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BRD4-PRC2 Represses Transcription of T-helper 2-Specific Negative Regulators during T-cell Differentiation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 14th Jun 2022

Dear Dr. Cheung,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees find the analysis interesting. However, they also find that some further analysis is needed to consider publication here. Should you be able to extend the analysis along the lines indicated by the referees then I would like to consider a revised version. We would need further support for that the BRD4-PRC2 complex is directly involved in the repression of some BRD4 target gene and also for the mechanism involved (via PRC2).

I am happy to discuss the raised points further and it would be helpful to do so via either email or a video call.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

I thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further with you.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

I have attached a document with helpful tips on how to prepare the revised version

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (12th Sep 2022).

As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

If you require more time to complete the revisions let me know as we can grant an extension.

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Zhao and colleagues nicely demonstrate a novel role for BRD4 in regulating Th2 differentiation through the recruitment of repressive cofactors. They show that Th2 differentiation and cytokine production are regulated by Brd4-dependent distinct mechanisms, with Brd4 acting to repress Foxp3 and Fbxw7 loci and activate Il4, Il5, and IL13. Repression of Foxp3 and Fbxw7 is important for maintaining the transcriptional and translational expression of Th2 regulator Gata3, which induces downstream cytokine genes. They further determine that Brd4 recruits G9a to the Foxp3 promoter and PRC2 to the Foxp3 and Fbxw7 promoters. They show that Brd4 interacts with EED of PRC2 through BD2 using selective inhibitors and identify acetylated lysines on EED that likely support this binding. This is a very nice and complete study of gene-specific regulation in Th2 differentiation through different functional co-factors of BRD4.

Two things would be nice additions:

1) (Essential) Figure 4H shows that the interaction can be competed using K19ac peptides of EED. These data would be further

strengthened by expressing the EED mutant form and seeing if this has weakened interaction with BRD4.
2) (Non-essential) There is a nice workup of BD2 interaction with BRD4. To further demonstrate the multifunctional relationships on BRD4, the same analysis would be good to show with G9a, as I don't see that analysis in prior literature cited by the authors. For example, the authors show that BRD4 interacts with G9a-is this BD-dependent? Can the authors determine whether BD1 or BD2 is required in this binding and putative acetyl sites on G9a?

Referee #2:

This is a very interesting study that described a non-canonical function for BRD4 in repressing expression of lineage inappropriate genes to maintain Th2 cell phenotypic stability. The study has impressive breadth ranging from studying the molecular interactions between protein complex members often in primary Th cells to in vivo models of allergic asthma. I have some concerns and a number of questions that a believe should be answered to support the author's conclusions.

Major questions:

Cell viability- the authors perform many experiments using primary lymphocytes which are well known to be sensitive to perturbation often resulting in cell death. I am concerned that cell death after treatment or overexpression may affect the interpretation of the results. Therefore, I think it is of upmost importance that they authors include information about cell viability after their treatments for example after JQ1 or shRNA knockdown of Brd4.
This applies not only to the in vitro studies but also the in vivo asthma model-there are very few Brd4-knockdown lymphocytes detected in the BAL-is that because there is a survival difference not an effect on differentiation?

Throughout the manuscript (for example in Figure 1 and Supp Figure 1) cells were treated with JQ1, however I couldn't find how long the cells were treated for. This is important because the authors suggest that many genes were surprisingly upregulated, but these could be secondary effects if the treatment of JQ1 was for a longer period. This information should be reported but regardless I think it is important to perform a kinetic of JQ1 treatment on Th2 cells to observe how rapidly Foxp3 and Fbxw7are upregulated.

Minor questions:

-Clarification of the asthma model used: The authors generate polyclonal control and Brd4 knockdown Th2 cells from C57BL/6 mice and then inject them into Rag1-/- mice that have been challenged with OVA protein. If the Th2 cells are not antigen specific how is an OVA-specific response generated?

-Figure 2D and E- Instead of the bar graphs with relative %. The IL4 and Gata3 data should be expressed as Mean Fluorescence Intensity (MFI).

-Please explain the 16-fold difference in il4 transcription after Fbxw7 knockdown (Fig. 2b) but only a modest difference in IL4 protein (Fig. 2d).

-Moreover, in Supp Fig 3I: In contrast to the differences shown by the FACS analysis, why is the Gata3 band after Fbxw7 knockdown so prominent compared to control Th2 cells? It suggests that Gata3 is lowly expressed in the control Th2 cells where it should be high. Please explain.

