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Peer Review Information

Journal: Nature Genetics
Manuscript Title: A unique bipartite Polycomb signature regulates stimulus response transcription during development
Corresponding author name(s): Professor Filippo Rijli

Editorial Notes:

Transferred manuscripts	This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Genetics.
Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data. Figures R1-R3 have been removed from the final rebuttal letter.

Reviewer Comments & Decisions:

Decision Letter, initial version:

14th Oct 2020

Dear Dr. Rijli,

Thank you for transferring your manuscript "A unique bipartite Polycomb signature regulates stimulus response transcription during development" to Nature Genetics.

I have now discussed the points of your appeal/rebuttal letter with my colleagues, and we would be willing to send the manuscript back to reviewers #1 and #4 for additional input. We therefore invite you to submit a revised manuscript along the lines that you propose.

When preparing a revision, please ensure that it generally complies with our editorial requirements for format and style; details can be found in the Guide to Authors on our website (http://www.nature.com/ng/).

Please be sure that your manuscript is accompanied by a separate letter detailing the changes you have made and your response to the points raised. At this stage we will need you to upload:
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[REDACTED]

With kind wishes,

Tiago

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Author Rebuttal to Initial comments

POINT-BY-POINT RESPONSE TO REVIEWER #1 and #4 COMMENTS NG-A56037-T Rijli

We thank all the reviewers for their insightful and constructive inputs which helped us to significantly improve our work. We were delighted that reviewer #2 and #3 were satisfied by our previous revision. On the other hand, referees #1 and #4 raised additional concerns, which we have now addressed, despite the very difficult conditions due to the coronavirus pandemics, with additional experiments, data analyses 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 have also accordingly revised the main text and improved readability and discussion. We believe that - and hope the reviewers agree – our manuscript is now much stronger. We apologize for some redundancies between the reviewer-specific responses.

Referee #1 (Remarks to the Author):

In their revised manuscript, Kitazawa et al address many technical and scientific comments raised in the previous round of review.

I reiterate the high-level concept – identification of a special chromatin state for immediate early response genes that maintains transcriptional quiescence while permitting rapid induction – is very attractive. The authors have offered a plethora of experiments that describe such a chromatin state at a small number of genes and they predict from their data that ultimately there are about 100 or 200 such genes in the genome in multiple tissues. This is an important contribution to the field.

I am left in the end to consider whether I am satisfied with the authors statements and data regarding how bipartite chromatin is established, maintained and how the barrier to productive elongation is released. In my opinion, new data and new visuals convince me that the mechanism for (1) barrier to productive elongation and (2) how the barrier is released are not provided in a satisfying manner here. I do not think additional chip/RNA experiments will help provide the answer. The TSA experiment and the brief and frankly uninspired discussion of this result are not at all compelling.

Rev1.1. Frankly, we were surprised in receiving these comments. We estimated that in our previous revision we had thoroughly addressed each of the reviewer's concerns and provided much more data and information than we had been asked. We were confident that this reviewer should have been satisfied with all the additional experimental data we had added to strongly support our conclusions, even data that s/he did not ask for in the first place.

Briefly, in the previous revised version, we had already managed, despite the coronavirus pandemics, to include a substantial amount of additional data and key experiments supporting our initial discovery of a special chromatin state for immediate early genes during development, and demonstrating:

1. How the bipartite signature is established, regulated, and resolved during development and how it regulates the rapidity and magnitude of the transcriptional response of inducible bipartite genes to relevant environmental stimuli (Figs. 3 and 4f, Extended Data Figs. 2c-e, 3g-I, 6, 8f,g, 9e and 10a,b of previous revised manuscript and **new Figs. 3, 5e-g, 7a,g and 8, Extended Data Figs. 3f,g, 6, 8f-h** of current manuscript; also please see **Rev1.3a**)

2. That the barrier to RNAPII elongation on bipartite genes is released upon removal of H3K27me3 (Fig. 4a-e, Extended Data Figs. 7d-g, 8a-e, 9a-c of previous revision and **new Figs. 4a,b, 5b-d, Extended Data Figs. 7a-g, 8a-e** of current revision).

3. That the bipartite signature still allows for very rapid induction of IEGs while the Polycomb marking of gene body, by inhibiting elongation, sets a transcriptional threshold preventing rapid response to non-relevant signals (Fig. 4f and Extended Data Figs. 8f,g and 9e of previous

revision; also please see **new Fig. 5e-g and Extended Data Fig. 8f-h** of current revision). This latter is another very important functional demonstration of the physiological importance of this chromatin signature on IEGs during development.

⇒ Nonetheless, despite the challenging conditions, we have been working tirelessly after we received the reviewer's comments and we can now provide important **new data** which bring significant additional mechanistic insight (please see below, ***Rev1.3b,c,*** **new Figs. 6, 7**). We do hope that this reviewer will consider favorably this substantial additional effort and that we have now provided in a satisfying manner all the answers to the remaining reviewer's concerns.

To my eye, the authors have provided a description of a chromatin state which restrains RNA polymerase and provides potential for rapid engagement.

Rev1.2. We feel that it would be fair to say that what we discovered it is not just merely the description of a chromatin state which restrains RNA polymerase, while still providing potential for rapid engagement. We did demonstrate that H3K27me3 on the gene body, despite IEG active promoters, is functionally required in restraining RNAPII and productive elongation - we demonstrated this by multiple approaches, namely by *Ezh2* and *Eed* KOs in neurons and ES cells, respectively, and by acute removal of H3K27me3 by dCas9-Utx in primary neurons (Fig. 4b-f and Extended Data Figs. 7d-f, 8a-e, 9a-e of previous revision and **new Figs. 5 and 6, Extended Data Figs. 7 and 8** of current manuscript).

They have not provided compelling evidence for how K27me3 presents a barrier to elongation (or results from a completely different regulatory mechanism restraining elongation). Nor do they provide compelling evidence for release. Chip for phosphorylated CREB shows accumulation AFTER pause release. I conclude that these interesting findings and provocative model are descriptive with little mechanistic insight.

Rev1.3. We believed we had already provided in the previous revised version important experiments which contributed to address the questions of *(1) how H3K27me3 presents a barrier to elongation (*please see ***Rev1.1,*** Fig. 4a-e, Extended Data Figs. 7d-g, 8a-e, 9a-c of previous revision and **new Figs. 4, 5b-d, Extended Data Figs. 7a-g, 8a-e** of current revision) and *(2) how the barrier is released* (***Rev1.3a,*** **Figure R1**; *please see below why we think the* phoshoCREB ChIP *data may have been involuntarily misinterpreted by this reviewer*). On the other hand, having discovered a special chromatin state for inducible IEGs during development and having demonstrated that H3K27me3 is required in restraining RNAPII productive elongation, while regulating the rapidity and magnitude of the transcriptional response to relevant signals (Fig. 4f of Extended Data Figs. 8f,g and 9e of previous manuscript and **new Fig. 5e-g, Extended Data Fig. 8f-h** of current revision), we feel that a full in-depth understanding of the biochemical mechanism of how Polycomb achieves that may well be beyond the scope of this study.

Nonetheless, as mentioned above, we can now provide in the current revised version important **new data** which bring significant additional **mechanistic insight**. Please see below, ***Rev1.3a,b*** **and new Fig. 7** regarding *how the barrier is released* and ***Rev1.3c*** **and new Fig. 6** regarding *how H3K27me3 presents a barrier to elongation.*

Below is the detailed list of changes and new evidence:

Rev1.3a. Mechanism of barrier release (1)

First of all, we were puzzled as to why the reviewer stated: '*Chip for phosphorylated CREB shows accumulation AFTER pause release*'. In fact, we showed quite the opposite. We nicely correlated the increase of phosphoCREB (pCREB) at bipartite IEG promoters and enhancers **with the increase of promoter and gene body H3K27ac and RNAPII release** (Fig. 3e of previous and current revised versions, also Extended Data Fig. 2c,e of previous version). But why this reviewer may have got this incorrectly? We then went back to our figures and realized that the reviewer may have made an involuntary mistake of interpretation of these data, due to our perhaps unclear figure layout. In fact, s/he may have wrongfully assigned the *Fos* and *Egr1* **E14.5 pCREB** ChIP profiles (at this stage these IEGs are bipartite, there is **NO** binding of pCREB, and RNAPII is pausing), to the **E18.5** stage when RNAPII is already released, IEGs are induced, and the bipartite state is resolved. (In the previous revised manuscript, we did not carry out ChIP of pCREB at E18.5, but only at E14.5 and P4 - see below). This may have happened in Fig. 3e (*Fos*) and Extended Data Fig. 2c (*Egr1*) of the previous revised version which we attach again here for the reviewer's perusal (**Figure R1**). We circled the specific **E14.5 pCREB** ChIP tracks which may have led the reviewer to unwitting misinterpretation. The E14.5 pCREB tracks were in fact positioned at the 'border' between the E14.5 and the E18.5 stage ChIP and mRNA profiles, thus constituting a potential source of missassignement. We have now revised both figures (**new Fig. 3e and Extended Data Fig. 6b** of the current revised manuscript).

In addition, to further clarify this point, we now carried out an additional pCREB ChIP-seq at E18.5 and added this to the genome browser profiles of *Fos* and *Egr1* (**new Fig. 3e, Extended Data Fig. 6b, d**). It can be nicely appreciated that pCREB starts to accumulate in promoter regions of these IEGs at E18.5 in accordance with mRNA expression.

