. Although the reported findings are clearly relevant in the context of craniofacial biology, conceptually speaking, the role of Polycomb in 3D chromatin organization in general as well as its potential role as a facilitator of long-range enhancer-promoter communication has been previously reported in other cell types (e.g. ESC, neural progenitors) and model organisms (e.g. *Drosophila*) (Bantignies et al., 2011; Denholtz et al., 2013; Kondo et al., 2014; Schoenfelder et al., 2015; Joshi et al., 2015; Cruz-Molina et al., 2017; Rhodes et al., 2020; Pachano et al., 2021; Denaud et al., 2024). Therefore, the main strength of the manuscript is that it shows that the role of Polycomb in 3D genome organization and as a facilitator of enhancer-promoter contacts, which in vertebrates has been mainly investigated using in vitro cellular systems, seems to be also important in vivo. Moreover, it also adds a slightly different angle to previous studies, as based on the findings for the *Hoxa2* locus, the Polycomb tethering elements they describe might not be necessarily closed to the enhancers they assist but up to 50 Kb away. However, I am afraid experimental work in additional loci and/or alternative experimental approaches based on gain-of-function/knock-in (e.g. insert tethering element nearby distal enhancers) strategies would be necessary to make stronger and more general claims about the relevance and mechanism of action of such PcG-tethering elements.

More specifically, I have the following concerns and suggestions:

1. Previous work (e.g. Rhodes et al., 2020) indicates that cohesin-mediated loop extrusion counteracts the long-range interactions between PcG-bound promoters. Therefore, one the increased short-range interactions that PcAct genes display in the absence of EZH2 could be mediated by cohesin, whose loading and extrusion within the affected TADs could be increased due to the overall increase in H3K27ac levels and chromatin accessibility. The authors could perhaps perform ChIP-seq for some cohesin subunits to address this possibility.

We thank the reviewer for this insightful suggestion. The proposed mechanism assumes that loss of *Ezh2* leads to increased H3K27ac levels and chromatin accessibility, thereby promoting enhanced cohesin loading and loop extrusion.

However, in our system we do not observe a global gain of H3K27ac upon *Ezh2* deletion. Although a modest genome-wide increase in H3K27ac is detected at promoters and DAEs that were enriched with H3K27me3 in WT cells (Supplementary Fig. 12a-c), we do not detect any significant gain of H3K27ac at the DAEs involved in the ectopic interactions, when comparing E11.5 *Ezh2*cKO CNCC subpopulations with WT (Supplementary Fig. 12j-n). Moreover, no specific gain in chromatin accessibility sites is observed in the absence of *Ezh2*, as reported in our previous work (fig. S17C; Minoux et al., 2017).

In addition, to assess potential changes in CTCF binding upon *Ezh2* deletion (see also the response to Reviewer 1 point 9), we performed CTCF ChIP-seq on E11.5 prominences (merged FNP, Mx, Md, and PA2) in both WT and *Ezh2*cKO conditions. Our analysis does not reveal major genome-wide redistribution or strengthening of CTCF binding, arguing against large-scale remodeling of CTCF-anchored loop architecture.

As described in the initial version of our manuscript, chromatin rewiring in *Ezh2*cKO CNCCs—marked by reduced long-range chromatin contacts and increased short-range ectopic interactions mainly within TADs—is consistently associated with stronger TAD insulation and higher TAD strength (Fig. 4i-k; Supplementary Fig. 14e-g). This pattern aligns more with the loss of long-range Polycomb-mediated interactions, which counteract TAD-level constraints, than with enhanced cohesin-driven loop extrusion, which would typically weaken insulation or alter CTCF-bound boundaries.

Furthermore, we show in the revised manuscript that DAEs involved in ectopic short-range interactions are enriched for CpG islands and CTCF binding sites (Fig. 4g,h), suggesting that intrinsic sequence features and pre-existing TAD architecture facilitate these contacts once Polycomb-mediated long-range interactions are reduced.

Together, our data support a model in which the chromatin rewiring observed in *Ezh2*cKO CNCCs primarily reflects the loss of Polycomb-dependent long-range interactions, thereby unmasking underlying TAD-level structural constraints, rather than increased cohesin loading or loop extrusion.

The text has been modified accordingly (pages 14,15, lines 316-336).

2. Similarly, the authors suggest that PcG tethering elements facilitate long-range interactions between PcAct genes and distal enhancers. Do these enhancers have any particular features?. E.g. Are they often located close to CTCF peaks, YY1 peaks or CpG islands that can somehow facilitate the establishment of long-range contacts once these enhancers become active?.

To determine whether CTCF binding sites or CpG islands overlap with E11.5 active Q4 DAEs, we performed a genome-wide enrichment analysis of CTCF ChIP-seq peaks and CpG islands within these

regions. For CTCF-binding sites, we used the CTCF ChIP-seq dataset generated from E11.5 WT facial prominences (merged FNP, Mx, Md, and PA2), as described above. For CpG islands, we used the NMI list derived from ES cells by Long et al. 2013, which is based on biochemical detection and provides a more inclusive set of CpG islands than computational predictions. Using permutation testing (1,000 iterations) in regioneR, we did not observe a significant enrichment of CTCF peaks or CpG islands within active Q4 DAEs compared with expectations based on randomized active DAEs.

Z-score meta-profiles illustrating the positional enrichment of oCGIs and CTCF signals at PTEs within E11.5 Q4 active DAEs, relative to randomized active DAE regions, are provided for the reviewer's reference (Fig.4_Reviewer 2.2) but are not included in the main manuscript.

With respect to YY1, during the revision we had to prioritize ChIP-seq in vivo experiments and attempts for low input material ChIP-seq (CUT&RUN) failed in our developmental context. Therefore, we could not directly assess YY1 enrichment in Q4 DAEs in our system. Publicly available datasets from other cell types are unlikely to reflect binding patterns in our developmental cellular background.

However, our results indicate that E11.5 active Q4 DAEs are not intrinsically enriched for canonical architectural features such as CTCF binding sites or CpG islands that would independently promote long-range contacts. This supports a model in which long-range engagement of these enhancers relies primarily on prior Polycomb-dependent regulatory topology rather than on inherent sequence-based architectural properties.

3. Regarding the Hoxa2 locus, I found surprising that the HIRE1 and HIRE2 enhancer clusters seem to be already active (based on H3K27ac) at E8.5 and, moreover, the levels of H3K27ac seem to be similar in PA2 and Md: how do the authors explain that the enhancers only contact the Hoxa2 promoter in PA2 but not in Md?

This point was discussed in detail in our previous study (Kessler et al., 2023). To avoid redundancy, we have not repeated the full discussion here; instead, we now explicitly refer to that work at relevant locations in the manuscript. Specifically, we added the citation “(discussed in Kessler et al., 2023)” to the sentences describing this aspect. Namely, in the Results section (page 20, lines 438-439): “In E8.5 pre-migratory progenitors, HIRE1 and HIRE2 already carried H3K27ac marks (discussed in²⁴), though they did not establish contacts with the *Hoxa2* promoter (Fig. 6a; Supplementary Fig. 19a).”; and, in the Discussion section (page 27, lines 612-613): “Interestingly, HIRE1 and HIRE2 are already active (H3K27ac-marked) at E8.5 yet they are not in contact with the Pc-marked *Hoxa2* promoter (discussed in²⁴)”.