-At the bottom of page 6 the authors state "which is due to induction of Fbxw7 by JQ1". The data shown only reveals a correlation with induction of Fbxw7 by JQ1 and Gata3 degradation. Therefore I suggest the authors either change this statement to reflect perform the direct experiment of performing JQ1 inhibition of Fbxw7 knockdown cells and studying Gata3 ubiquitination and degradation etc.

-No axis labels on the FACs plots in Supp Figure 1A.

Referee #3:

The authors have studied a potential role of BRD4 in gene repression and have tried to provide mechanistic insights into such role. To this extent they have addressed the role of BRD4 in the regulation of T-helper cell differentiation. By gene expression analysis the authors find that the inhibition of BRD4 results in both decreased and increased expression of genes. The authors are surprised by the latter finding and therefore perform experiments to study how BRD4 can work as transcriptional repressor. First, they show that BRD4 can be found associated with some of the genes with increased expression. Thereafter, they show that BRD4 can associate with the Polycomb Repressive Complex 2 and that the interaction occurs through the binding of the BRD4 BD2 domain to acetylated lysine 19 of EED. This is the main new finding of the manuscript and the remaining

experiments have been performed to support this observation.

The potential main novelty of the manuscript is the mechanistic elucidation of how BRD4 can work as a transcriptional repressor, and that this involves the binding of BRD4 to acetylated lysine 19 of EED. The authors propose that BRD4 in this way recruits PRC2 to target genes leading to their transcriptional repression.

The observations in the manuscript are novel and could be of interest to publish in EMBO Journal, however, before this can be recommended the authors should address the critical points as listed below:

Specific Major Concerns

1. Correlations or mechanistic insights?
Although the authors try to show that a BRD4-PRC2 complex is directly involved in the repression of some BRD4 target genes, the provided experiments do not convincingly support this.
a. It is not clear from any of the experiments how long the cells were treated with JQ1 before samples were harvested for RNA-sequencing or any of the other assays presented in the manuscript. The authors should perform time-course studies to understand the kinetics of down- versus up-regulated genes using JQ1. Moreover, it is strongly suggested that the authors consider generating cells in which BRD4 is tagged with a degradation tag, so better and clearer time-resolved experiments can be done. The fact that the expression of some genes is increased after treatment with JQ1 after some time is not surprisingly at all.
b. The authors provide results suggesting that PRC2 and BRD4 are in a complex and later show that the interactions between BRD4 and the two PRC2 components SUZ12 and EZH2 is dependent on DNA (Benzonase treatment). Surprisingly a similar experiment is not provided for EED, and the authors should do that.
c. The authors map a potential site of binding to EED to acetylated lysine 19, however, disappointingly they do not introduce an EEDK19R mutant into their cellular system to address the importance of this interaction for recruitment of PRC2 to repressed genes or the importance of this residue for transcriptional repression. Such an experiment is key for the acceptance of the manuscript.

2. Model
The authors propose that BRD4 recruits PRC2 to genes where BRD4 works as a transcriptional repressor. How do the authors envision that will work? Is EED recruited independently of the remaining part of the PRC2 complex? How is BRD4 recruited to these genes? Some kinetic data to support the working model would be important for the manuscript.

Other concerns
1. The methods section does not contain any description of how RNA-seq and ChIP-seq data were analyzed.
2. BRD4-PRC2 interactions: Figure 3D should be supported with mass spec data. How many proteins do the authors observe binding to BRD4 with the used experimental conditions? Do the authors suggest that all the observed interactions are specific?
3. Several figures are lacking negative controls:
• Fig. 4B to the right and bottom lack a negative control for a nuclear protein precipitated by the Flag antibody and is HA tagged.
• Figure 4D lacks a negative control, which could be HA-EEDK19R.

Point-to-Point Responses to Reviewer's Comments (*EMBOJ* MS# 2022-111473)

A. Major New Experimental Data Added in the Revision

A.1. We performed detailed studies of BRD4 interaction with EED-WT and EED-K19R. We demonstrated that BRD4 interacts with EED-WT but not EED-K19R mutant. Importantly, we further demonstrated that BRD4 and EED-WT (not K19R) synergistically repress *Fbxw7* promoter activity using luciferase assay.
A.2. We performed additional kinetic studies of BRD4-repressed genes in Th2 cells. We demonstrated that *Nr4a2*, *Foxp3* and *Fbxw7* mRNA are rapidly upregulated 6-24 hours post-treatment of JQ1 and MZ1 in Th2 cells that have been differentiated for 3 days.
A.3. We included new data showing that shRNA knockdown of BRD4 do not lead to more Th2 cell death compared to control shRNA.
A.4. We performed new IP study to demonstrate that BRD4-G9a interaction is not BrD-dependent, as JQ1 has no effect on their interaction in Th2 cells.
A.5. We performed new IP study to demonstrate that benzonase treatment has no effect on BRD4-EED interaction.