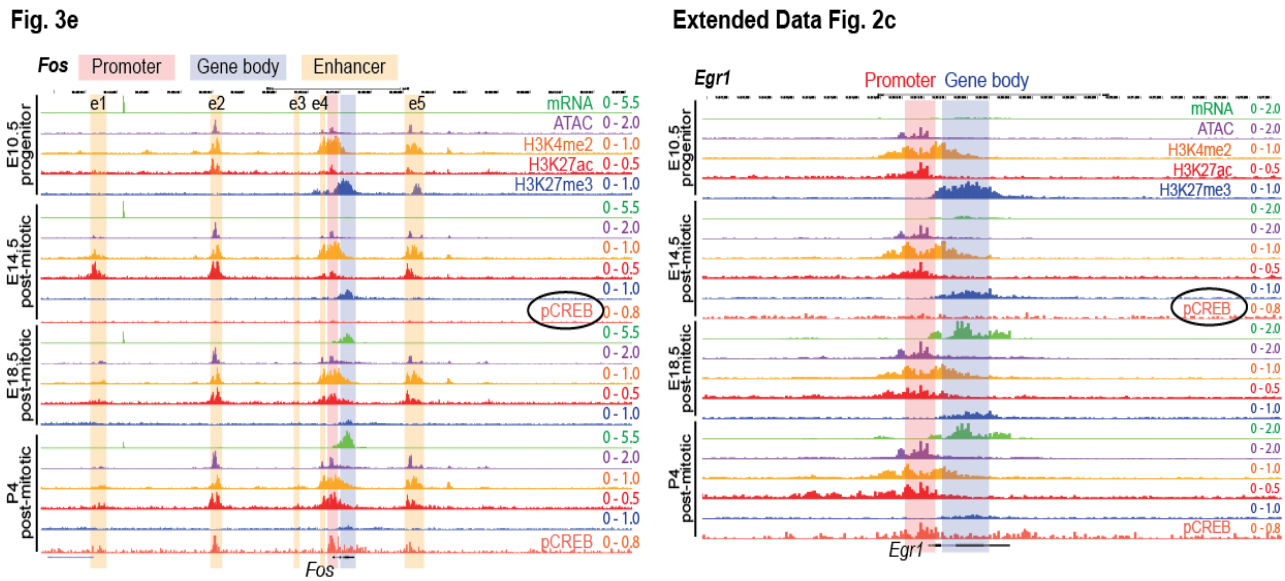

Figure R1: Genome browser views from previous manuscript revision, Fig. 3e (*Fos*, left) and Extended Data Fig. 2c (*Egr1*, right). The E14.5 phosphoCREB (pCREB) tracks that may have been mistaken as E18.5 are highlighted by circles.

Rev1.3b. Mechanism of barrier release (2)

In addition, to help the reviewer to further evaluate our effort to provide mechanistic insight into how **the resolving of bipartite chromatin and release of the barrier are achieved as a consequence of stimulus-dependent promoter activation**, we now provide **new data**, not yet available at the time of submission of the previous revised version, that further support our model and **directly address the impact of stimulus-dependent induction on the change of the bipartite epigenetic state** (**new Figs. 7b-g, 8a)**. Namely:

1. By treating E12.5 short-term cultured hindbrain neurons with KCl, an inducing stimulus of neuronal activity that causes phosphorylation of CREB, H3K27 acetylation and rapid transcriptional induction of IEGs (West et al., 2011, PMID: 21555405; Kim et al., *Nature* 2010, PMID:20393465), we did observe **the stimulus-dependent removal of the H3K27me3 mark from the gene bodies of bipartite IEGs (new Fig. 7b, d-f)**.

2. We found that GSK-J4, an inhibitor of H3K27me3 methyltransferases (i.e. Utx, Jmjd3), **prevented neuronal activity-dependent gene body H3K27me3 removal (new Fig. 7b)**, indicating that H3K27me3 is removed through stimulus-dependent **active demethylation**. Moreover, by additionally carrying out ChIP-seq of H3K27me3 in E18.5 wild-type and Jmjd3KO hindbrain *in vivo*, we confirmed that inactivation of *Jmjd3*

inhibited, at least partially, removal of the gene body H3K27me3 mark from the E14.5 bipartite genes that become active at peri/postnatal (P4) stages (**new Fig. 7c**).

3. We found that treatment of embryonic neurons with a cocktail of **neuronal activity blockers** (TDN cocktail = TTX + D-AP5 + NBQX) **prevented the removal of H3K27me3 from IEG gene bodies** in long-term hindbrain neuron culture (**new Fig. 7d**; long-term culture normally causes the progressive removal of H3K27me3 from IEG gene bodies, see new Extended Data Fig. 8a).

⇒ These results strongly indicate that in developing neurons **the removal of the H3K27me3 barrier on IEG gene bodies is stimulus-dependent, and induced by neuronal activity**.

4. We next tested the contribution of *de novo* promoter H3K27 acetylation in activity-dependent release of the gene body H3K27me3 barrier. We treated E12.5 short-term cultured neurons with KCl in the presence of A-485, an inhibitor of H3K27 acetyltransferase p300/CBP, and found that A-485 inhibits KCl-dependent increase of mRNA and promoter H3K27ac levels (**new Fig. 7e,** RNA and H3K27ac), and importantly also prevented the removal of the H3K27me3 mark from bipartite IEG gene bodies (**new Fig. 7e**, H3K27me3), indicating that **gene body H3K27me3 removal requires stimulus-dependent de novo promoter H3K27 acetylation**.

5. We next asked whether the KCl-dependent gene body H3K27me3 removal is the consequence of transcriptional elongation or rather it is driven by promoter acetylation per se. We treated E12.5 short-term cultured neurons with KCl in the presence of flavopiridol, a Cdk9 inhibitor that works as a RNAPII transcriptional elongation blocker. Flavopiridol treatment caused a complete block of the KCl-dependent de novo transcription of IEGs, while de novo promoter H3K27ac was not prevented (**new Fig. 7f**, RNA and H3K27ac). Interestingly, we found that flavopiridol did not prevent the KCl-dependent removal of H3K27me3 from bipartite IEG gene bodies (**new Fig. 7f**, H3K27me3), indicating that **the gene body H3K27me3 mark is removed by KCl-induced neuronal activity regardless of mRNA transcriptional elongation, provided that de novo promoter H3K27 acetylation occurs**.

6. We had already provided in the previous revised version new evidence indicating that a competitive balance between H3K27ac at promoters and H3K27me3 in gene bodies maintains the bipartite signature and provides a barrier to elongation (Extended Data Fig. 7b,c in the previous revision, **new Fig. 7g** in the current version). Briefly, by treating E12.5 short-term cultured hindbrain neurons with histone deacetylase (HDAC) inhibitor trichostatin A (TSA), we observed TSA-dependent increase of mRNA levels and spreading of H3K27ac into the gene bodies of bipartite genes, accompanied by the

removal of the H3K27me3 mark (**new Fig. 7g**, left). As a representative example, we also showed a genome browser view of *Fos* (**new Fig. 7g**, right): TSA-treatment caused the resolution of the bipartite signature at this locus into an active state.

⇒ Together with the analysis of *Ezh2cKO* E14.5 hindbrain cells showing the replacement of H3K27me3 by H3K27ac in gene bodies of bipartite genes (Fig. 4b of previous revision; **new Fig. 5a** of current version), and the **new evidence** described in points **1-6**, these compelling results provide a rationale to understand how H3K27me3 provides a barrier to stimulus-dependent elongation (please see also below our **new data**, **Rev1.3c**) and provide direct evidence for our proposed mechanism. We demonstrate that **stimulus-dependent increase of promoter H3K27ac causes active H3K27me3 removal from the gene body and elongation barrier release shifting from a bipartite to an active state (summary diagram, new Fig. 8a).** We believe these new data bring substantial mechanistic insight in understanding the regulation of the bipartite signature.

We do hope the reviewer will be satisfied by these compelling findings.

Rev1.3c. ***Mechanism of elongation barrier***

In the previous revised manuscript (Fig. 4c-e, Extended Data Figs. 7d-g, 8a-e, 9a-c), we provided evidence of barrier to RNAPII elongation on bipartite genes which is released upon removal of H3K27me3. We now provide **new data** addressing **how the H3K27me3 mark may block transcriptional elongation of bipartite genes**, by an additional analysis of PRC2 KO ESCs which was not included in the previous revised version of the manuscript (**new Fig. 6**). Namely:

1. We revealed the contribution of the negative elongation factor (NELF) complex in blocking H3K27me3-dependent elongation. NELF is known to negatively regulate transcriptional elongation by pausing RNAPII in TSSs (Chen et al., *Nat Rev Mol Cell Biol*, 2018, PMID: 29740129). Inducing stimulus-dependent removal of NELF from IEG promoters causes release of paused RNAPII into elongation (Schaukowitch et al., *Mol Cell* 2014, PMID: 25263592; Adelman et al., *PNAS*, PMID:19820169). Here, we hypothesized that H3K27me3 on gene body may inhibit transcriptional elongation in bipartite genes by interfering with stimulus-dependent NELF release. By ChIP-seq of NELF-b, a core component of the NELF complex, we found that **NELF-b levels in bipartite gene promoters are decreased in *EedKO* as compared to wild-type ESCs** (**new Fig. 6a,** left). Importantly, this was accompanied by the **release of paused RNAPII-S5P from promoter regions** (Extended Data Fig. 9b of previous revision; **new Fig. 6a,** right), phenocopying the consequences of NELF knock-down experiments (Muse et al., *Nat Genet*, 2007, PMID: 17994021; Saha et al., *Nat Neurosci,* 2011, PMID: 21623364).