Excerpt from the Discussion addressing this point from Kessler *et al.*, 2023:

“Intriguingly, HIRE1 and HIRE2 are active, i.e., enriched with H3K27ac, both in *Hoxa2*-expressing PA2 and *Hox*-free Md CNCCs, yet the contacts between HIRE1 and HIRE2 and *Hoxa2* only occur in PA2 CNCCs (Fig. 3a, b). In *Hox*-free CNCCs, the *Hoxa2* promoter may not be available for HIRE1/HIRE2 interaction since, together with the whole *Hoxa* cluster, it is embedded in a large repressive Polycomb domain¹⁶ which may segregate in a repressive nuclear compartment distinct from the active HIRE1/HIRE2. Indeed, Polycomb binding at *Hoxa* promoters in developing limbs can prevent their interaction with active enhancers⁹⁷. In PA2 CNCCs, local patterning signals may activate proximal enhancers so that the *Hoxa2* locus is “singled out” from the Polycomb repressed *Hoxa* cluster and transcriptionally induced, followed by removal of H3K27me3 and switched to H3K27ac deposition¹⁶; this might, in turn, allow rapid interaction with the inter-TAD SEs boosting *Hoxa2* expression to full transcriptional output. Similarly, *Hoxa3* might become collinearly connected to HIRE1/HIRE2 in PA3 CNCCs, where *Hoxa3* is transcriptionally induced and *Hoxa2* is expressed as well.

Moreover, tissue-specific 3D chromatin conformation can also contribute to enhancer activity and specificity⁶. For instance, the *Pen* enhancer shows activity in both developing forelimbs and hindlimbs,

but it only controls *Pitx1* transcription in hindlimbs. This restricted enhancer activity is associated with a 3D chromatin configuration allowing *Pen* and *Pitx1* to interact only in hindlimbs, whereas enhancer and promoter are maintained physically separated in forelimbs⁶. Structural chromatin variants can however convert the inactive into an active 3D conformation, thereby inducing *Pitx1* misexpression in forelimbs⁶. Moreover, forced chromatin looping of strong enhancers to developmentally silenced promoters can be sufficient to stimulate transcription^{98,99}. Thus, HIRE1/HIRE2-driven transcriptional regulation of *Hoxa2* may be allowed by a PA2-specific 3D chromatin configuration.”

4. Upon close inspection, and as the authors briefly mention, there is a putative DAE next to the Pc-Tethering element that displays high H3K4me2 and H3K27ac levels, particularly in PA2. Moreover, this DAE also has a clear CTCF peak. Could this DAE act together with the tethering element in controlling Hoxa2 expression as previously reported for enhancers associated with orphan CpG islands?. As this DAE displays increased H3K27ac specifically in PA2, I think the authors should consider deleting it alone and together with the tethering element.

We thank the reviewer for raising this important point. Indeed, as stated in the original version of our manuscript, a putative H3K27ac-marked enhancer is located immediately 3' to the *Hoxa2*PTE region (now termed *Hoxa2*PTE-36kb). This 5.5 kb element (chr6:51,168,636–51,174,159), which we named Regulatory Element 5.5 (RE5.5), displays features of a poised enhancer in E8.5 progenitors, being marked by H3K27me3 and H3K4me2 (Supplementary Fig. 20d).

In the original manuscript, we focused on the *Hoxa2*PTE-36kb region because it retains the H3K27me3 mark observed at the pre-migratory stage across all four post-migratory CNCC subpopulations. Due to the potential role of RE5.5 as an active enhancer in post-migratory CNCCs and due to its partial overlap with the TAD boundary, its functional analysis was initially not pursued to avoid potentially confounding effects.

Following the reviewer's request to delete RE5.5 alone and in combination with the adjacent *Hoxa2*PTE-36kb region, we first expanded the description of this locus.

The revised text now reads as follows (pages 20-21, lines 443-460):

“...we analyzed the genomic landscape surrounding HIRE1 and HIRE2 at E8.5. This analysis identified an H3K27me3-enriched domain proximal to the 5' end of HIRE1, approximately 1Mb from the *Hoxa2* promoter and spanning its TAD border, as defined by CTCF profiles and TAD insulation scores (Fig. 6a, 7a; Supplementary Fig. 19a, 20d). This region interacts with *Hoxa2* in E8.5 pre-migratory progenitors (Fig. 6a; Supplementary Fig. 19a) and can be divided into two sub-regions of 36 kb and 5.5 kb, respectively, based on their distinct epigenetic dynamics at post-migratory stages. The 36 kb element (chr6:51,173,844-51,209,776), referred to as the *Hoxa2* Polycomb Tethering Element-36kb (*Hoxa2*PTE-36kb), retains the H3K27me3 mark observed at the pre-migratory stage across all four post-migratory CNCC subpopulations, with additional enrichment towards its 5' side (Fig. 6a, 7a, Supplementary Fig. 19a, 20d). This element also contains two CTCF binding sites at its 5' end (hereafter CTCF1 and CTCF2) (Fig. 7a, Supplementary Fig. 20d). The 5.5kb element (chr6:51,168,636-51,174,159), termed Regulatory Element 5.5 (RE5.5), exhibit features of a poised enhancer⁴⁵ in E8.5 progenitors, being marked by H3K27me3 and H3K4me2 (Supplementary Fig. 20d). In post-migratory CNCC subpopulations, including PA2, RE5.5 loses H3K27me3 and gains active H3K4me2 and H3K27ac marks (Fig. 7a, Supplementary Fig. 20d). RE5.5 is also highly enriched in orphan CpG islands (oCGIs,^{51,53}) and spans the TAD boundary separating *Hoxa2* and HIRE1/HIRE2 (Fig. 6a, 7a, Supplementary Fig. 19a, 20d; Methods).”

To address the reviewer's question and assess the contribution of RE5.5 to *Hoxa2* regulation in PA2, we generated a targeted deletion of this element.

Moreover, to respond to Reviewer 1, point 9, regarding the role of CTCF in the PTE context, we produced two additional sub-deletions: (i) a deletion encompassing the CTCF1 and CTCF2 sites within the *Hoxa2*PTE-36kb region, and (ii) a deletion spanning the CTCF3 and CTCF4 sites. Notably, the deletions of CTCF3+4 and RE5.5 partially overlapped, generating a shared deletion that included CTCF3.

As mentioned in the revised Results section (pages 22, 23, lines 503-510):

“None of the deletions significantly reduced *Hoxa2* expression in PA2 at E10.5, as assessed by qPCR, nor caused morphological defects in E18.5 PA2-derived structures of *CTCF1+2*^{del/del}, *RE5.5*^{del/del} and *CTCF3+4*^{del/del} homozygous mutants (Supplementary Fig. 21a-c). However, heterozygous deletions of *CTCF1+2*, *RE5.5*, or *CTCF3+4* on a *Hoxa2* haploinsufficient background generated mild skeletal abnormalities at E18.5, mainly affecting the styloid process (Supplementary Fig. 21d-o). These defects were much less severe than those in *Hoxa2*PTE-36kb^{del/wt};*Hoxa2*^{EGFP/wt} trans-heterozygous mutants, indicating that each element individually contributes only modestly to *Hoxa2* regulation.”

At the reviewer’s request, to determine whether *Hoxa2*PTE-36kb and RE5.5 act together in regulating *Hoxa2*, we deleted the 42 kb region (hereafter *Hoxa2*PTE-42kb) encompassing both elements and the adjacent CTCF-binding site (CTCF4).

We found that:

- this deletion strongly weakened *Hoxa2* interactions with HIRE1 and HIRE2 in E11.5 PA2, while increasing shorter-range contacts (Fig. 7b), resembling the effects of the *Hoxa2*PTE-36kb deletion (Fig. 7b).
- at the transcriptional level, *Hoxa2* expression decreased by about 70% in E10.5 *Hoxa2*PTE-42kb^{del/del} PA2 compared with WT (Fig. 7d).
- at E18.5, homozygous *Hoxa2*PTE-42kb^{del/del} and trans-heterozygous *Hoxa2*PTE-42kb^{del/wt};*Hoxa2*^{EGFP/wt} mutants displayed severe malformations of all PA2-derived skeletal structures (Fig. 7e-n). In the trans-heterozygous mutants, several structures were partially transformed into Md-like elements, such as duplicated incus and malleus, and a smaller, dysmorphic, pinna phenocopying aspects of the *Hoxa2*^{-/-} (Rijli et al., 1993) and *HIRE1*^{del/del} (Kessler et al., 2023) phenotypes (Fig. 7m-r).
- as observed with the *Hoxa2*PTE-36kb deletion, *Hox*-negative CNCCs showed no significant transcriptional or morphological changes in E18.5 *Hoxa2*PTE-42kb^{del/del} nor *Hoxa2*PTE-42kb^{del/wt};*Hoxa2*^{EGFP/wt} mutants (Fig. 7d-f,h,i,k,m).