B. Major changes in Figures and Supplementary Figures

Figure 4

Fig. 4F– We performed detailed overexpression and reciprocal IP studies of BRD4 interaction with EED-WT and EED-K19R in HEK293 cells.
Fig. 4G– We performed luciferase assay of *Fbxw7* promoter activity using overexpression of BRD4, EED-WT and EED-K19R.

Figure S1

Fig. S1G– We provided additional gating strategies of live/dead cells followed by IL-4 expressing cells in Th2 cells treated with shRNA control and shRNA targeting BRD4.
Fig. S1H– We provided additional cell viability data of Th2 cells treated with shRNA control and shRNA targeting BRD4, as assessed by PI/Annexin V staining.

Figure S3

Fig. S3F– Th2 cells were differentiated for 3 days, then treated with JQ1 and de-repression kinetic studies of *Nr4a2, Foxp3* and *Fbxw7* were performed.
Fig. S3G– Th2 cells were differentiated for 3 days, then treated with MZ1 and de-repression kinetic studies of *Nr4a2, Foxp3* and *Fbxw7* were performed.
Fig. S3K– We provided a new set of Gata3 expression in Th2 cells treated with shRNA control and shRNA targeting Fbxw7, with more obvious Gata3 expression in sh-control treated Th2 cells.

Figure S4

Fig. S4C– We performed IP study of BRD4 interaction with G9a in Th2 cells, treated with or without JQ1.
Fig. S4E– We performed additional overexpression and IP studies of BRD4 interaction with EED in HEK293 cells, of which lysates were treated with benzonase.

C. Referee #1 (Remarks to the Author):

C.1. *"This is a very nice and complete study of gene-specific regulation in Th2 differentiation through different functional co-factors of BRD4."*

Response: We thank the reviewer's recognition of the novelty of our findings on the role of Brd4-PRC2 complex in gene-specific repression during Th2 cell differentiation.

C.2. "*(Essential) Figure 4H shows that the interaction can be competed using K19ac peptides of EED. These data would be further strengthened by expressing the EED mutant form and seeing if this has weakened interaction with BRD4."*

Response: We thank the reviewer for the suggestion of BRD4-EED interaction through EED-K19 site, which we addressed in the revision. Specifically, we demonstrated that EED-K19R mutant fails to interact with BRD4 (**Fig. 4F**). The expression of EED-K19R mutant also rescued BRD4-EED-mediated repression of *Fbxw7* promoter activity in our established luciferase assay system (**Fig. 4G**). Therefore, our data strongly support the functional role of BRD4-EED(K19) in gene repression.

C.3. *"(Non-essential) There is a nice workup of BD2 interaction with BRD4. To further demonstrate the multifunctional relationships on BRD4, the same analysis would be good to show with G9a, as I don't see that analysis in prior literature cited by the authors. For example, the authors show that BRD4 interacts with G9a-is this BD-dependent? Can the authors determine whether BD1 or BD2 is required in this binding and putative acetyl sites on G9a?"*

Response: We thank the reviewer for the interest in BRD4-G9a interaction. We showed that BRD4-G9a interaction is not BrD-dependent, as JQ1 has no effect on their interaction (**Fig. S4C**).

D. Referee #2 (Remarks to the Author):

D.1. *"This is a very interesting study that described a non-canonical function for BRD4 in repressing expression of lineage inappropriate genes to maintain Th2 cell phenotypic stability. The study has impressive breadth ranging from studying the molecular interactions between protein complex members often in primary Th cells to in vivo models of allergic asthma. I have some concerns and a number of questions that a believe should be answered to support the author's conclusions."*

Response: We appreciate this reviewer's appraisal of the novelty and breath of this study on molecular interactions between protein complex in Th2 cells. *Overall*, our study has been greatly strengthened by taking this reviewer's helpful suggestion of including cell viability data and performing kinetic studies. Please see below our detailed responses to the reviewer's specific questions.