In addition, we had already shown in previous revision that PRC2-removal from bipartite gene bodies is accompanied by increased levels of mRNA (Fig. 4c, e, f of previous revision; **new Fig. 5b, d, e**), elongation marks (Extended Data Fig. 8e and 9a,c of previous version; **new Extended Data Fig. 7e-g**) and fraction of gene body transcripts (Fig. 4d of previous version; **new Fig. 5c**).

⇒ These results provide **evidence that H3K27me3 in bipartite gene bodies inhibits stimulus-dependent RNAPII release and elongation through maintenance of NELF and interference with its removal**.

2. Next, we assessed **the role of PRC-dependent chromatin compaction in bipartite gene bodies.** We found that the H3K27me3 removal caused de-compaction of bipartite gene bodies. Namely, by analyzing public datasets of ATAC-seq in wild-type and *Ezh1/Ezh2KO* mouse ESCs (Lavarone et al., *Nat Commun*, 2019, PMID: 30976011), we found that while the *Ezh1/Ezh2* PRC2 KO did not affect promoter accessibility, **gene body accessibility was significantly increased** (**new Fig. 6c**). Moreover, there is evidence that PRC1 interferes with transcriptional elongation through chromatin compaction (Schuettengruber et al., Cell, 2017, PMID: 28938122; Simon and Kingston, *Nat Rev Mol Cell Biol*, 2009, PMID: 19738629). One unique feature of the bipartite signature that we have shown in the previous revised manuscript is high-level deposition of Ring1b, a core component of PRC1, in bipartite gene bodies (previous and **new Fig. 4a**). Even though gene body H3K27me3 levels are slightly lower in bipartite genes as compared to bivalent genes (**new Figs. 2b and 4a** - H3K27me3), Ring1b levels are higher in bipartite genes as compared to bivalent genes (**new Fig. 4a** - Ring1b). To assess **the role of H3K27me3 for Ring1b bipartite gene body deposition** we analyzed ChIP-seq of Ring1b in wild-type and *Ezh1/Ezh2KO* mouse ESCs from the above mentioned public datasets (Lavarone et al., *Nat Commun*, 2019, PMID: 30976011). ChIP-seq of Ring1b showed that *Ezh1/Ezh2* removal caused a reduction of gene body Ring1b levels in bipartite genes (**new Fig. 6b**), indicating that the **gene body Ring1b deposition on bipartite genes is H3K27me3-dependent**.

3. We next asked if the de-compaction (increased accessibility) of bipartite genes in PRC2 KO was primarily caused by **the removal of H3K27me3 and Ring1b per se or whether it was merely a consequence of increased transcription.** We carried out experiments to quantify chromatin compaction of bipartite IEGs (i.e. *Fos*, *Egr1*) using wild-type and *EedKO* ESCs in serum-starved condition (new **Fig. 6d, e**). As already shown in the revised manuscript (old Extended Data Fig. 9e; new Fig. 5f), while these IEGs showed a modest increase of expression in *EedKO* ESCs, as compared to wild type, in the serum-containing medium (**new Fig. 6d mRNA**), in the serum-starved condition their expression levels did not change between mutant and wild-type (**new Fig. 6e mRNA**) - i.e. they are basically not expressed, likely due to lack of inducible stimulus. We found

that, even in the serum-starved condition, bipartite IEGs showed increased accessibilities in *EedKO* ESCs (**new Fig 6e, ATAC**) indicating that the **de-compaction of bipartite gene bodies in PRC2 KO was not merely correlative with increased transcription, but was rather primarily caused by the removal of H3K27me3 and Ring1b**.

⇒ In summary, **these results strongly indicate that H3K27me3 mark causes compaction of bipartite gene bodies, partly through Ring1b deposition, which in turn hampers transcriptional elongation**.

I suggest to the authors that their model visuals should be included in the main text of their manuscript, they are not simple and having the visual in the primary manuscript, not buried online, would facilitate understanding of their thought process to a general readership.

Rev1.4. We thank the reviewer for this suggestion. We agree and included them in **new Figs. 5g and 8a, b**. We also prepared new summary visuals to integrate the new data conclusions.

We do hope that this reviewer will be fully satisfied by these new compelling results and revisions.

Referee #4 (Remarks to the Author):

In this manuscript the authors describe a new mechanism regulating IEG expression in vivo during neuronal development. They identify a new epigenetic "bipartitic" signature that characterizes activity-regulated IEGs and identify the role of PRC2 in maintaining low transcription levels.
The authors have provided an extensive revision of the previous reviewer's comments and performed an impressive number of new experiments therefore I will comment mostly on more general issues related to the manuscripts. Most issues raised by the reviewers have been addressed either with new experiments or by changing the text. However, despite the remarkable tour de force, the novelty of the findings and the potential appeal to a broad readership remain questionable.

Rev4.1. We respectfully disagree. We believe that our findings presented in the previous and current revisions of the manuscript are particularly appealing to a broad readership, because:

1. We discovered a novel chromatin state for inducible immediate early genes during development. We discovered that the bipartite chromatin signature is not only limited to

developing neurons but it is widely present in developing tissues/cell types as well (e.g. we identified the signature in developing heart, liver, and neural crest cells), and in ES cells (**new Figs. 1, 2, Extended Data Figs. 2, 3**). By extensively carrying out functional analysis using PRC2-KO neurons and ESCs, we revealed that the bipartite chromatin signature provides a **general epigenetic mechanism** regulating the specificity, rapidity, and magnitude of stimulus-dependent transcriptional response of immediate early genes during development (**new Figs. 5, 6, Extended Data Figs. 7, 8**). We further clarified the broad importance of the findings in the revised abstract, introduction, and discussion sections and **new Figs. 2, 5, 6**.

2. We demonstrated how the bipartite signature is established, regulated, and resolved during development and how it regulates the specificity, rapidity and magnitude of the transcriptional response of inducible bipartite genes to relevant environmental stimuli (**new Figs. 3-7**, **Extended Data Figs. 5-8**, please also see **Rev4.2b**.).

3. We discovered a barrier to RNAPII elongation on bipartite genes, which is released upon removal of H3K27me3 from gene body. We demonstrated this by **multiple functional approaches**, namely by *Ezh2* and *Eed* KOs in neurons and ES cells, respectively, and by acute removal of H3K27me3 by dCas9-Utx in primary neurons (**new Figs. 4-6, Extended Data Figs. 7, 8**).

4. We demonstrated that the bipartite signature still allows for very rapid induction of IEGs while the Polycomb marking of gene body, by inhibiting elongation, sets a transcriptional threshold preventing rapid response to non-relevant/spurious signals (**new Fig. 5e-g, Extended Data Fig. 8f-h**). This latter is another important functional demonstration of the physiological importance of this chromatin signature on IEGs during development (please see below).

First of all, although the manuscript is very dense due to large number of experiments performed, the authors often overlook the physiological significance of their findings. The issue of cause/effects is unfortunately a problem for any epigenetic study, however in this particular case the link between sensory stimulation and the laying of the epigenetic marks that define the bipartitic status is very thin. The authors provide a detailed description of the correlation between transcriptional levels and bipartitic vs bivalent genes but whether the findings go beyond correlation and the impact of neuronal activity (and intracellular calcium levels, for example) on the change of epigenetic status is unclear. Overall, the manuscript is technically remarkable and the findings of a new epigenetic signature potentially regulating the yet unclear mechanism at the core of IEG rapid expression are certainly interesting. However, unfortunately the manuscript lacks a clear mechanistic explanation of how this correlates with neuronal maturation and the establishment of the neuronal circuitry underlying IEG expression in response to sensory stimulation.

Rev4.2. Since this referee did not review the first version of our study, we focused our revision on addressing all the concerns of reviewers 1-3. Therefore, in the previous revised version of the manuscript, we had not put too much emphasis on the link between sensory stimulation and bipartite chromatin regulation, nor on the correlation between activity-dependent resolution of the bipartite signature and neuronal development/maturation.

This choice was obligate and due to the direction the study had taken after the first round of revision, and the fact that we needed to add to the paper an extensive number of new experiments to address the reviewer 1-3 concerns.

Nonetheless, the previous revised version already included key data directly addressing, at least in part, the points raised by this reviewer as well (please see below ***Rev4.2a***).

⇒ In addition, despite the challenging conditions due to the coronavirus pandemics, we have now included **new data** in the current revised version that we believe should fully address all the remaining concerns of this reviewer (please see ***Rev4.2b and Rev4.3***). We do hope the reviewer will agree and will be satisfied by this significant effort.

Namely:

Rev4.2a. Firstly, by in vivo ChIP-seq experiments we discovered that increase of CREB phoshorylation (pCREB) and binding significantly correlates with the release and elongation of RNAPII and productive transcriptional induction of IEG bipartite genes during perinatal/early postnatal barrelette neuron refinement (previous revision: Fig. 3e, Extended Data Figs 2c, e and 7a-d; current revision: **new Figs. 3e and 7a, Extended Data Fig. 6a,b,d,e**; please see also ***Rev1.3a***). As the reviewer knows, pCREB has been long known to be one of the main readouts of sensory stimulation and increase of intracellular calcium levels (e.g. Yap et al., Neuron 2018, PMID: 30359600).