This led us to make the following conclusion in the revised text (page 24, lines 527-530):

“Altogether, these findings reveal that the *Hoxa2*PTE-42kb region integrates multiple regulatory features and suggest that Pc, CTCF, and oCGIs cooperate to position the Polycomb-marked *Hoxa2* promoter near its long-range enhancers, enabling efficient enhancer-promoter communication in PA2 CNCCs.”

5. The authors should perform H3K27ac ChIP-seq experiments in PA2 upon deletion of the Pc-tethering element. Is the activation of the HIRE1 and HIRE2 enhancer clusters affected or is it just their communication with the *Hoxa2* promoter disrupted? Similarly, are H3K27ac levels affected for the DAE located next to the deleted tethering element?

We thank the reviewer for this comment. This control was indeed important. To address the reviewer’s question, we performed H3K27ac ChIP-seq on PA2 dissected from E11.5 WT and *Hoxa2*PTE-36kb^{del/del} mutant embryos. As shown in Supplementary Fig. 19c, H3K27ac levels at HIRE1 and RE5.5 are comparable between *Hoxa2*PTE-36kb^{del/del} mutant and WT embryos.

The manuscript has been revised accordingly and now states (page 21, lines 475-478):

“We then deleted the *Hoxa2*PTE-36kb element using CRISPR-Cas9 to assess its function. In E11.5 PA2, this deletion markedly reduced interactions between *Hoxa2* and its enhancers HIRE1, HIRE2, and RE5.5 (Fig. 7b), without affecting H3K27ac levels at these elements (Supplementary Fig. 19c).”

These results indicate that the reduced interactions between HIRE1/RE5.5 and the *Hoxa2* promoter in PA2 of *Hoxa2*PTE-36kb mutants cannot be attributed to a decrease or loss of enhancer activation, as measured by H3K27ac.

6. Do the *Hox2* tethering element as well as those similar polycomb-marked anchors often overlap with orphan CpG islands? To test this, the authors should use broad definitions of CpG islands based on biochemical rather than purely computational properties (e.g. NMI as defined by Long et al. 2013) as well as evaluate the CG and CpG content of those tethering elements. This would reveal to what extent the proposed tethering elements are genetically and functionally distinct from orphan CpG islands that were previously proposed to act as tethering elements facilitating enhancer-promoter communication (Pachano et al., 2021), as also described in *Drosophila* (Batut et al., 2022).

We thank the reviewer for this important suggestion. To address this point, we performed a genome-wide identification of putative PTEs using the same analytical framework applied to the *Hoxa2* locus (see also our response to Reviewer 1, point 8). A full description of the computational workflow has been added to the Methods section. The genomic coordinates of all identified putative PTEs are provided in Supplementary Table 7.

To assess whether PTEs are enriched for orphan CpG islands (oCGIs), we carried out a genome-wide enrichment analysis. As requested by the reviewer, we used the NMI CGI list from ES cells reported by Long et al. (2013), which is based on biochemical detection and provides a more inclusive set of CGIs than computational predictions. We filtered this list to exclude CGIs overlapping annotated promoters, thereby retaining only oCGIs. The analysis was repeated using a computationally derived oCGI annotation, yielding similar enrichment profiles and confirming the robustness of the results. Permutation testing (1,000 iterations) implemented in *regioneR*, using all E8.5 WT Polycomb domains as the background, revealed a significant enrichment of oCGI overlap within PTEs ($p < 0.05$) (Fig. 5m). In the response to Reviewer 1, point 9, we further show that PTEs are significantly enriched for CTCF binding sites (Fig. 5n).

The manuscript has been revised accordingly and now states (page 24-25, lines 546-552):

“As for the *Hoxa2*PTE-42kb (see above), we observed that PTEs are significantly enriched for CTCF binding sites and oCpG islands^{51,53} (Methods; Fig. 5m,n). Altogether, these findings suggest that distal Polycomb-marked anchors in pre-migratory progenitors integrate architectural and sequence-based regulatory features that cooperate with Pc to function as essential structural elements enabling the later recruitment of long-range active enhancers required for proper gene regulation in post-migratory CNCCs (Fig. 7s).”

The discussion was also revised as follows (Page 27, lines 597-600):

“Furthermore, we extend prior ESC studies showing PRC1/PRC2-mediated pre-formed contacts between Pc-promoters and poised enhancers⁴⁵⁻⁴⁷ and demonstrate that Pc-marked P-DAE contacts can also facilitate recruitment of long-distance active enhancers that were not previously in contact with their target promoters.”

Reviewer #3 (Remarks to the Author):

Joshi et al. focused on the dynamics of 3D chromatin architecture in the regulation of gene expression in cranial neural crest cells (CNCCs). They investigated how Polycomb proteins repress the expression of specific genes in undifferentiated CNCCs, and how this repression is lifted as CNCCs migrate and differentiate, allowing the formation of enhancer-promoter interactions that activate gene expression. Furthermore, the authors described in detail how the loss of the *Ezh2* gene disrupts both these repressive networks and activating interactions, ultimately impairing proper gene expression. In this study, the authors employed various genomic datasets including RNA-seq, ChIP-seq, and capture

Hi-C (cpHi-C). By classifying different types of chromatin interactions based on epigenetic modification states, they meticulously analyzed the transitions during differentiation and in the Ezh2 knockout context. In the first part of the paper, they present a classification of promoter-DAE interactions and general trends resulting from Ezh2 loss. In the latter part, they propose a model in which PcG-dependent long-range loops regulate the long-distance enhancer-promoter interactions and gene activation of Hoxa2. This connects the generalized phenomena observed in the first part to concrete, gene-specific outcomes and mechanisms. In particular, the latter part explains how the global changes in chromatin contacts seen in Figure 3a lead to detrimental effects at specific gene loci.

The finding that the Ezh2 knockout leads to inappropriate activation (de-repression) of genes that should normally remain repressed highlights an important function of Polycomb proteins: beyond merely acting as transcriptional repressors, they also help organize chromatin architecture to physically block unwanted ectopic enhancer interactions. This underscores a crucial role for Polycomb in ensuring gene expression specificity and sheds light on a new functional aspect of Polycomb biology.

In summary, this study is highly novel and certainly of interest to the fields of Polycomb and chromatin biology. While the manuscript is well-structured and generally easy to follow, there are some points where the interpretation of data remains unclear, making the manuscript as it currently stands difficult to accept. The addition of the following data and experiments would likely make the manuscript more acceptable.

The authors have performed a comprehensive and multifaceted analysis of developmental processes in mouse CNCCs, and the biological phenomena they target are highly novel; overall, the study is fundamentally acceptable. However, there are some concerns regarding transparency. Specifically, much of the data are presented in a highly quantitative manner, with relatively few figures such as heatmaps or track views that show data at individual loci or genomic regions. This leaves the reader wishing to see more of the actual raw data.

In addition, although the study features a wealth of informative, detailed analyses, the volume and presentation of the data are rather busy and difficult to follow. In particular, the supplementary figures include excessive data that are not directly central to the main narrative, which may confuse readers. From the perspective of novelty and data robustness, I believe this manuscript would even be acceptable in a higher-tier journal such as Nature Communications. However, I suspect that prior reviewers may have issued negative evaluations largely because of how the data are presented and expressed, rather than because of any substantive flaw.

The revisions we have proposed can be broadly summarized in three areas: consistency of data across different cell subtypes; clarifying the timing of chromatin structural changes following Ezh2 knockout; and further characterization of loop anchor features. We consider these to be reasonable requests that should not require excessive additional effort from the authors. If the authors can adequately address at least the major points raised, I believe the manuscript would be acceptable for this journal, and we submit these comments as our review response.