D.2. *"Cell viability- the authors perform many experiments using primary lymphocytes which are well known to be sensitive to perturbation often resulting in cell death. I am concerned that cell death after treatment or overexpression may affect the interpretation of the results. Therefore, I think it is of upmost importance that they authors include information about cell viability after their treatments for example after JQ1 or shRNA knockdown of Brd4. This applies not only to the in vitro studies but also the in vivo asthma model-there are very few Brd4-knockdown lymphocytes detected in the BAL-is that because there is a survival difference not an effect on differentiation?"*

Response: We provided a large body of new data in the revision showing that shRNA knockdown of BRD4 do not lead to more cell death as compared to shRNA control, assessed by live/dead cell gating and PI/Annexin staining (**Fig. S1G,H**). Note that we gate on live cells only for IL-4^{+} cells, which excludes the possibility that decrease in Th2 cells is due to cell viability. The same applies for the cells prepared for *in-vivo* study. We sort GFP-expressing (shControl/shBRD4) naïve T cells and

differentiate them to Th2 for 6 days, and we inject equal number of cells into $Rag^{-/-}$ mice. In *in-vivo* asthma model, eosinophils contribute to the elevation in the total leukocytes recruited to the airways [1]. Our data indicate a lack of infiltration of lymphocytes and eosinophils in the BAL fluid at the end of the study, which is 7 days after initial transfer of the Th2 cells. This suggests an initial failure of shBRD4 Th2 cells to secret IL-4/13 precludes later eosinophilic infiltration and amplification of lymphocyte recruitment. This remains to be tested, however, is not the focus of this study. In this study, we have shown that the BRD4 in Th2 cells is required for the induction of *in-vivo* asthma model, potentially through reduced eosinophilic-lymphocyte infiltration mediated by reduced secretion of Th2 cytokines.

D.3. *"Throughout the manuscript (for example in Figure 1 and Supp Figure 1) cells were treated with JQ1, however I couldn't find how long the cells were treated for. This is important because the authors suggest that many genes were surprisingly upregulated, but these could be secondary effects if the treatment of JQ1 was for a longer period. This information should be reported but regardless I think it is important to perform a kinetic of JQ1 treatment on Th2 cells to observe how rapidly Foxp3 and Fbxw7are upregulated."*

Response: We thank the reviewer for the helpful suggestion. We have now made it clear that naïve $CD4^{+}$ T cells are differentiated to Th2 cells for 6 days before analysis, and JQ1 is added on Day 0 unless otherwise specified. We agree with the reviewer's concern that upregulated genes might be secondary effects due to long period of culture time. Therefore, we have performed additional kinetic studies of JQ1 treatment on Th2 cells. Th2 cells were cultured for 3 days, then treated with BRD4-inhibitor JQ1 and -degrader MZ1 for 6, 24, and 48 hours and mRNA de-repression of *Nr4a2*, *Foxp3* and *Fbxw7* were examined (**Fig. S3F,G**). *Foxp3* and *Nr4a2* expression are upregulated in 6 hours, and *Fbxw7* de-repression kinetics is slower. We further used a luciferase assay to confirm that BRD4-EED-WT but not EED-K19R represses *Fbxw7* promoter activity (**Fig. 4G**), supporting that the repressive function is direct. Collectively, our data demonstrate that JQ1 directly de-repress *Foxp3* and *Fbxw7* gene expression, the two critical negative regulators in Th2 cells as shown in our study.

D.4. *"Minor points. Clarification of the asthma model used: The authors generate polyclonal control and Brd4 knockdown Th2 cells from C57BL/6 mice and then inject them into Rag1-/- mice that have been challenged with OVA protein. If the Th2 cells are not antigen specific how is an OVA-specific response generated?"*

Response: We thank the reviewer for the request of clarification. We choose to adoptively transfer Th2 cells into $Rag^{-/-}$ to determine the direct effect of BRD4 in Th2 cells-induced airway inflammation. We used OVA to further sensitize the mice to induce allergic airway inflammation, as this has been shown to be Th2-dependent with high eosinophilic-IgE [1-3]. Although the response is not OVA-specific, this model demonstrates that BRD4 in Th2 cells mediates allergic inflammation (IgE as marker) in OVA-sensitized mice.

D.5. "*Minor points.* Figure 2D and E- Instead of the bar graphs with relative %. The IL4 and Gata3 data should be expressed as Mean Fluorescence Intensity (MFI)."