In addition, we now added a **new** pCREB ChIP-seq at E18.5 (in addition to E14.5 and P4) and added this to the genome browser profiles of *Fos* and *Egr1* (**new Fig. 3e, Extended Data Fig. 6b,d**). It can be nicely appreciated that pCREB starts to accumulate in promoter regions of these bipartite IEGs at E18.5 in accordance with stimulus-induced mRNA expression (please see also below).

Rev4.2b. Secondly, and we believe quite importantly, we now provide **new data that directly address the impact of neuronal stimulation on the change of the bipartite epigenetic state (new Fig.7b-f; please see also *Rev1.3b*)**.

We treated E12.5 short-term cultured hindbrain neurons with KCl and assessed its effect on the epigenetic change at bipartite IEGs. KCl-mediated depolarization of cultured neurons causes the

increase of intracellular calcium signaling and phosphorylation of CREB on IEG promoters and has been widely used to mimic the genomic response to a wide range of sensory stimuli (e.g. Kim et al., *Nature* 2010, PMID:20393465; Malik et al., *Nat Neurosci* 2014, PMID:25195102; Tyssowski et al., *Neuron* 2018, PMID:29681534).

We revealed that:

1. In addition to causing rapid and expected transcriptional induction, **KCl-mediated neuronal activity induces the removal of the H3K27me3 mark from the bipartite IEG gene bodies (new Fig.7b, d-f)**.

2. We found that GSK-J4, an inhibitor of H3K27me3 methyltransferases (i.e. Utx, Jmjd3), **prevented neuronal activity-dependent gene body H3K27me3 removal (new Fig.7b)**, indicating that H3K27me3 is removed through **active demethylation**. Moreover, by additionally carrying out ChIP-seq of H3K27me3 in E18.5 wild-type and *Jmjd3KO* hindbrain *in vivo*, we confirmed that inactivation of *Jmjd3* inhibited, at least partially, removal of the gene body H3K27me3 mark from the E14.5 bipartite genes that become active at peri/postnatal (P4) stages (**new Fig. 7c**).

3. We found that treatment of embryonic neurons with a cocktail of **neuronal activity blockers** (TDN cocktail = TTX + D-AP5 + NBQX) **prevented the removal of H3K27me3 from IEG gene bodies** in long-term hindbrain neuron culture (**new Fig. 7d;** long-term culture normally causes the progressive removal of H3K27me3 from IEG gene bodies, new Extended Data Fig. 8a).

⇒ These results strongly indicate that in developing neurons **the removal of the H3K27me3 barrier on IEG gene bodies is stimulus-dependent, and induced by neuronal activity**.

4. We next tested the contribution of *de novo* promoter H3K27 acetylation in activity-dependent release of the gene body H3K27me3 barrier. We treated E12.5 short-term cultured neurons with KCl in the presence of A-485, an inhibitor of H3K27 acetyltransferase p300/CBP, and found that A-485 inhibits KCl-dependent increase of mRNA and promoter H3K27ac levels (**new Fig. 7e mRNA and H3K27ac**), and notably also prevented the removal of the H3K27me3 mark from bipartite IEG gene bodies (**new Fig. 7e H3K27me3**), indicating that **gene body H3K27me3 removal depends on stimulus-dependent de novo promoter H3K27 acetylation**.

5. We next asked whether the KCl-dependent gene body H3K27me3 removal is the consequence of transcriptional elongation or rather it is driven by promoter H3K27 acetylation per se. We treated E12.5 short-term cultured neurons with KCl in the presence of flavopiridol, a Cdk9 inhibitor that works as a RNAPII transcriptional

elongation blocker. Flavopiridol treatment caused a complete block of the KCl-dependent de novo transcription of IEGs, while de novo promoter H3K27 acetylation was not prevented (**new Fig. 7f mRNA and H3K27ac**). Interestingly, flavopiridol did not prevent the KCl-dependent removal of H3K27me3 from bipartite IEG gene bodies (**new Fig. 7f H3K27me3**), indicating that **the gene body H3K27me3 mark is removed by KCl-induced neuronal activity regardless of mRNA transcriptional elongation, provided that de novo promoter H3K27 acetylation occurs**.

⇒ These and the extensive additional data described in ***Rev1.3b,c,*** nicely support our conclusions and provide direct evidence for our proposed mechanism. We demonstrate that **stimulus-dependent increase of promoter H3K27ac causes active H3K27me3 removal from the gene body and RNAPolII elongation barrier release shifting from a bipartite to an active state (summary diagram, new Fig. 8a).** We believe these new data bring substantial additional mechanistic insight in understanding the regulation of the bipartite signature.
We do hope the reviewer will be satisfied by these compelling findings.

6. We also note that we already extensively demonstrated in the previous revised manuscript the functional relevance of bipartite chromatin in rapid transcriptional response of IEGs to inducing stimuli using *Ezh2KO* neurons and *EedKO* ESCs. Namely, the bipartite signature still allows for very rapid induction of IEGs while the Polycomb marking of gene body, by inhibiting elongation, sets a transcriptional threshold preventing rapid response to non-relevant signals (**new Fig. 5e-g, Extended Data Fig. 8f-h**).

⇒ Thus, together with the new data above shown in **new Fig.7a-f**, we believe that we clarified **the cause/effect link** between neuronal activity, the resolution of the bipartite chromatin signature, and rapid induction in response to relevant stimuli during development.

Rev4.3. Moreover, we further investigated **the relevance of maintaining IEGs in a bipartite state during early development and its role in preventing precocious activity-dependent neuronal development/maturation.**
To this aim, we took advantage of the knowledge from a recent study, published after we submitted our revised version (Stroud et al., *Neuron* 2020, PMID: 32589877), and generated **new data** for our study that were not included in the previous revised manuscript but we have now integrated in the current version. These authors showed that activity-regulated AP1 family transcription factors (e.g. Fos, Jun) mediate the maturation process of early postnatal neurons through the *de novo* activation of AP1-specific enhancers in a neuronal subtype-specific manner. We hypothesized that the precocious activation of bipartite IEGs, including Fos, in immature (i.e. E14.5 early postmitotic) neurons by removal of the gene body H3K27me3 mark (new Fig.

5d, e) may lead to precocious opening and activation of the early postnatal Fos-specific enhancer maturation program.

Firstly, among the enhancers that normally become open only in postnatal barrelette neurons (3967 enhancers), we identified 85 neuronal activity-regulated Fos-binding enhancers (Malik et al., *Nat Neurosci* 2014, PMID:25195102). By carrying out ATAC-seq using short-term cultured E12.5 Drg11-positive trigeminal neurons treated with 55mM KCl for 1 hour, we confirmed that strong neuronal stimulation can precociously open these 85 enhancers in immature sensory neurons (**Figure R2**). Next, we carried out ATAC-seq in the **E14.5 *Ezh2cKO* neurons** and found that the **85 activity-regulated Fos-binding enhancers that normally become open only in early postnatal (E18.5/P4) barrelette neurons acquire precocious accessibility already at E14.5 (new Fig. 5h, Extended Data Fig. 8i)**. This strongly indicates that the precocious removal of the H3K27me3 mark on bipartite IEGs, leading to their ectopic transcriptional activation in embryonic sensory neurons (Fig. 4e,f of previous manuscript**;** Fig. 5d,e of current manuscript**),** has a direct impact on normal *in vivo* neuronal prenatal development and maturation, through the likely ectopic activation of secondary response genes. These findings further support the physiological importance of the bipartite chromatin for the appropriate regulation of stimulus-dependent transcriptional response of bipartite IEGs during development.

⇒ In summary, we believe that, altogether, the additional experiments, data analyses and revisions to the text described in ***Rev1.3a,b,c***, ***Rev4.2*** and ***Rev4.3*** responses**,** fully address: *(1) the impact of neuronal activity (and intracellular calcium levels, for example) on the change of bipartite epigenetic status and (2) provide a mechanistic explanation of how this correlates with neuronal maturation and the establishment of the neuronal circuitry underlying IEG expression in response to sensory stimulation.*

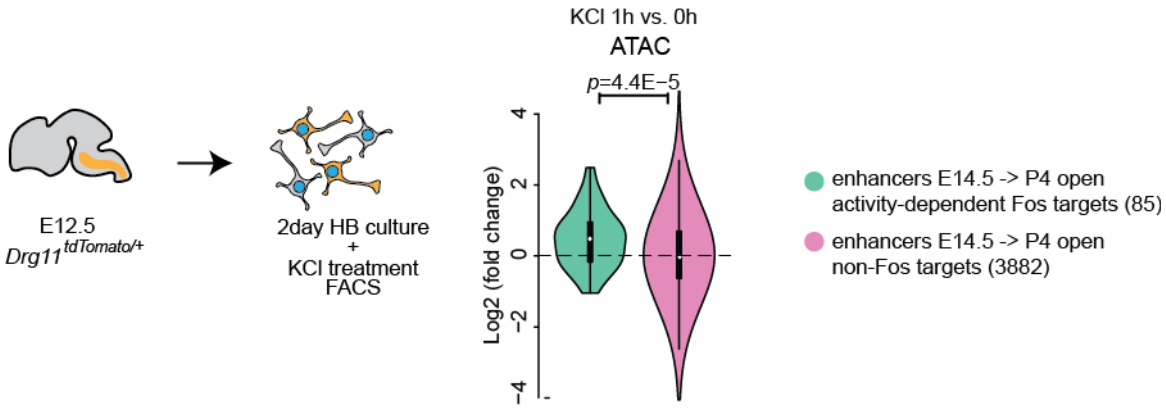

Figure R2: Left, *Drg11*$^{tdTomato/+}$ hindbrains were ex vivo cultured for a short time course. After 55mM KCl treatment for 1 hour, Drg11+ immature trigeminal neurons were FACS-isolated for ATAC-seq

analysis. Right, violin plots visualizing log2 fold changes of enhancer chromatin accessibilities in 1hour KCl-treated neurons as compared to non-treated control neurons. Increased accessibility is selectively detected in KCl-treated neurons at activity-dependent Fos-binding enhancers that normally become open only at P4 (green, n=85) (right, all the remaining non-Fos enhancers that gain accessibilities only at P4, n=3882). The *p*-value is from a two-sided Wilcoxon test. We present this figure only for the reviewer's perusal, but we are also happy to include it in current revised manuscript depending on the reviewer's recommendation.