Major comments

1. The authors present results from four distinct CNCC subtypes spanning E8.5 to E11.5. However, the datasets shown in the main figures do not appear to be unified across the same cell type, making it difficult to believe that these phenomena are truly generalizable. For example, Figure 2c presents data only from Md, Figure 3 only from PA2, and Figure 4 only from FNP. Are these findings similarly observed across all cell types at E11.5? Would it be possible to consolidate the main figures to show data from a single cell type, and summarize the data from other cell types separately in supplementary figures?

We thank the reviewer for this important comment. Our intention in the main figures was to present representative datasets from different facial prominences, rather than focusing on a single CNCC subtype, in order to demonstrate that the observed phenomena are not restricted to one specific population.

Importantly, the same trends are observed across all CNCC subpopulations, and the corresponding datasets were already included in the Supplementary Figures and remain unchanged.

Specifically:

- former Fig. 2c (now Fig. 3c) shows E11.5 Md; the equivalent data for the other E11.5 CNCC subpopulations, as well as for the E10.5 populations (FNP, Mx, Md, and PA2), are provided in Supplementary Fig. 8i (unchanged).
- former Fig. 3c,d (now Fig. 4c,d) present PA2, while the other prominences were shown in former Supplementary Fig. 11d–i (now Supplementary Fig. 12d–i);
- former Fig. 4a–d (now Fig. 5b–f) present FNP, with the remaining prominences shown in former Supplementary Fig. 14a–i (now Supplementary Fig. 15a–i).

Thus, the findings are consistent across all examined CNCC subtypes. In the revised manuscript, we have ensured that all relevant supplementary panels are explicitly cited in the main text to guide the reader to the corresponding datasets.

2. To reveal the dual regulatory mechanisms of Polycomb, the authors use Ezh2 knockout and explore how the resulting changes in 3D chromatin architecture affect gene expression at later differentiation stages (E10.5 and E11.5 post-migratory CNCCs). However, once PRC2 is knocked out, it would not be surprising for transcriptional changes in various genes to occur immediately at that time point. This raises concern over whether the observed chromatin architecture changes and phenotypes are truly due to PcG-dependent chromatin organization. To address this, could the authors provide data showing the expression of PcG subunit proteins and H3K27me3 enrichment in each cell type at E8.5, E10.5, and E11.5 after Ezh2 knockout? Clarifying at which stage PcG is completely depleted, and checking for gene expression changes at E8.5 (the time point knockout is initiated), would strengthen the interpretation.

We thank the reviewer for raising this important point.

To address this question, we performed *in-situ* hybridization and immunostainings on sections on control (*Wnt1::Cre; RosaRFP*) and *Ezh2cKO* (*Wnt1::Cre; ROSA^{RFP}; Ezh2^{lox/lox}*) embryos at E8.5, E9.5, E10.5, and E11.5. These experiments include (i) *in situ* hybridization using an *Ezh2* antisense probe; (ii) combined *Ezh2* *in situ* hybridization with anti-RFP immunostaining; (iii) anti-RFP immunostaining with DAPI counterstaining; (iv) anti-H3K27me3 immunostaining; and (v) anti-EED immunostaining.

Schematics of embryos at each stage, indicating the planes of section corresponding to the images, are also provided. These data are presented in Supplementary Fig. 11a–l.

In addition, we performed H3K27me3 ChIP–seq on merged FNP/Mx/Md CNCCs from E10.5 WT and *Ezh2cKO* embryos. The results are shown in Supplementary Fig. 11m–n and include (i) a scatter plot showing the correlation of H3K27me3 ChIP enrichment ($\log_2[\text{CPM} + 0.1]$) at promoters in WT (x-axis) versus *Ezh2cKO* (y-axis) merged CNCCs, and (ii) a genome browser view of chromosome 6 (50.5–52.5 Mb) displaying H3K27me3 profiles in WT and *Ezh2cKO* samples.

As indicated in the main text (pages 13,14, lines 289-302), The *Wnt1::Cre* transgene drives Cre recombinase expression in pre-migratory CNCC progenitors⁵², leading to the loss of *Ezh2* expression in CNCCs as early as E8.5 (approximately 10-12 somites) (Supplementary Fig. 11a-c). At this stage, global H3K27me3 levels by immunohistochemistry remained comparable to those in WT embryos, indicating that Pc target genes were not yet affected. Between E9.5 and E11.5, however, H3K27me3 levels progressively decrease in *Ezh2cKO* CNCCs (Supplementary Fig. 11d-l). By E11.5, H3K27me3 is nearly undetectable by immunostaining. To assess the consequences of *Ezh2* deletion on 3D chromatin organization and gene expression, we then focused on E11.5 *Ezh2cKO* embryos. Interestingly, EED protein levels remained unchanged in *Ezh2cKO* mutants, at all analyzed stages (Supplementary Fig. 11a-l).”

Together, these results show that conditional deletion of *Ezh2* in CNCC progenitors leads to a progressive loss of H3K27me3 from E9.5 to E11.5, with the mark being almost completely depleted by E11.5.

3. In Figure 6a, even though *Hoxa2* expression emerges normally in *Ezh2*-deficient cells at E10.5, it decreases at E11.5. Does this suggest that *Ezh2* is necessary to maintain the established contacts between HIRE1/2 and the *Hoxa2* promoter? The manuscript appears to imply that contact formation between HIRE1/2 and the *Hoxa2* promoter requires prior contact with the *Hoxa2* promoter-proximal PTE. Is it possible to generate capture promoter Hi-C data at E10.5? Since *Hoxa2* is already expressed at E10.5, does this mean that the contacts between HIRE1/2 and the *Hoxa2* promoter are formed in *Ezh2*-deficient cells at that stage? Alternatively, another possibility is that at E10.5, *Ezh2* is not yet completely depleted. As noted above, data showing the time course of H3K27me3 ChIP-seq depletion and expression of Polycomb subunit proteins are essential. In addition, could the authors provide a ChIP-seq snapshot of the *Hoxa2* locus at E10.5?

We thank the reviewer for these important and insightful comments, which overlap in part with those raised by Reviewer 1 (see our response to point 10) and that we fully address below:

About the *Hoxa2* expression dynamics at E10.5 and E11.5, WT embryos exhibit a physiological increase in *Hoxa2* expression between E10.5 and E11.5, as illustrated by RNA measurements at the *Hoxa2* locus (Fig. 6b and Kessler et al., 2023; Fig. 2). This increase reflects expansion of the *Hoxa2*-positive CNCC population and strengthening of enhancer activity during this developmental interval.

In *Ezh2cKO* CNCCs, *Hoxa2* transcript levels are already reduced in PA2 at E10.5 compared with WT (Fig. 6a), demonstrating that a regulatory impairment is already detectable at this stage. However, the reduction becomes more pronounced at E11.5.

Concerning the temporal dynamics of H3K27me3 depletion in *Ezh2cKO* CNCCs, H3K27me3 depletion is progressive. At E8.5, global H3K27me3 levels are comparable between WT and *Ezh2cKO* embryos (Supplementary Fig. 11). We have assessed that a gradual decline in H3K27me3 levels occurs between E9.5 and E11.5 (see response to point 2), and by E11.5, H3K27me3 is nearly undetectable. Thus, at E10.5, residual PRC2-dependent chromatin marking is still present, whereas depletion is more pronounced at E11.5. This temporal progression is critical for interpreting the stage-specific transcriptional phenotype.

Therefore, the more pronounced reduction of *Hoxa2* expression observed at E11.5 in *Ezh2cKO* CNCCs occurs in the context of: i) further progressive depletion of H3K27me3; and, ii) increased transcriptional demand at the locus. Together, these data indicate a progressive impairment of regulatory output rather than an abrupt failure of contact maintenance.