Response: We thank the reviewer for the suggestion to show data as MFI. However, in this study, we aim to show that knockdown of *Fbxw7* has *consistent effect* on IL-4 and Gata3 expression, therefore we choose to use relative % to demonstrate the trend with statistical significance.

D.6. *"Minor points. Please explain the 16-fold difference in il4 transcription after Fbxw7 knockdown (Fig. 2b) but only a modest difference in IL4 protein (Fig. 2d)."*

Response: The *Il4* mRNA represents an average mRNA level of a mix of Th2 cells and cells that are not fully differentiated, whereas the flow cytometry analysis specifically indicates the percentage of

cells that are expressing IL-4 protein, which we believe leads to the difference. *Notably*, we have shown consistently that Fbxw7 knockdown induces a marked increase of *Il4* mRNA and IL-4 protein expression levels.

D.7. *"Minor points. Moreover, in Supp Fig 3I: In contrast to the differences shown by the FACS analysis, why is the Gata3 band after Fbxw7 knockdown so prominent compared to control Th2 cells? It suggests that Gata3 is lowly expressed in the control Th2 cells where it should be high. Please explain."*

Response: Previously, we used a very short exposure time to demonstrate that knockdown of shFbxw7 can upregulated Gata3 protein expression with prominent contrast. We have now provided updated data in **Fig.S3K**, with a more appropriate exposure time to reflect the Gata3 expression in Th2 cells. Note that Fbxw7 knockdown induces about 3-fold of Gata3 protein induction in Th2 cells by Western blotting (**Fig.S3K**), which is consistent with FACS analysis (**Fig. 2D**).

D.8. *"Minor points. At the bottom of page 6 the authors state "which is due to induction of Fbxw7 by JQ1". The data shown only reveals a correlation with induction of Fbxw7 by JQ1 and Gata3 degradation. Therefore I suggest the authors either change this statement to reflect perform the direct experiment of performing JQ1 inhibition of Fbxw7 knockdown cells and studying Gata3 ubiquitination and degradation etc."*

Response: We thank the reviewer for this comment. We have changed the statement to "which correlated with the induction of *Fbxw7* by JQ1" as suggested to reflect a correlation but not causal relationship.

D.9. *"Minor points. No axis labels on the FACs plots in Supp Figure 1A"*

Response: We have updated the axis labels on the FACS plots in **Fig. S1A** as suggested.

E. Referee #3 (Remarks to the Author):

E.1. *"The authors have studied a potential role of BRD4 in gene repression and have tried to provide mechanistic insights into such role. To this extent they have addressed the role of BRD4 in the regulation of T-helper cell differentiation. By gene expression analysis the authors find that the inhibition of BRD4 results in both decreased and increased expression of genes. The authors are surprised by the latter finding and therefore perform experiments to study how BRD4 can work as transcriptional repressor. First, they show that BRD4 can be found associated with some of the genes with increased expression. Thereafter, they show that BRD4 can associate with the Polycomb Repressive Complex 2 and that the interaction occurs through the binding of the BRD4 BD2 domain to acetylated lysine 19 of EED. This is the main new finding of the manuscript and the remaining experiments have been performed to support this observation.*

The potential main novelty of the manuscript is the mechanistic elucidation of how BRD4 can work as a transcriptional repressor, and that this involves the binding of BRD4 to acetylated lysine 19 of EED. The authors propose that BRD4 in this way recruits PRC2 to target genes leading to their transcriptional repression.

The observations in the manuscript are novel and could be of interest to publish in EMBO Journal, however, before this can be recommended the authors should address the critical points as listed below:"

Response: We thank the reviewer's recognition of the novelty of our study. We also appreciate the reviewer's constructive critique about the details of this study. During the revision of our manuscript, we have obtained compelling new data using kinetic studies, luciferase assay, and EED-K19R mutant (see below) that have greatly facilitated our ability to confirm that BRD4-PRC2 complex is directly involved in repression of some BRD4-target genes, and the functional importance of EED-K19ac in interaction with BRD4 and repression of genes. Additionally, we have provided substantial amount of new results to further strengthen and validate our mechanistic model, which are described in detail below in response to this reviewer's specific comment.