Rev4.4. Lastly, we would like to note that identifying specific genes downstream of IEGs that drive the neuronal maturation process in developing barrelette neurons would be clearly outside the scope and topic of this particular study. We hope that this reviewer agrees. Moreover, we believe that going along that path would not bring, in our opinion, any major novelty to the study. Downstream IEG targets have been extensively identified in other sensory stimulation paradigms, and belong to the secondary response gene class which drives neuronal and synaptic maturation (e.g. Malik et al., *Nat Neurosci* 2014, PMID:25195102; Su et al., *Nat Neurosci* 2017, PMID:28166220; Tyssowski et al., *Neuron* 2018, PMID:29681534; Stroud et al., *Neuron* 2020, PMID: 32589877).

In this respect, we show that in Kir2.1-silenced barrelette neurons IEGs are not induced (**new Fig. 1b**) and that barrelette neurons do not form a whisker-related map (**new Extended Data Fig. 1g, h**), due to abnormal activity-dependent refinement of dentritic arbors of barrelette neurons (**new Extended Data Fig. 1i-p**).

We do hope that this reviewer will be fully satisfied by these new compelling results and revisions.

Decision Letter, first revision:

3rd Nov 2020

Dear Filippo,

Your Article, "A unique bipartite Polycomb signature regulates stimulus response transcription during development" has now been seen by 2 referees.

Unfortunately, reviewer #1 declined to comment on the revised manuscript. So, I decided to send it to a new reviewer (#5), who is an expert on Polycomb function.

You will see from the reviewers' comments below that while reviewer #4 finds your work improved

and has no additional concerns, some important points are raised by reviewer #5. Indeed, this reviewer thinks that the main results are of broad interest but that there are many overstatements that need to be carefully addressed, i.e., either removed or significantly toned down.

We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We therefore invite you to revise your manuscript taking into account all reviewer comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact me if you have any questions or concerns regarding the reviewer's comments.

When revising your manuscript:

*1) Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response may be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available
here.
Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf
It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
A revised checklist is essential for re-review of the paper.

Please be aware of our guidelines on digital image standards.

Please use the link below to submit your revised manuscript and related files:

[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised manuscript within four to eight weeks. If you cannot send it within this time, please let us know.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Tiago

Tiago Faial, PhD
Senior Editor
Nature Genetics
https://orcid.org/0000-0003-0864-1200

Reviewers' Comments:

Reviewer #4:
Remarks to the Author:
The authors have provided a compelling revised manuscript and have addressed my major concerns, especially regarding the physiological significance of their findings. The revised manuscript is now suitable for publication in Nature Genetics.

Reviewer #5:
Remarks to the Author:
The revised manuscript "A Unique Bipartite Polycomb Signature Regulates Stimulus Response Transcription during Development" by Rijli and colleagues describes a previously unreported "bipartite" arrangement of histone post-translational modifications over gene promoters and gene bodies in neuronal and other developing cell types, explores the functional impact of this bipartite configuration on immediate early gene activation in response to environmental signals, and dissects the series of events that accompany the transition from bipartite to active states. The authors report a novel phenomenon that should be of broad interest to developmental and chromatin biologists, and provide substantial understanding of its mechanistic underpinnings and likely functional significance.

Despite these qualities, the manuscript is plagued by overstatements and exaggerations that cannot be left unedited. The paper can therefore be considered a reasonable candidate for publication as long as some critical modifications are made.

Major comments:

1. The sentence in lines 143-145 states a conclusion that lacks any demonstration whatsoever at this early point in the manuscript and must therefore be removed: "Thus, developing neurons at prenatal stages have the potential to respond to activity-dependent stimuli and such plasticity is maintained by Pc-dependent transcriptionally poised chromatin organization." At this stage, the authors have not shown any evidence of a "Pc-dependent transcriptionally poised chromatin organization," nor have they provided any evidence of "plasticity" being maintained by such a phenomenon.

2. The sentence in lines 210-211 ("Altogether, these results indicate that the bipartite signature is widely used during development and could regulate rapid IEG transcriptional inducibility") must be rephrased, because the results presented until then do not "indicate" that the bipartite signature could regulate rapid inducibility. Perhaps they "raise the intriguing possibility" that that might be the case, but anything more definitive at this stage is simply inaccurate.

3. The authors conclude that the bipartite state allows for promoter-enhancer contacts despite the lack of productive elongation based on a 4C experiment with 3 viewpoints around a single gene (Extended Data Fig. 6c). This is an exaggeration and the authors must more explicitly acknowledge that this conclusion is based only on Fos.

4. The authors claim in lines 350-351 that bipartite genes have higher deposition of CDK9 on their promoters than mRNA low genes, but the violin plot in Fig. 4a shows no obvious difference. The statement should therefore be removed from the manuscript.

5. In lines 355-358 it is stated that levels of RNAPII-S2P are on average "much lower" around the TESs of E14.5Bip genes than around the TESs of E14.5AcP genes, which is a clear exaggeration of the data. They are significantly lower in a statistical sense, perhaps, but not "much lower" for any reasonable reader of the paper.

6. The statement in lines 453-456, based on the data presented in Extended Fig. 8g, that "the bipartite signature may still allow for rapid inducibility of IEGs, whereas the bivalent state constrains IEGs to a much slower response and only in the presence of prolonged stimulation" represents a major extrapolation and must therefore be reworded. The authors test the behavior of two bipartite genes and one bivalent gene in this assay; this is not sufficient to draw conclusions about the general behavior of the two gene categories.

7. The authors falsely state that the "stimulated versus unstimulated transcript ratio (fold change) was much higher in Ezh2 homozygous mutants than controls" in response to KCl or forskolin stimulation. The data in fact show that transcript levels are higher in Ezh2 homozygous mutants in both unstimulated and stimulated conditions, without much difference in the stimulated versus unstimulated ratio. We can conclude that in these cells Ezh2 acts to limit the expression of these genes regardless of the stimulation conditions, but not to regulate the amplitude of upregulation upon stimulation. The alternative interpretation that the authors seem to be making is outright misleading and must be changed.

8. In lines 514-515 the authors write that bipartite genes show higher levels of Ring1b enrichment than bivalent genes, but the violin plot in Fig. 4a shows a difference that is so modest as to make the authors' statement devoid of any biological meaning. This sentence should be removed from the manuscript.

9. The authors interpret the data presented in Fig. 6e as showing that chromatin accessibility is increased in the absence of Eed independently of Fos and Egr1 transcription (serum-starved condition). However, they should note that the increase in accessibility in EedKO versus wild-type is of a noticeably smaller magnitude in the serum-starved condition, in particular for Egr1. The authors therefore need to be less categorical in their conclusion here and allow that transcription may partially contribute to changes in gene body chromatin accessibility. The authors go on to state on line 534 that "H3K27me3 causes compaction," a serious exaggeration of what their data actually show. All that one can reasonably conclude is that H3K27me3 (or more precisely Eed) is required for full compaction.

10. On a related note, it is highly misleading to state that changes in chromatin accessibility were dependent on Ring1b given that this wasn't even tested.

11. Furthermore, it is stated in the discussion (lines 713-716) that Pc-compacted gene body chromatin likely contributes to preventing Pol II elongation at bipartite genes, but this is in fact neither supported nor strongly suggested by the data. At no time do the authors show that increasing chromatin accessibility at bipartite gene bodies is, on its own, sufficient to drive transcription elongation; they only show in Fig. 6e that elongation is not required for Eed deletion to increase chromatin accessibility. The authors therefore need to revise lines 713-716.

12. In the experiment whose results are shown in Fig. 7b, does inhibition of the demethylases have an effect on gene induction at the mRNA level? I suspect not, given the results shown in Fig. 7e, wherein inhibition of histone acetyltransferases dramatically impairs H3K27me3 removal but only modestly reduces mRNA induction. Overall, it appears that in these hindbrain cultures removal of gene body H3K27me3 is sufficient to de-repress transcription (Fig. 5d) but not necessary for a fair degree of transcription to take place (Fig. 7e). This is a glaring omission in the authors' reporting of their experimental results and needs to be discussed explicitly in order to accurately represent the mechanisms at play. It is (or can be) more complicated than what the authors currently suggest in lines 587-589 (K27ac deposition leading to K27me3 removal leading to elongation).