Moreover, concerning enhancer–promoter architecture and timing, our promoter capture Hi-C data show that: i) in E8.5 progenitors and in *Hox*-negative CNCCs, the repressed *Hoxa2* promoter predominantly interacts with the *Hoxa2*PTE-36kb region (Supplementary Fig. 19a); and, ii) in PA2 CNCCs, where *Hoxa2* is selectively activated, contacts shift away from the *Hoxa2*PTE-36kb toward HIRE1/HIRE2 and the H3K27ac-marked RE5.5 enhancer (Fig. 6a; Supplementary Fig. 19a). Importantly, in PA2 CNCCs the *Hoxa2*PTE-36kb region no longer directly contacts the *Hoxa2* promoter, indicating that active enhancer–promoter engagement at E11.5 is not mediated by persistent direct PTE–promoter looping.

A ChIP-seq snapshot of the *Hoxa2* locus, as well as promoter capture Hi-C data for the *Hoxa2* locus at E10.5 in WT PA2 CNCCs are available in Kessler et al. (2023) (Fig. 3). Performing promoter capture Hi-C on E10.5 *Ezh2cKO* CNCCs was technically unfeasible because this experiment requires a large number of cells, which cannot be obtained in sufficient quantities due to the Mendelian frequency of the relevant mutants.

However, given: i) the progressive decline of H3K27me3 between E9.5 and E11.5 in *Ezh2cKO* CNCCs; ii) the partial reduction of *Hoxa2* expression already present at E10.5 in *Ezh2cKO* CNCCs; and, iii) the architectural shift from PTE-dependent contacts to HIRE1/HIRE2-driven contacts upon activation in WT

CNCCs, the data are most consistent with a model in which Polycomb-dependent topology established at earlier stages facilitates robust enhancer–promoter engagement. As H3K27me3 levels decline progressively, this regulatory architecture becomes increasingly destabilized, leading to a gradual reduction in transcriptional output that is more evident at E11.5. Thus, the temporal expression pattern does not indicate a strict requirement for PRC2 in maintaining already established active contacts, but rather supports a model of progressive destabilization of a developmentally established regulatory configuration.

4. In Figure 5, Ezh2 knockout leads to a failure to establish proper enhancer contacts and instead creates interactions with short-range contacts. Regarding these new loop anchors, do they tend to form within specific types of chromatin domains? Are there particular trends associated with epigenetic modifications, CGI content, or binding of CTCF and transcription factors? I am very interested in whether a hierarchy emerges in the placement of these second- and third-choice loop anchors once PcG interactions are lost.

We thank the reviewer for this insightful comment. To determine whether CTCF binding sites or CpG islands overlap with the proximal ectopic interactions that arise in *Ezh2cKO* CNCCs, we performed a genome-wide enrichment analysis of CTCF ChIP–seq peaks and CpG islands within these regions. For CTCF-binding sites, we used CTCF ChIP–seq dataset generated from E11.5 WT facial prominences (merged FNP, Mx, Md, and PA2). For CpG islands, we used the NMI list derived from ES cells, from Long et al., 2013, which is based on biochemical detection and provides a more inclusive set of CpG islands than computational predictions.

In short, we found that when Polycomb-dependent long-range contacts are lost, interactions preferentially collapse onto pre-existing architectural features such as CTCF-bound sites and CGI-rich regions within the same TAD.

Namely, we found that DAEs participating in shorter-range ectopic interactions are enriched for CpG islands (CGIs; NMI (Long et al., 2013) and CTCF binding sites compared with DAEs not involved in such interactions (Fig. 4g,h), indicating that intrinsic sequence features and pre-existing TAD architecture contribute to the formation of these ectopic loops.

Consistently, as noted in the initial version of our manuscript, chromatin rewiring in *Ezh2cKO* CNCCs – characterized by a reduction in long-range chromatin contacts and a concomitant increase in shorter-range ectopic interaction, predominantly within TADs – is accompanied by increased TAD strength and more prominent TAD boundary loops (Fig. 4i-k; Supplementary Fig. 14e-g).

The text has been modified accordingly (pages 14,15, lines 316-330).

Minor Comments

1. In Figure 4a, is it possible to show representative loci in Q1–Q4, especially in Q2 and Q3? While the data convincingly suggest general trends, providing concrete examples of specific loci would help the reader better visualize the phenomenon.

We have clarified in the text that *Hoxa2* in PA2 *Ezh2cKO* CNCCs exemplifies the regulatory dynamics of Q4/Q3 shared promoters, whereas in *Hox-negative Ezh2cKO* CNCCs it displays the regulatory dynamics characteristic of Q1/Q2 shared promoters.

The revised text now reads :

“In E11.5 *Ezh2cKO* PA2 CNCCs, *Hoxa2* expression was decreased by 64% relative to control cells (Fig. 6b). This reduction was accompanied by two major changes in chromatin architecture. First, interactions between the *Hoxa2* promoter and HIRE1/HIRE2 were nearly completely lost (Fig. 6a; Supplementary Fig. 19a,b). Second, novel shorter-range ectopic contacts formed primarily within the *Hoxa2* TAD (pink

arrows, Fig. 6a). This rewiring exemplifies the regulatory dynamics described for Q4/Q3 shared promoters (Fig. 5e).” (page 19, lines 416-421)

“In contrast, in *Hox*-negative CNCCs, *Ezh2cKO* resulted in a modest up-regulation of *Hoxa2* (Supplementary Fig. 20a) along with interaction patterns typical of Q1/Q2 shared promoters, namely reduced contacts between *Hoxa2* and Pc-marked regions, including the *Hoxa* cluster (black arrows, Supplementary Fig. 20b,c), together with a concomitant increase in short-range interactions near the *Hoxa2* promoter (Supplementary Fig. 20b, pink arrows).” (page 19, lines 423-428)

In addition, to address the reviewer's request, we now include two representative PcAct genes essential for craniofacial development, *Hand2* and *Lhx8*. In Md CNCCs, where *Hand2* and *Lhx8* are highly expressed in WT embryos, both genes show reduced expression and impaired long-range promoter–enhancer interactions in *Ezh2cKO* CNCCs, accompanied by increased short-range contacts (Supplementary Fig. 17), consistent with the behaviour of Q4/Q3 shared promoters.

Finally, the supplementary table 6 now lists the genomic coordinates (mm10) of all E11.5 active Q4 DAEs in each CNCC subpopulation, as well the corresponding target promoters at E11.5, with their PcAct or PcActTF classification. The distance to the target promoter and the interaction type are also mentioned.

2. It is difficult to understand what Figure 2b is meant to show, and the authors' intended message is not clearly conveyed.

We thank the reviewer for this comment and apologize for the lack of clarity in the original presentation. The goal of Figure 2b (now Figure 3b) is to demonstrate that promoter–enhancer interaction dynamics during the E8.5 to E11.5 transition are tightly coupled to epigenetic state changes.

We separated PcAct promoters into two groups at E11.5: those that remain Polycomb-repressed (left panel) and those that become transcriptionally active (right panel). For each group, we quantified whether promoter–DAE interactions were inherited, lost, or newly acquired, and stratified these interactions according to the E11.5 epigenetic state of the DAEs (active or Pc-marked).

The key finding is that interaction rewiring follows epigenetic identity. Namely: i) promoters that remain Polycomb-repressed preferentially retain interactions with Pc-marked DAEs and lose interactions with DAEs that are active at E11.5; ii) in contrast, promoters that become active preferentially lose interactions with Pc-marked DAEs and acquire interactions with DAEs that are active at E11.5.

Thus, the heatmaps illustrate that promoter–enhancer contacts are reorganized in a coordinated manner according to epigenetic state transitions, rather than changing randomly during CNCC differentiation. In other words, promoters tend to interact with enhancers that share their regulatory state.