E.2. *"Correlations or mechanistic insights?*
Although the authors try to show that a BRD4-PRC2 complex is directly involved in the repression of some BRD4 target genes, the provided experiments do not convincingly support this.

a. It is not clear from any of the experiments how long the cells were treated with JQ1 before samples were harvested for RNA-sequencing or any of the other assays presented in the manuscript. The authors should perform time-course studies to understand the kinetics of down- versus up-regulated genes using JQ1. Moreover, it is strongly suggested that the authors consider generating cells in which BRD4 is tagged with a degradation tag, so better and clearer time-resolved experiments can be done. The fact that the expression of some genes is increased after treatment with JQ1 after some time is not surprisingly at all."

Response: We thank the reviewer for the helpful suggestion. We have now made it clear that naïve $CD4^+$ T cells are differentiated to Th2 cells for 6 days before analysis, and JQ1 is added on Day 0 unless otherwise specified. We agree with the reviewer's concern that upregulated genes might be secondary effects due to long period of culture time. Therefore, we have performed additional kinetic studies of JQ1 treatment of Th2 cells. Th2 cells were cultured for 3 days, then treated with BRD4-inhibitor JQ1 for 6, 24, and 48 hours and mRNA de-repression of *Nr4a2*, *Foxp3* and *Fbxw7* were examined (**Fig. S3F**). To address the reviewer's suggestion to generate cells in which BRD4 tagged with a degradation tag, we used a BRD4 PROTAC degrader MZ1 that has been shown to degrade BRD4 protein and demonstrated similar results to JQ1 treatments in kinetic studies (**Fig. S3G**). *Foxp3* and *Nr4a2* expression are upregulated in 6 hours, and *Fbxw7* de-repression kinetics is slower. We further used a luciferase assay to confirm that BRD4-EED-WT but not EED-K19R represses *Fbxw7* promoter activity (**Fig. 4G**), supporting that the repressive function is direct. Overall, our studies support a direct role of BRD4-PRC2 complex in gene transcriptional repression of *Foxp3*, *Fbxw7* and *Nr4a2*.

E.3 *"b. The authors provide results suggesting that PRC2 and BRD4 are in a complex and later show that the interactions between BRD4 and the two PRC2 components SUZ12 and EZH2 is dependent on DNA (Benzonase treatment). Surprisingly a similar experiment is not provided for EED, and the authors should do that"*

Response: We thank the reviewer for raising this important concern. We showed that BRD4-EED interaction is not dependent on DNA, as benzonase treatment has no effect on their interaction (**Fig. S4E**).

E.4 *"c. The authors map a potential site of binding to EED to acetylated lysine 19, however, disappointingly they do not introduce an EEDK19R mutant into their cellular system to address the importance of this interaction for recruitment of PRC2 to repressed genes or the importance of this residue for transcriptional repression. Such an experiment is key for the acceptance of the manuscript."*

Response: Per this reviewer's helpful suggestion to "introduce EEDK19R mutant into our cellular system to address the importance of this residue for transcriptional repression", we have now clearly

demonstrated that EED-K19R mutant fails to interact with BRD4 (**Fig. 4F**). We have previously established a direct repression effect of BRD4 dependent on the bromodomains on the promoter activity of *Fbxw7* (**Fig. S3E**). Using this cellular system, we further extend our findings by introducing EEDK19R mutant. We found that the expression of BRD4+EED synergistically represses, while EED-K19R mutant rescues BRD4-EED-mediated repression of *Fbxw7* promoter activity (**Fig. 4G**). Therefore, our data has demonstrated a direct role of BRD4-EED(K19) interaction in gene transcription repression.

E.5 *"2. Model*
The authors propose that BRD4 recruits PRC2 to genes where BRD4 works as a transcriptional repressor. How do the authors envision that will work? Is EED recruited independently of the remaining part of the PRC2 complex? How is BRD4 recruited to these genes? Some kinetic data to support the working model would be important for the manuscript."

Response: We have demonstrated that BRD4 is a master regulator of PRC2 complex through different mechanisms, as our data suggest that BRD4 interacts with DNA/RNA (benzonase study) to recruit Ezh2 and Suz12 to chromatin, while BRD4 interacts with EED(K19ac) directly, to coordinately ensure the intact PRC2 complex on gene loci for transcriptional repression.

To address how BRD4 is recruited to the repressed genes, we demonstrated that YY1 and Gata3 recruits BRD4 to repressed genes, using shRNA targeting YY1 and Gatat3 (**Fig. 3B**), and PRC2 complex binding to repressed gene loci decreased upon JQ1 treatment. Therefore, we have clearly established that TFs recruits BRD4-PRC2 complex to represses Th2-negative regulators during Th2 cell differentiation.