13. Similarly, instead of implying (perhaps inadvertently) that A-485 treatment inhibits KCl-dependent increases in mRNA and H3K27ac levels to a similar degree in Fig. 7e, the authors must acknowledge that A-485 has a dramatic effect on H3K27ac but only a modest effect on mRNA induction, thus revealing that H3K27ac is only partially necessary for mRNA induction, perhaps because its role of promoting H3K27me3 removal from the gene body is not essential (see previous comment).

14. Regarding the apparent compatibility of transcriptional induction of bipartite genes with failure to remove much or all of the gene body H3K27me3, it is striking that the instances in which H3K27me3 removal is observed in KCl-treated hindbrain cultures involve a ChIP after 1 hour of treatment (Fig. 7), whereas robust transcriptional induction is observed in E14.5 hindbrain neurons after only 8 minutes of treatment. Until the authors show that H3K27me3 removal occurs on that shorter timescale, they must refrain from concluding that active H3K27me3 removal is important for facilitating bipartite gene induction. The available evidence suggests that it is dispensable.

15. The conclusions drawn from the results described in lines 602-607 (that Fos-binding enhancers in E14.5 hindbrain neurons gain accessibility in the absence of Ezh2 and that this indicates that untimely transcription of bipartite IEGs has a direct developmental impact) are not properly supported. Deleting Ezh2 can lead to massive alterations in the expression of developmental regulators and it is not clear that the changes in accessibility at the Fos-binding enhancers are a direct result of the loss of a bipartite signature at the Fos gene.

Minor comments:

16. The classification of activity-regulated genes as those upregulated in E18.5 versus E14.5 wild-type vPrV neurons and expressed at lower levels in Kir-overexpressing versus wild-type E18.5 vPrV neurons seems to completely ignore the possibility that some of the genes might be expressed at much lower levels in Kir-overexpressing neurons at both timepoints, but still subject to an activity-dependent increase.

17. Activity-regulated genes (ARGs) show an enrichment for H3K27me3+/H3K4me2+/ATAC+ signatures, but it's not clear whether this is specific to ARGs or whether the same enrichment would be observed for the broader category of genes whose expression is upregulated during this developmental time window.

18. The phrase "epigenetic features" in line 259 (and similar use of the term "epigenetic" elsewhere in the manuscript) to denote chromatin features is not a very precise use of language, but unfortunately this distinction is rarely made anymore in the literature.

19. Is NELF-b present at the promoters of Fos and Egr1?

Author Rebuttal, first revision:

POINT-BY-POINT RESPONSE TO REVIEWERS COMMENTS NG-A56037R RIJLI

We were delighted that reviewer #4 was satisfied by our previous revision. Moreover, we wish to sincerely thank reviewer #5 for her/his insightful and constructive inputs which helped us to improve our work. Please find below a detailed account of the changes to the text and experimental additions to the current revision of the manuscript. In particular, the suggestion to look at the correlation between stimulus-dependent H3K27me3 removal from gene body and (fast) transcriptional induction of IEGs (please see responses to points 12-14) led to exciting new results strongly supporting our previous conclusions. We believe that our manuscript is now even stronger and, again, we appreciate the reviewer's time and effort in thoroughly assessing this work.

Reviewer #4:

Remarks to the Author:

The authors have provided a compelling revised manuscript and have addressed my major concerns, especially regarding the physiological significance of their findings. The revised manuscript is now suitable for publication in Nature Genetics.

We wish to thank this reviewer for the very positive assessment and all her/his input which helped to make the conclusions of our work even stronger.

Reviewer #5:

Remarks to the Author:

The revised manuscript "A Unique Bipartite Polycomb Signature Regulates Stimulus Response Transcription during Development" by Rijli and colleagues describes a previously unreported "bipartite" arrangement of histone post-translational modifications over gene promoters and gene bodies in neuronal and other developing cell types, explores the functional impact of this bipartite configuration on immediate early gene activation in response to environmental signals, and dissects the series of events that accompany the transition from bipartite to active states. The authors report a novel phenomenon that should be of broad interest to developmental and chromatin biologists, and provide substantial understanding of its mechanistic underpinnings and likely functional significance. Despite these qualities, the manuscript is plagued by overstatements and exaggerations that cannot be left unedited. The paper can therefore be considered a reasonable candidate for publication as long as some critical modifications are made.

We were delighted that reviewer #5 appreciated the novelty and broad interest of this work, as well as our effort to unravel the mechanistic underpinning and functional significance of the newly discovered Polycomb-dependent bipartite chromatin signature. In this revised manuscript, we revised the manuscript carefully following the reviewer's suggestions. Furthermore, we added important new data strongly supporting the functional relevance of the removal of gene body H3K27me3 on the rapid stimulus-dependent transcriptional induction of bipartite IEGs during development.

We wish to thank this reviewer for her/his insightful and constructive inputs which helped us to improve the manuscript and make our conclusions even stronger.

Major comments:

1. The sentence in lines 143-145 states a conclusion that lacks any demonstration whatsoever at this early point in the manuscript and must therefore be removed: "Thus, developing neurons at prenatal stages have the potential to respond to activity-dependent stimuli and such plasticity is maintained by Pc-dependent transcriptionally poised chromatin organization." At this stage, the

authors have not shown any evidence of a "Pc-dependent transcriptionally poised chromatin organization," nor have they provided any evidence of "plasticity" being maintained by such a phenomenon.

We agree. We removed the sentence. Please see lines 144-146 of the revised manuscript.

2. The sentence in lines 210-211 ("Altogether, these results indicate that the bipartite signature is widely used during development and could regulate rapid IEG transcriptional inducibility") must be rephrased, because the results presented until then do not "indicate" that the bipartite signature could regulate rapid inducibility. Perhaps they "raise the intriguing possibility" that that might be the case, but anything more definitive at this stage is simply inaccurate.
We agree. We rephrased the sentence as suggested by the reviewer. Please see lines 210-212 of the revised manuscript. Please see also responses to points #12-14.

3. The authors conclude that the bipartite state allows for promoter-enhancer contacts despite the lack of productive elongation based on a 4C experiment with 3 viewpoints around a single gene (Extended Data Fig. 6c). This is an exaggeration and the authors must more explicitly acknowledge that this conclusion is based only on Fos.

We agree. We rephrased the sentences to explicitly state that our conclusion is based only on *Fos*. Please see lines 310-311 and 321-325 of the revised manuscript.

4. The authors claim in lines 350-351 that bipartite genes have higher deposition of CDK9 on their promoters than mRNA low genes, but the violin plot in Fig. 4a shows no obvious difference. The statement should therefore be removed from the manuscript.

We acknowledge the point of the reviewer and removed the statement. Please see lines 350-352 of the revised manuscript.

On the other hand, we would like to point out that the ChIP-seq experiments summarized in Fig. 4 are very challenging experiments as they are limited by the amount of cells that can be possibly isolated from primary tissues. The dynamic range as well as the signal-to-noise ratio that can be expected from such experiments will be much lower than what can be achieved for experiments based on cell cultures, especially for some antibodies that are known to produce only moderate IP enrichments. To provide an indication of the sometime small signal range that can be observed in these ChIP-seq experiments (e.g. for Cdk9 and RNAPII-S2P, please also see our response to point 5), we have included the distributions for the 30%-lowest and -highest expressed genes. In this light, we think it is still valuable to present our results, even though the shifts are small and may not be an indication of functional associations when taken individually, but are very unlikely to be based on random noise as they occur in a coherent fashion across the different measured variables.

5. In lines 355-358 it is stated that levels of RNAPII-S2P are on average "much lower" around the TESs of E14.5Bip genes than around the TESs of E14.5AcP genes, which is a clear exaggeration of the data. They are significantly lower in a statistical sense, perhaps, but not "much lower" for any reasonable reader of the paper.

We agree with the reviewer and rephrased "much lower" into "significantly lower". Please see lines 354-358 of the revised manuscript.

Again, we wish to mention that our statement that RNAPII transcripts of bipartite genes are not efficiently elongated is also supported by ChIP-seq of the H3K36me3 mark, an established readout of productive RNAPII-S2P elongation in gene body regions, which carries a much broader dynamic range as compared to RNAPII-S2P (Fig. 4b). Please also see our response to point 4 above.

6. The statement in lines 453-456, based on the data presented in Extended Fig. 8g, that "the bipartite signature may still allow for rapid inducibility of IEGs, whereas the bivalent state constrains IEGs to a much slower response and only in the presence of prolonged stimulation" represents a major extrapolation and must therefore be reworded. The authors test the behavior of two bipartite genes and one bivalent gene in this assay; this is not sufficient to draw conclusions about the general behavior of the two gene categories.

We acknowledge the point of the reviewer. We reworded the sentence accordingly. Please see lines 451-454 of the revised manuscript. Please see also our responses to points 12-14.