3. In Figure 5, contacts between the Hoxa2 promoter and Hoxa2 PTE remain even in Ezh2-KO cells (E11.5 PA2 Ezh2-KO). What do the authors think these contacts depend on? Could they be maintained by residual PRC1?

The chromatin contact that persists in E11.5 *Ezh2cKO* PA2 CNCCs is consistent with an interaction between the active *Hoxa2* promoter, and the active regulatory element located immediately adjacent to *Hoxa2*PTE-36kb (now designated RE5.5), potentially facilitated by CTCF-binding sites.

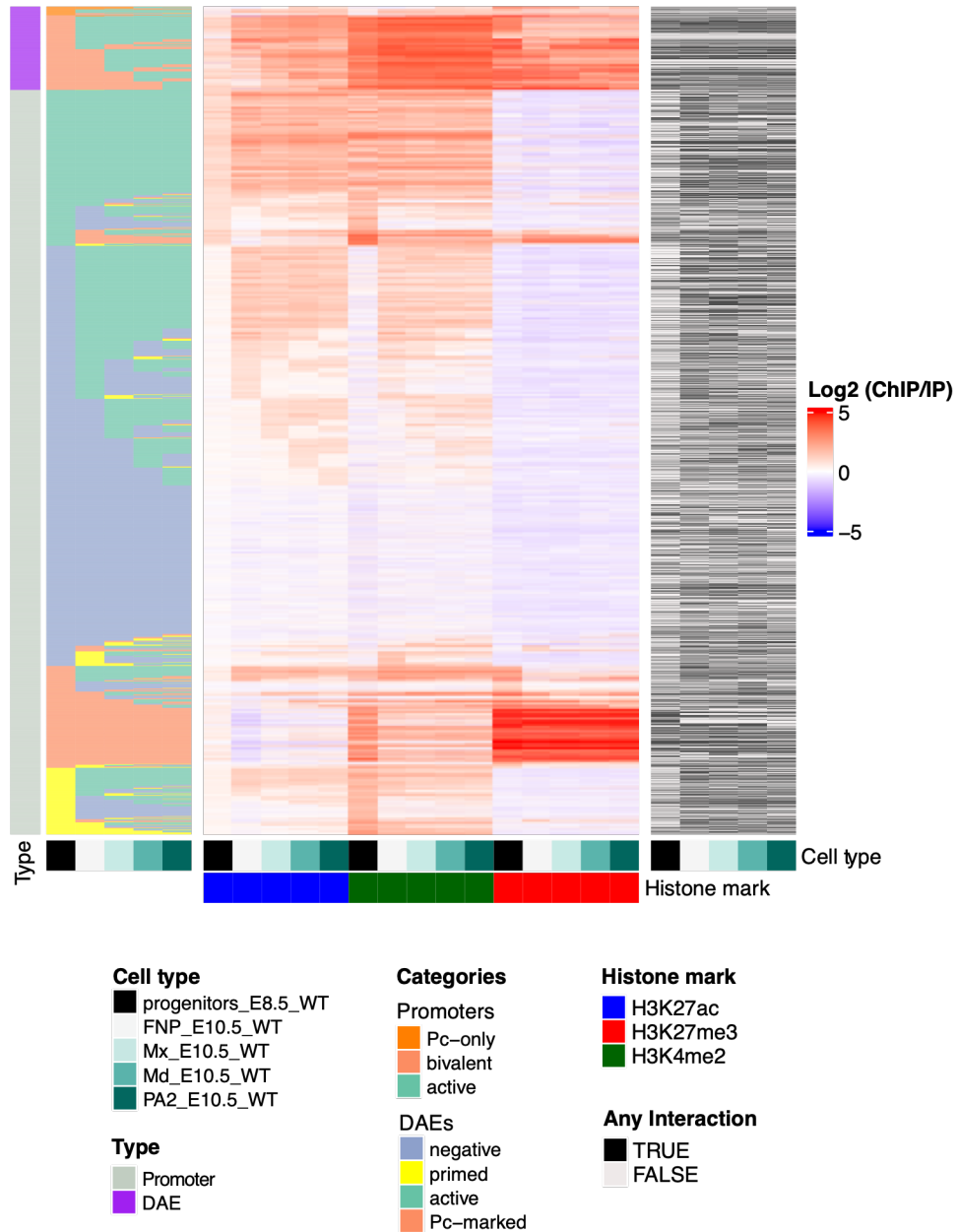
4. Figures 6a and 6b might fit better within Figure 5, which would help readers understand the relationship between gene expression and chromatin interactions as an integrated figure.

We agree with the reviewer. Figs. 6 and 7 (formerly Figs. 5 and 6) have been completely restructured. Figure 6 now presents the *Ezh2cKO* condition, whereas Figure 7 focuses on the *Hoxa2*PTE deletions (*Hoxa2*-36 kb and *Hoxa2*-42 kb).

5. *Is it possible to provide a simple schematic diagram showing the timing of knockout induction and sample collection in a supplementary figure would make it easier for readers to grasp the actual experimental design.*

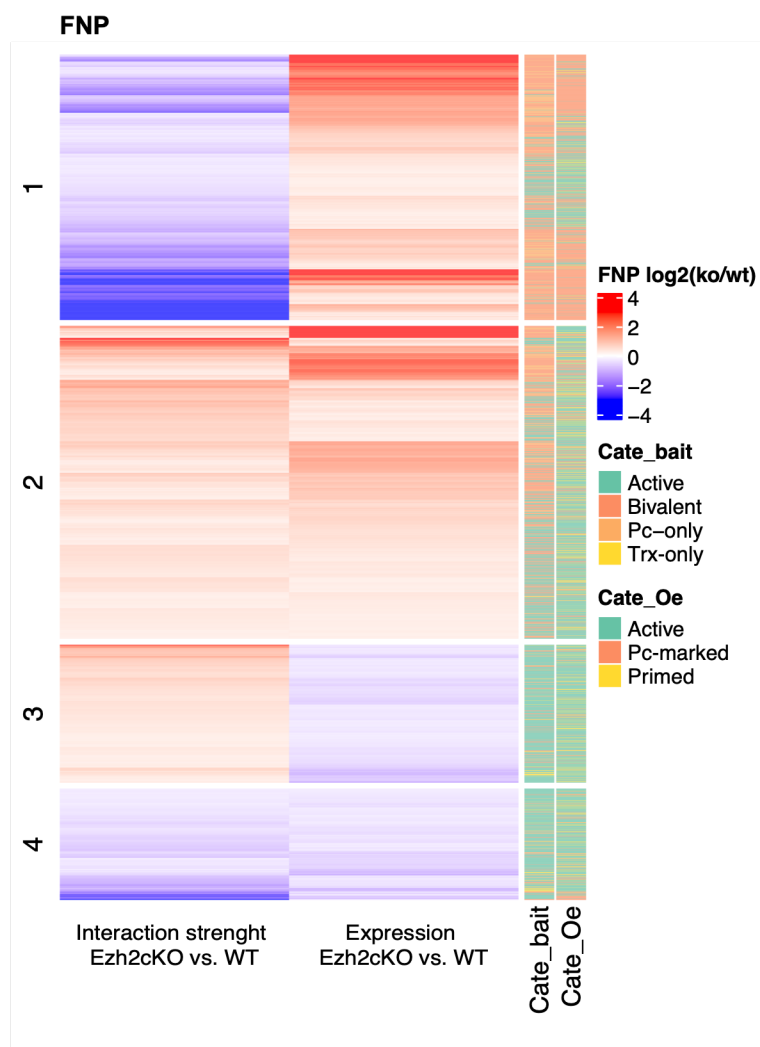
Fig. 1a now presents the experimental workflow, including the timing of sample collection. The time-course analysis of H3K27me3 loss in *Ezh2cKO* CNCCs is provided in Supplementary Fig. 11.