However, we believe that kinetic studies to determine the sequence of TFs-BRD4-PRC2 complex formation on repressed genes will likely not generate significant insights, as we envision that the chromatin binding of these complexes would not be sequential.

E.6. *"Other concerns*
1. The methods section does not contain any description of how RNA-seq and ChIP-seq data were analyzed."

Response: We now updated our methods section to contain description of RNA-seq and ChIP-seq analysis.

E.7. *"BRD4-PRC2 interactions: Figure 3D should be supported with mass spec data. How many proteins do the authors observe binding to BRD4 with the used experimental conditions? Do the authors suggest that all the observed interactions are specific?"*

Response: We thank the reviewer's suggestion of using mass spec data to support our **Fig. 3D** showing BRD4 interaction with Ezh2, Suz12, EED and G9a. *Importantly,* our **Fig. 3D** showed that BRD4 interacts with G9a and Ezh2, which have been confirmed by recent papers using IP approaches [4, 5]. In another study it has also been confirmed that BRD4 could pulldown Suz12/Ezh2 using mass spectrometry approach [6]. Collectively, the previous literature has confirmed the validity of **Fig. 3D** and strengthened our discovery of the novel BRD4-EED interaction. Notably, our data indicated that all interactions are specific, as IgG has no pulldown.

E.8. "3. Several figures are lacking negative controls:
• Fig. 4B to the right and bottom lack a negative control for a nuclear protein precipitated by the Flag antibody and is HA tagged.• Figure 4D lacks a negative control, which could be HA-EEDK19R."

Response: We thank the reviewer for the concern for negative controls. In **Fig. 4B**, IgG is the negative control. In **Fig. 4D**, The HA-EED-K19R would not be a good negative control, as EED-K19ac is not the only acetylated site. Our IP-MS data have shown that K19, K221, K251 sites are all acetylated. However, the study of EED-compound mutant will be unnecessary to determine that the EED is actually acetylated, as this has been confirmed by IP-MS.

References:

1. Whitehead, G.S., et al., *Allergen-induced airway disease is mouse strain dependent.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(1): p. L32-42.

2. Kumar, R.K., C. Herbert, and P.S. Foster, *The "classical" ovalbumin challenge model of asthma in mice.* Curr Drug Targets, 2008. **9**(6): p. 485-94.

3. Brewer, J.P., A.B. Kisselgof, and T.R. Martin, *Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice.* Am J Respir Crit Care Med, 1999. **160**(4): p. 1150-6.

4. Sakamaki, J.I., et al., *Bromodomain Protein BRD4 Is a Transcriptional Repressor of Autophagy and Lysosomal Function.* Mol Cell, 2017. **66**(4): p. 517-532 e9.

5. Zhou, D., et al., *FACT subunit SUPT16H associates with BRD4 and contributes to silencing of interferon signaling.* Nucleic Acids Res, 2022. **50**(15): p. 8700-8718.

6. Lambert, J.P., et al., *Interactome Rewiring Following Pharmacological Targeting of BET Bromodomains.* Mol Cell, 2019. **73**(3): p. 621-638 e17.

1st Revision - Editorial Decision 21st Dec 2022

Dear Kalung,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below. As you can see the referees appreciate the added data and are supportive of publication here. Referee #3 has a few minor points that should be fairly easy to address.

When you submit the revised version please also address the following editorial points:

- Please upload high resolution single figure files

- I think that the SFig 1-5 are intended to be EV figures. Please rename the figures as EV Figure 1 etc, correct figure call outs in text, remove the legend from the figure files and add them to the main MS file. I see that Figure S1 runs over two pages - please make it into two separate EV files. We normally can only have 5 EV figures, but in this case, I can grant an exception to have 6.

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Almost there!

Best Karin

Karin Dumstrei, PhD
Senior Editor
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Referee #2:

The authors have clearly responded to my comments.

Referee #3:

As mentioned in my previous review, the potential main novelty of the manuscript is the mechanistic elucidation of how BRD4

can work as a transcriptional repressor, involving the binding of BRD4 to acetylated lysine 19 of EED.