7. The authors falsely state that the "stimulated versus unstimulated transcript ratio (fold change) was much higher in Ezh2 homozygous mutants than controls" in response to KCl or forskolin stimulation. The data in fact show that transcript levels are higher in Ezh2 homozygous mutants in both unstimulated and stimulated conditions, without much difference in the stimulated versus unstimulated ratio. We can conclude that in these cells Ezh2 acts to limit the expression of these genes regardless of the stimulation conditions, but not to regulate the amplitude of upregulation upon stimulation. The alternative interpretation that the authors seem to be making is outright misleading and must be changed.

We acknowledge this point. However, we believe our previous statement was perhaps over-stated but not false, as the stimulated versus unstimulated transcript ratio (fold change) was higher (admittedly not 'much higher') in *Ezh2* homozygous mutants than controls in the *in vivo* experiment previously presented in Fig. 5e. Nonetheless, as our conclusion that Polycomb on gene body not only acts to limit the expression of bipartite stimulus response genes but indeed regulates the amplitude of upregulation upon stimulation is largely supported by data in *EedKO* ESCs (old Fig. 5f, new Fig. 5e) and by our new data added to this revision (please see our

response to points 12-14, and new Fig. 7b, f, h) and other data presented (new Fig. 7), we decided to remove the previous Fig. 5e and related statements from the current manuscript. However, if the reviewer would like us to reinstate these experiments and the figure, we will be happy to include them as part of the Extended Data Figures.

Finally, we edited the text accordingly. Please see lines 455-468 and 480-481 of the revised manuscript.

8. In lines 514-515 the authors write that bipartite genes show higher levels of Ring1b enrichment than bivalent genes, but the violin plot in Fig. 4a shows a difference that is so modest as to make the authors' statement devoid of any biological meaning. This sentence should be removed from the manuscript.

We removed the sentence. Please see lines 510-513 of the revised manuscript.

9. The authors interpret the data presented in Fig. 6e as showing that chromatin accessibility is increased in the absence of Eed independently of Fos and Egr1 transcription (serum-starved condition). However, they should note that the increase in accessibility in EedKO versus wild-type is of a noticeably smaller magnitude in the serum-starved condition, in particular for Egr1. The authors therefore need to be less categorical in their conclusion here and allow that transcription may partially contribute to changes in gene body chromatin accessibility. The authors go on to state on line 534 that "H3K27me3 causes compaction," a serious exaggeration of what their data actually show. All that one can reasonably conclude is that H3K27me3 (or more precisely Eed) is required for full compaction.

This is also a well taken point. We have edited the manuscript text toning down our conclusion along the lines suggested by the reviewer. Please see lines 527-533 in the results section and also lines 753-762 in the discussion section of the revised manuscript.

10. On a related note, it is highly misleading to state that changes in chromatin accessibility were dependent on Ring1b given that this wasn't even tested.

We agree. We have edited the manuscript text, toning down our conclusion along the lines suggested by the reviewer. Please see lines 520-522, as well as 527-533 in the result section and also lines 753-762 in the discussion section of the revised manuscript.

11. Furthermore, it is stated in the discussion (lines 713-716) that Pc-compacted gene body chromatin likely contributes to preventing Pol II elongation at bipartite genes, but this is in fact neither supported nor strongly suggested by the data. At no time do the authors show that increasing chromatin accessibility at bipartite gene bodies is, on its own, sufficient to drive

transcription elongation; they only show in Fig. 6e that elongation is not required for Eed deletion to increase chromatin accessibility. The authors therefore need to revise lines 713-716.
We agree that what we stated in the discussion was rather a speculation. We have now revised the manuscript text according to the reviewer's suggestion. Please see lines 753-762 of the revised manuscript.

12. In the experiment whose results are shown in Fig. 7b, does inhibition of the demethylases have an effect on gene induction at the mRNA level? I suspect not, given the results shown in Fig. 7e, wherein inhibition of histone acetyltransferases dramatically impairs H3K27me3 removal but only modestly reduces mRNA induction. Overall, it appears that in these hindbrain cultures removal of gene body H3K27me3 is sufficient to de-repress transcription (Fig. 5d) but not necessary for a fair degree of transcription to take place (Fig. 7e). This is a glaring omission in the authors' reporting of their experimental results and needs to be discussed explicitly in order to accurately represent the mechanisms at play. It is (or can be) more complicated than what the authors currently suggest in lines 587-589 (K27ac deposition leading to K27me3 removal leading to elongation).
We thank the reviewer for pointing out this important issue which is also linked to the next two points of revision (i.e. #13 and #14, please see below). These reviewer's comments led us to further experimentally investigate this aspect which, indeed, is key to understand the functional importance of the H3K27me3 gene body mark for the regulation of rapid bipartite IEG induction during development. We are very pleased with the results which we include in a new figure (Fig. 7b, f) and schematically summarize in Fig. 7i.

Namely, we further investigated how inducing stimuli regulate the rapidity of gene body H3K27me3 removal and how inhibition of H3K27me3 mark removal from gene body may prevent rapid stimulus-dependent transcriptional induction of bipartite IEGs (e.g. *Fos* and *Egr1*).

To this aim, we firstly assessed the time course of inducing stimulus (i.e. 55mM KCl)-dependent bipartite gene body H3K27me3 removal in short-term cultured E12.5 hindbrain neurons (please also see point 14). Notably, we found that the decrease of the H3K27me3 mark is detectable as early as **8 minutes** after KCl treatment (new Fig. 7b), showing that significant H3K27me3 removal starts very rapidly after exposure to the inducing stimuli.

Next, we assessed how the H3K27 demethylase inhibitor (Gsk-J4) treatment affects transcriptional induction of bipartite IEGs after short (8 minutes) or prolonged (60 minutes) exposure to KCl (new Fig. 7f). Strikingly, we found that Gsk-J4 treatment, i.e. inhibition of H3K27me3 removal, **prevents the rapid induction** of bipartite IEGs after 8-minute exposure to the KCl stimulus (new Fig. 7f and scheme in Fig. 7i). Taken together with our previous observation that, in the absence of the H3K27me3 mark in *EedKO* ESCs, the amplitude of the rapid bipartite IEG transcriptional response upon short exposure (8 minutes) to inducing stimuli (i.e. FCS) is enhanced as compared to wild-type control (new Fig. 5e), these results strongly

indicate that the stimulus-dependent gene body H3K27me3 mark removal is essential to achieve rapid and sizeable transcriptional induction of bipartite IEGs.

In the Discussion section, we speculate that this mechanism might be most effective during development in preventing inappropriate induction by acute exposure to weak or non-physiologically relevant signals. In fact, based on our results, it is likely that short exposure to an inducing signal could achieve fast bipartite IEG transcriptional induction only if signal levels are sufficiently high to induce fast increase of promoter H3K27 acetylation and fast gene body H3K27me3 removal (lines 714-719 of revised manuscript).

On the other hand, after prolonged exposure (i.e. 60 minutes) to the KCl stimulus even Gsk-J4 treated neurons showed transcriptional up-regulation of bipartite IEGs as pointed out by the reviewer. Nonetheless, mRNA levels remained significantly lower as compared to control neurons (new Fig. 7f). This indicates that, in the event of incomplete H3K27me3 mark removal from the gene body, while rapid bipartite IEG mRNA induction is impaired, transcripts can nonetheless accumulate over time upon prolonged stimulation, albeit they never reach optimal levels under such a condition. However, during in vivo development it is unlikely that cells will be ever confronted with such a scenario.

We thank the reviewer again for raising this important point. In the revised manuscript, we explicitly presented and discussed these observations. Please see lines 546-556 and 565-579.

13. Similarly, instead of implying (perhaps inadvertently) that A-485 treatment inhibits KCl-dependent increases in mRNA and H3K27ac levels to a similar degree in Fig. 7e, the authors must acknowledge that A-485 has a dramatic effect on H3K27ac but only a modest effect on mRNA induction, thus revealing that H3K27ac is only partially necessary for mRNA induction, perhaps because its role of promoting H3K27me3 removal from the gene body is not essential (see previous comment).

We thank the reviewer for raising this important point. Please also see our response to point 12. To assess the effect of acetyltransferase inhibitor (A-485) treatment on KCl-dependent transcriptional induction of bipartite IEGs, we additionally quantified mRNA levels of control and A-485 treated E12.5 short-term cultured hindbrain neurons after short (i.e. 8 minutes) exposure to 55mM KCl.

Notably, we found that the A-485 treatment also **prevents the rapid induction** of bipartite IEGs after short-time (i.e. 8 minutes) exposure to KCl (new Fig. 7h and scheme in Fig. 7i). These results and those discussed in our response to point 12, strongly support the conclusion that fast bipartite IEG transcriptional induction requires *de novo* H3K27 acetylation and rapid removal of the gene body H3K27me3 mark through active de-methylation.

On the other hand, as described in the previous Fig. 7e (new Fig. 7g), and as pointed out by the reviewer, after prolonged exposure (i.e. 60 minutes) to the KCl stimulus even A-485 treated

neurons showed transcriptional up-regulation of bipartite IEGs. Nonetheless, mRNA levels remained significantly lower as compared to control neurons (new Fig. 7g).

This indicates that, even in the event of lack or reduction of *de novo* H3K27 acetylation at the bipartite IEG promoters, the existing H3K27ac levels maybe sufficient to allow the mRNA of bipartite IEGs to accumulate (but it cannot be fast-induced) provided that stimulation is maintained over time, albeit their transcripts do not reach optimal levels under such a condition.

We revised the manuscript accordingly. Please see lines 586-599.