Figure 1_Reviewer 1.1



Heat map generated for PcAct genes and their interacting DAEs across all analyzed cell populations, showing epigenetic categories, histone mark enrichment profiles, and chromatin interaction status. The map displays histone mark enrichment at PcAct gene promoters and their associated DAEs, together with interaction status (TRUE/FALSE) indicating whether promoters or DAEs participate in detectable chromatin interactions. Data are shown for E8.5 pre-migratory progenitors and E10.5 post-migratory cranial neural crest cell prominences (FNP, Mx, Md, and PA2). TRUE interactions are defined by a CHiCAGO score ≥ 5 .

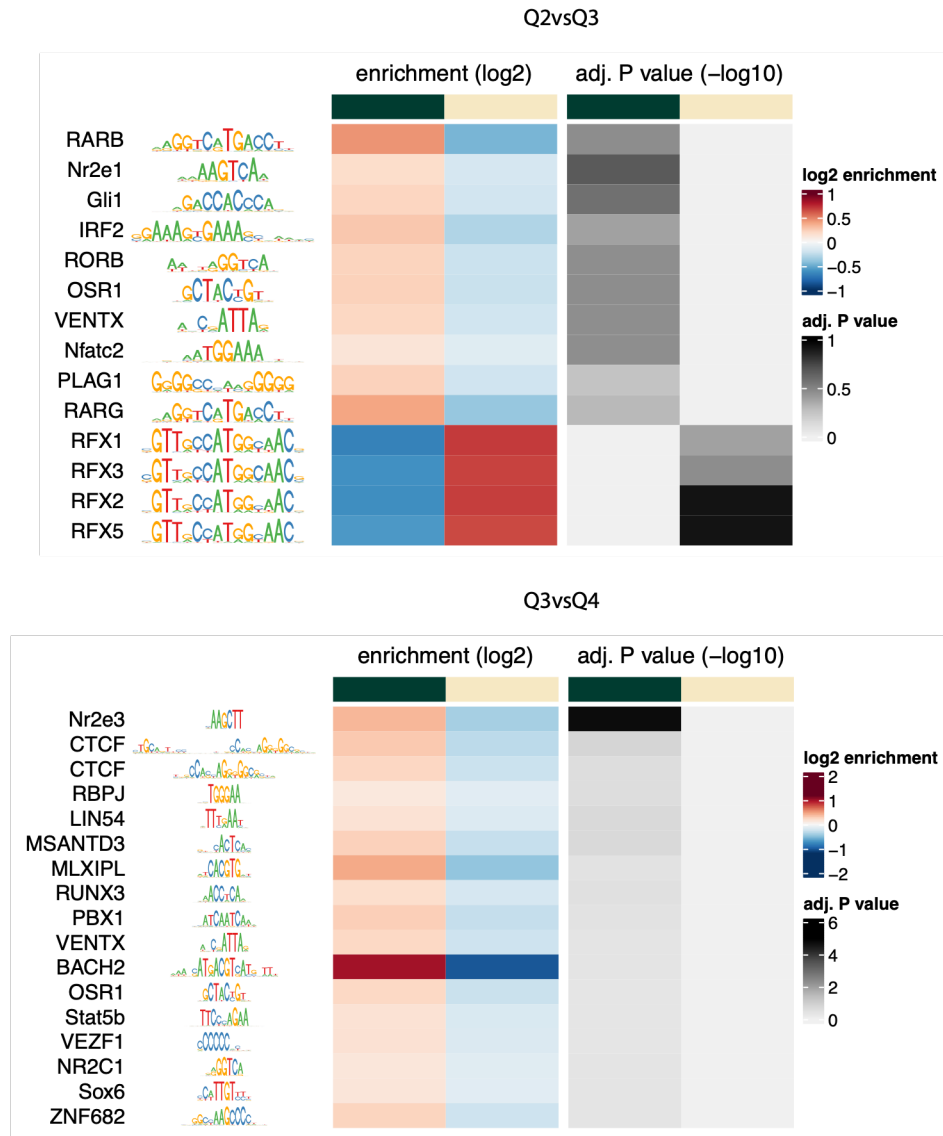
Figure 2_Reviewer 1.3



Heat map summarizing the relationship between changes in chromatin interaction strength and gene expression between *Ezh2cKO* and WT in E11.5 FNP CNCCs.

Promoter–DAE pairs were clustered into four groups corresponding to the four quadrants (Q1–Q4) defined in the scatter plot (Fig. 5b), reflecting distinct combinations of interaction and expression changes. The heat map displays log2 fold changes in interaction strength and gene expression (*Ezh2cKO* vs. WT), together with the epigenetic state of promoters and associated DAEs in E11.5 WT FNP. Epigenetic categories are indicated for both promoter and DAE elements.

Figure 3_Reviewer 1.7

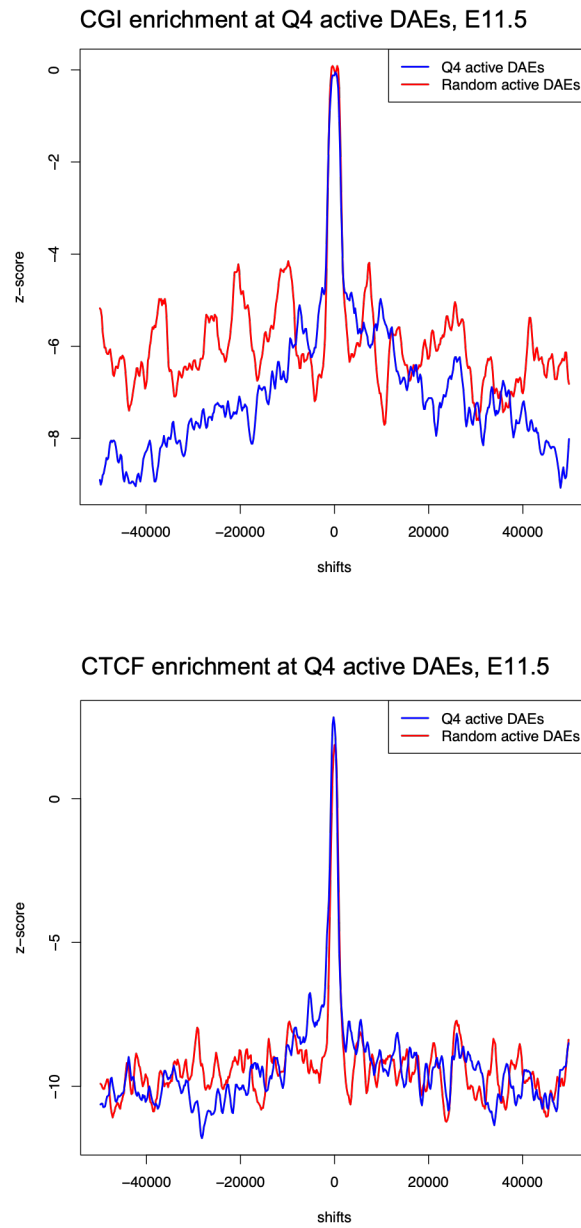


Motif enrichment analysis of Q2 vs. Q3 and Q3 vs. Q4 DEAs.

Note that an adjusted p -value threshold of $-\log_{10}(p_{adj}) > 1.3$ was considered significant.

In the Q3 vs. Q4 comparison, the apparent statistical significance of *Nr2e3* in the heatmap is attributable to its correspondence to a HindIII restriction fragment, the enzyme used in promoter Capture Hi-C.

Figure 4_Reviewer 2.2



Z-score meta-profiles of CpG island (CGI) enrichment and CTCF signals at Q4 active DAEs, compared to a random set of active DAEs matching H3K27ac levels of Q4 active DAEs.

POINT-BY-POINT RESPONSE TO REVIEWERS' COMMENTS NCOMMS-25-41946A

We are grateful to all three reviewers for their very positive feedback on our revised manuscript. While Reviewers 2 and 3 are fully satisfied with our revision, we address below (in blue) the remaining minor comments raised by Reviewer 1.