In the revised manuscript, the authors have addressed most of my main concerns. They have added kinetic studies to further address the mechanism by which BRD4 can work as a transcriptional repressor, and they have performed some extra control experiments. Although the manuscript is improved, I have a few concerns that the authors should address:

1. It is still not clear in most of the experiments (e.g. Figures 1 and 3) for how long the authors treated the cells with JQ1 or the other inhibitors used in the study. Moreover, it is assumed that Day 0 is the start of the experiment after 6 days of differentiation. The authors should make that clear in the figure legends.

2. In the authors model, BRD4 recruits EED and the PRC2 complex to BRD4 repressed genes by binding to acetylated K19 at EED. The BD2 domain of BRD4 is required for this binding. In the kinetic studies, now provided by the authors (6, 24, 48 hours, Fig S3FG), they show a minor increase in mRNA levels of the three tested genes at 6 hours (for Fbxw7, this increase is not significant). The lack of increase could be explained by a slow removal of H3K27me3 from the three genes, however, the authors do not address that in the manuscript. To strengthen the kinetic observations, the authors could perform ChIP experiments of BRD4, EED and H3K27me3 at the promoters for the three genes 6, 24 and 48 hours after JQ1 treatment. I would actually like to see an earlier timepoint as well (at one hour) to know the early effects of JQ1 on BRD4 and EED binding.

3. The authors claim that BRD4 binding overlaps with GATA3 binding, however, this does not appear to be the case on the Fbxw7 gene (Fig. 3A). The authors should comment on that.

Point-to-Point Responses to Reviewer's Comments (*EMBOJ* MS# 2022-111473)

A. Referee #2 (Remarks to the Author):

A.1. "The authors have clearly responded to my comments."

Response: We appreciate the reviewer's constructive comments.

B. Referee #3 (Remarks to the Author):

B.1. "It is still not clear in most of the experiments (e.g. Figures 1 and 3) for how long the authors treated the cells with JQ1 or the other inhibitors used in the study. Moreover, it is assumed that Day 0 is the start of the experiment after 6 days of differentiation. The authors should make that clear in the figure legends."

Response: We thank the reviewer for the helpful suggestion. We have made it very clear in all the experiments and figure legends (data information) that "Mouse naïve $CD4^{+}$ T cells were cultured in Th2 polarization condition and treated with or without inhibitors on Day 0 and were differentiated for 6 days before analysis, unless otherwise specified".

B.2. "In the authors model, BRD4 recruits EED and the PRC2 complex to BRD4 repressed genes by binding to acetylated K19 at EED. The BD2 domain of BRD4 is required for this binding. In the kinetic studies, now provided by the authors (6, 24, 48 hours, Fig S3FG), they show a minor increase in mRNA levels of the three tested genes at 6 hours (for Fbxw7, this increase is not significant). The lack of increase could be explained by a slow removal of H3K27me3 from the three genes, however, the authors do not address that in the manuscript. To strengthen the kinetic observations, the authors could perform ChIP experiments of BRD4, EED and H3K27me3 at the promoters for the three genes 6, 24 and 48 hours after JQ1 treatment. I would actually like to see an earlier timepoint as well (at one hour) to know the early effects of JQ1 on BRD4 and EED binding."

Response: We thank the reviewer for the helpful suggestion. To strengthen the kinetic observations, we have performed ChIP experiments of BRD4 and EED on the regulatory regions of *Fbxw7* and *Foxp3* 6, 24 and 48 hours after JQ1 treatment, which correlate with the de-repression of mRNA level of *Fbxw7* and *Foxp3* (**Fig. EV4F, G and Fig. EV5G**).

B.3. "The authors claim that BRD4 binding overlaps with GATA3 binding, however, this does not appear to be the case on the Fbxw7 gene (Fig. 3A). The authors should comment on that."

Response: We thank the reviewer for the request of clarification. We have now updated the sentence "Gata3 co-localized with Brd4 on Foxp3 and Fbxw7, in addition to Il4, Il5 and Il13 gene loci (**Fig. 3A**)" to "Gata3 binds closely to or co-localizes with Brd4 binding sites on Foxp3 and Fbxw7, in addition to Il4, Il5 and Il13 gene loci (**Fig. 3A**)".

2nd Revision - Editorial Decision 11th Jan 2023

Dear William,

Thank you for submitting your revised manuscript. I have now had a chance to take a careful look at the revised version and all looks good.

I am therefore very pleased to accept the MS for publication here.

Congratulations on a nice study

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are tests one-sided or two-sided?
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Please complete ALL of the questions below.
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