14. Regarding the apparent compatibility of transcriptional induction of bipartite genes with failure to remove much or all of the gene body H3K27me3, it is striking that the instances in which H3K27me3 removal is observed in KCl-treated hindbrain cultures involve a ChIP after 1 hour of treatment (Fig. 7), whereas robust transcriptional induction is observed in E14.5 hindbrain neurons after only 8 minutes of treatment. Until the authors show that H3K27me3 removal occurs on that shorter timescale, they must refrain from concluding that active H3K27me3 removal is important for facilitating bipartite gene induction. The available evidence suggests that it is dispensable.

Again, we thank the reviewer for raising this valuable point which we have now addressed and presented above in our response to point #12. Briefly, we now show that significant H3K27me3 removal occurs on a short timescale, even after only 8 minutes of stimulation. Our new evidence (please see new Fig. 7b and scheme in Fig. 7i) strongly supports our conclusion that active and rapid H3K27me3 removal is important for facilitating bipartite gene fast induction.

We revised the manuscript accordingly. Please see lines 546-552 in the revised manuscript.

15. The conclusions drawn from the results described in lines 602-607 (that Fos-binding enhancers in E14.5 hindbrain neurons gain accessibility in the absence of Ezh2 and that this indicates that untimely transcription of bipartite IEGs has a direct developmental impact) are not properly supported. Deleting Ezh2 can lead to massive alterations in the expression of developmental regulators and it is not clear that the changes in accessibility at the Fos-binding enhancers are a direct result of the loss of a bipartite signature at the Fos gene.

We also agree with this point. However, as the AP-1 family consists of several transcription factors sharing binding motifs, it would not have been realistic nor proficient to carry out AP-1 factor functional experiments *in vivo*, due to potentially confounding functional redundancies (also, we felt it would be beyond the scope of this study- we acknowledge that the reviewer is not asking for that). We rather decided to use the *Ezh2cKO* model as an admittedly indirect 'proxy' of bipartite IEG deregulation, though looking directly at the effect on the accessibility of AP-1-specific binding sites genome-wide.

Nonetheless, following the reviewer comments, we decided to try and further validate our findings by a complementary approach. We asked whether the identified 85 Fos-binding enhancers would also gain accessibility in response to neuronal stimulation of E12.5 short-term cultured hindbrain trigeminal sensory neurons, therefore likely as a direct consequence of IEG induction. This is indeed what we observed; we have now added a figure with these new data (Extended Data Fig. 8i).

Lastly, to take into full account the reviewer's comments we have carefully revised this section. Please see lines 634-646 of the revised manuscript.

Minor comments:

16. The classification of activity-regulated genes as those upregulated in E18.5 versus E14.5 wild-type vPrV neurons and expressed at lower levels in Kir-overexpressing versus wild-type E18.5 vPrV neurons seems to completely ignore the possibility that some of the genes might be expressed at much lower levels in Kir-overexpressing neurons at both timepoints, but still subject to an activity-dependent increase.

We thank the reviewer for pointing this out. Indeed, when selecting the 56 barrelette sensory activity response genes (bsARGs), we chose genes that are up-regulated from E14.5 to E18.5 in vPrV barrelette neurons, and down-regulated from E18.5 wild-type to E18.5 Kir-overexpressing neurons, without considering the E14.5 Kir-overexpressing condition. However, we additionally required that selected genes have low or no expression in E14.5 wild-type neurons (RPKM < 3 at E14.5, please see Methods), which makes it unlikely that these genes are further down-regulated by Kir-overexpression at E14.5.

Furthermore, we decided not to include the E14.5 Kir-overexpressing data for the definition of the bsARGs, in order to focus only on the sensory stimulus experience-dependent neuronal activity regulated genes, and not on genes which respond to spontaneous firing of neuronal activity before sensory experience kicks in (Spitzer, *Nature*, 2006, PMID: 17151658; Blankenship et al., *Nat Rev Neurosci*, 2010, PMID: 19953103). The barrelette neurons receive sensory information from the whiskers at birth (E18.5) which guides the refinement of the barrelette map. At E14.5 the map has not yet fully formed as there is no connection with the periphery yet, but there is spontaneous firing of the neurons. We thus avoided including genes that are induced by spontaneous neuronal activity at E14.5, which are not the focus in the current study and will be the topic of a subsequent study.

To address the reviewer's comment, we have now included a figure only for the reviewer's perusal (Fig. R1). It shows MA plots comparing bsARGs expression in the Kir-overexpressing, as compared with wild-type, neurons at both E18.5 (Fig. R1a) and E14.5 (Fig. R1b) stages, respectively. Indeed, the bsARGs do not show changes in expression in Kir-overexpressing

neurons at E14.5. Because it is known that spontaneous neuronal activity regulates gene expression without inducing typical IEGs (Madjen et al., *Nature Neurosci,* 2006, PMID: 16582906), it is not very surprising that spontaneous neuronal activity (E14.5) and sensory-driven neuronal activity (E18.5) do not share most of the activity-response genes during neuron development. However, the underlying molecular logic regulating spontaneous activity-driven gene transcription is largely unknown.

17. Activity-regulated genes (ARGs) show an enrichment for H3K27me3+/H3K4me2+/ATAC+ signatures, but it's not clear whether this is specific to ARGs or whether the same enrichment would be observed for the broader category of genes whose expression is upregulated during this developmental time window.

The reviewer is right to point out that the H3K27me3+/H3K4me2+/ATAC+ signatures is not unique to bsARGs, but also generally observed in developmentally regulated genes. By analyzing genes that become up-regulated in E18.5 vPrV barrelette neurons compared with E14.5 neurons (646 genes), we confirmed that these developmental genes are enriched with H3K27me3+/H3K4me2+/ATAC+ signatures at E14.5 (Fig. R2). This is nicely in keeping with previous studies showing that bivalent poised chromatin signature regulates dynamics and plasticity of gene expression during development (e.g. Minoux et al., *Science*, 2017; Bernstein et al., *Cell*, 2006).

We present Fig. R2 only for the reviewer's perusal.

18. The phrase "epigenetic features" in line 259 (and similar use of the term "epigenetic" elsewhere in the manuscript) to denote chromatin features is not a very precise use of language, but unfortunately this distinction is rarely made anymore in the literature.

We agree. We replaced it with 'chromatin features' in lines 258-261 in the revised manuscript. In addition, we have replaced 'epigenetic' with 'chromatin' wherever we felt appropriate (please see the revised manuscript).

19. Is NELF-b present at the promoters of Fos and Egr1?

To address this point, we carried out new ChIP-seq of NELF-b using E14.5 hindbrain cells. Even though this *in vivo* ChIP-seq is relatively noisy particularly for *Egr1* (please also see our response to point 4), we could confirm that bipartite *Fos* and *Egr1* are targeted by NELF-b (Fig. R3). Furthermore, we also added a genome browser view of *Nr4a3*, another example of bipartite IEGs in E14.5 barrelette neurons (Fig. R3).

We present Fig. R3 only for the reviewer's perusal.

20. On line 552, "methyltransferases" should read "demethylases."

Thank you. We corrected it. Please see line 557 of the revised manuscript.

Decision Letter, second revision:

Our ref: NG-A56037R1

3rd Dec 2020

Dear Filippo,

Thank you for submitting your revised manuscript "A unique bipartite Polycomb signature regulates stimulus response transcription during development" (NG-A56037R1). It has now been seen by Reviewer #5 and their comments are below. The reviewer finds that the paper has improved in revision, and therefore we will be happy in principle to publish it in Nature Genetics, pending minor revisions to comply with our editorial and formatting guidelines.

** Note that I will send you a checklist detailing these editorial and formatting requirements in about a week. Please do not finalize your revisions or upload the final materials until you receive this additional information.**

In recognition of the time and expertise our reviewers provide to Nature Genetics's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "A unique bipartite Polycomb signature regulates stimulus response transcription during development". For those reviewers who give their assent, we will be publishing their names alongside the published article.

While we prepare these instructions, we encourage the Corresponding Author to begin to review and collect the following:

-- Confirmation from all authors that the manuscript correctly states their names, institutional affiliations, funding IDs, consortium membership and roles, author or collaborator status, and author contributions.

-- Declarations of any financial and non-financial competing interests from any author. For the sake of transparency and to help readers form their own judgment of potential bias, the Nature Research Journals require authors to declare any financial and non-financial competing interests in relation to the work described in the submitted manuscript. This declaration must be complete, including author initials, in the final manuscript text.

If you have any questions as you begin to prepare your submission please feel free to contact our Editorial offices at genetics@us.nature.com. We are happy to assist you.

Thank you again for your interest in Nature Genetics.

Sincerely,

Tiago

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Senior Editor
Nature Genetics
https://orcid.org/0000-0003-0864-1200

Reviewer #5 (Remarks to the Author):

Our comments were addressed in the revised manuscript.

ORCID

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Final Decision Letter:

19th Jan 2021

Dear Filippo,

I am delighted to say that your manuscript "A unique bipartite Polycomb signature regulates stimulus-response transcription during development" has been accepted for publication in an upcoming issue of Nature Genetics.

Prior to setting your manuscript, we may make minor changes to enhance the lucidity of the text and with reference to our house style. We therefore ask that you examine the proofs most carefully to ensure that we have not inadvertently altered the sense of your text in any way.

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