Reviewer #1 (Remarks to the Author):

1. Quantification of Network Features

We thank the authors for providing Supplementary Tables 4 and 5, which contain the requested quantitative information (number of interacting promoters/DAEs, genomic distances, and ChIP-seq signal intensities) for the PcActTF gene promoters. While these tables are comprehensive, the data would be more accessible to readers if presented in a graphical format.

We thank the reviewer for the positive comment. Supplementary Tables 4 and 5 were added at a previous reviewer's request to provide the quantitative details underlying our network diagrams — including the number of interacting promoters/DAEs, genomic distances, and ChIP-seq signal intensities. This information is therefore already represented visually in Figures 1 and 2 and Supplementary Figures 10 and 11. We agree that these visualizations improve accessibility and complement the quantitative data in the tables.

We thank the authors for their thoughtful explanation and the revised presentation of the network comparisons.

OK. Thank you.

2. Visualization of Differential Interactions

We thank the authors for addressing this point thoroughly.

OK. Thank you.

3. Ternary Plot Limitations

We acknowledge the generation of the additional heatmap (Fig.1_Reviewer 1.1) comparing pre- and post-migratory states. While the heatmap is element-centric and does not directly capture interaction dynamics, it provides complementary information on individual promoter and DAE states. We suggest including this heatmap as a supplementary figure (e.g., Supplementary Fig. X) to make it accessible to readers who may wish to examine element-level epigenetic states across stages. We noticed that the color key for promoters and DAEs in this heatmap appears to be reversed; please ensure this is corrected upon incorporation.

We thank the reviewer for this comment. In response, we have added the heatmap as Supplementary Fig. 8. We also thank the reviewer for identifying the inverted color key for promoters and DAEs, which has been corrected in the revised figure.

4. H3K27me3 Signal in Active Promoters

We thank the authors for their detailed and thoughtful explanation regarding the residual

H3K27me3 signal observed in active promoters. With these explanations, we consider this point fully addressed.

OK. Thank you.

4. Correlation Analysis (Figure 4)

We thank the authors for providing the heatmap (Fig.2_Reviewer 1.3). This visualization is much clearer than the scatterplots alone, and we recommend including it as a supplementary figure. Importantly, the heatmap reveals an intriguing pattern: in Q2 (upregulated genes), the most strongly expressed genes—many of which are bivalent in WT—do not show the strongest gains in promoter–DAE interactions. This suggests that bivalent genes can be robustly activated upon PRC2 loss without extensive enhancer rewiring, possibly reflecting their poised chromatin architecture. We suggest that the authors to briefly discuss its biological implications (e.g., bivalent promoters may be primed for activation without requiring new enhancer contacts).

We thank the reviewer for this comment and have included the heatmap as Supplementary Figure 17. We agree that the robust activation of bivalent genes upon PRC2 loss is an interesting observation, likely reflecting their poised chromatin architecture. This was indeed a central finding of our previous study (Minoux et al., Science 2017), where we discussed its biological implications in detail.

In the present manuscript, we had re-emphasized these concepts both in the Introduction (lines 83–90): “*At the chromatin level, CNCC plasticity and morphogenetic potential are maintained by a H3K27me3/H3K4me2 bivalent Polycomb (Pc) chromatin repressive signature, already present in pre-migratory progenitors. This signature maintains accessible and transcriptionally poised promoters of genes required at (post-)migratory stages for craniofacial morphogenesis^{12,35}. In response to local environmental cues, Pc target genes switch from a poised to an active state in specific CNCC subpopulations, enabling these cells to acquire their morphogenetic identities. In contrast, these genes remain transcriptionally silent in CNCCs lacking the specific inducing signals^{12,35}.*” ; and in the Discussion (lines 593–596): “*This dual role further highlights the functional significance of promoter bivalency, where H3K27me3/H3K4me2 marking not only poises repressed genes for activation but also encodes a specific 3D organization that supports precise spatiotemporal induction.*”

5. Dynamic Epigenetic Trajectories

We thank the authors for generating the Sankey diagram (now Supplementary Fig. 7e), which effectively visualizes the epigenetic and interaction dynamics of DAEs associated with activated PcAct genes from E8.5 to E11.5. This added panel significantly improves the presentation of temporal transitions and addresses our concern.

OK. Thank you.

6. Q3/Q4 Interaction Paradox

We thank the authors for performing the motif enrichment analysis and for clarifying the features of ectopic loops gained in Ezh2cKO CNCCs. While the absence of specific TF motifs that distinguish Q2 vs. Q3 or Q3 vs. Q4 is noted, the finding that DAEs involved in ectopic

interactions are enriched for CpG islands and CTCF binding sites (new Fig. 4g,h) is valuable and provides insight into the structural basis of these loops. This remains an interesting but unresolved observation that would benefit from further discussion and analysis.

We thank the reviewer for this positive comment. We agree that the enrichment of CpG islands and CTCF binding sites at DAEs involved in ectopic interactions in *Ezh2cKO* CNCCs offers important clues about the structural features underlying these loops, even in the absence of clearly discriminating TF motifs between Q2 vs. Q3 or Q3 vs. Q4. A full mechanistic dissection of these observations is beyond the scope of the present study, and we have acknowledged this in the Discussion with the following sentence: "While additional analyses will be required to fully elucidate the underlying mechanisms, these findings..." (lines 648–649).

7. PTE Proximity Hypothesis

We thank the authors for their efforts to extend the PTE analysis genome-wide and for providing Supplementary Table 7 with candidate PTE coordinates. While these tables are comprehensive, the data would be more accessible to readers if presented in a graphical format.

*The identification of 174 putative PTEs using a defined operational framework, along with the examples of *Hand2* and *Lhx8* (Supplementary Fig. 17), suggests that the regulatory logic observed at *Hoxa2* may indeed apply more broadly.*

We thank the reviewer for this positive feedback. Regarding the suggestion to present the data graphically, we believe the tabular format is most appropriate here, as the information — particularly the precise genomic coordinates in Supplementary Table 7 — is too detailed to be meaningfully conveyed in a figure.

8. CTCF's Role in PTE Context

*We thank the authors for the comprehensive response and the new data addressing the role of CTCF in the PTE context. The additional CTCF ChIP–seq experiments in both WT and *Ezh2cKO* conditions, together with the enrichment analyses and the updated genome browser views at the *Hoxa2* locus, substantially strengthen the manuscript.*

OK. Thank you.

9. HIRE1-Hoxa2 Interactions (Supplementary Figure 16)

We thank the authors for this thorough response. The additional experiments, particularly H3K27me3 ChIP–seq in PTE mutants and qPCR at early stages, provide compelling evidence that PRC2 is required for establishing regulatory architecture at the pre-migratory stage rather than maintaining active loops after differentiation. We suggest briefly highlighting this developmental priming model in the Discussion.

We thank the reviewer for this positive feedback. The developmental priming model is presented in Figure 7 and discussed in the manuscript in the context of PRC2's role in establishing regulatory architecture at the pre-migratory stage. As stated in lines 559–562: "*We find that a pre-established, *Ezh2*-dependent, repressive chromatin topology in pre-migratory progenitors enables the later selective recruitment of long-range enhancers to activate subpopulation-specific patterning programs.*".

Reviewer #2 (Remarks to the Author):

I would like to thank the authors for their efforts in addressing my previous concerns, including the generation of new transgenic animals with additional deletions, as well as new ChIP-seq experiments. Overall, I believe the authors have successfully addressed all of my main concerns. Therefore, I congratulate them on their interesting and comprehensive work, which I recommend to be published in Nature Communications.

Reviewer #3 (Remarks to the Author):

I would like to thank the authors for their thorough response and for addressing the points raised in the previous review.

The additional experiments and clarifications provided in the revised manuscript have successfully addressed my concerns. Specifically, the new data regarding the timing and hierarchy of PcG-mediated 3D chromatin architecture in higher-order genome structure formation provides the necessary evidence to support the authors' claims.

I believe the manuscript has been significantly improved and is now suitable for publication.