THE EMBO JOURNAL

An alternative NURF complex sustains acute myeloid leukemia by regulating the accessibility of insulator regions.

Kristian Helin, Aliaksandra Radzisheuskaya, Isabel Pena Rømer, Eugenia Lorenzini, Richard Koche, Yingqian Zhan, Pavel Shliaha, Alexandra Cooper, Daria Shlyueva, Jens Johansen, Ronald Hendrickson, and Zheng Fan
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Transaction Report:

(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 **1st Jun 2023**

Dear Dr. Helin,

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise for the protracted assessment process due to delays in referee report submission. We have now received comments from two reviewers, which are included below for your information.

As you will see from the reports, all reviewers find the proposed role of alternative NURF complex in sustaining AML of interest. However, reviewer 2 also raises several points that would need to be addressed, including further validation of the direct role of NURF in the alteration of insulator accessibility and a more thorough investigation of the role of MYC. I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

Based on the interest expressed in the reports, I invite you to address these issues in a revised version of the manuscript. I should also add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

We generally allow three months as standard revision time, which can be extended to six months in the case of major revisions. 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, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this deadline, please let us know in advance to discuss an extension.

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. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to discussing your revision.

Yours sincerely,

Cornelius Schneider

Cornelius Schneider, PhD
Editor
The EMBO Journal
c.schneider@embojournal.org

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)

- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (30th Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

Referee #1:

Radzisheuskaya et al report the results of a CRISPRi screen to identify chromatin associated factors required for survival of MLL-rearranged AML cells. The authors discovered that BPTF depletion provides vulnerability to both THP-1 and MOLM-13 cells, which carry Mll-Af9 fusions. Moreover, the authors discover that in these cells, BPTF is part of a novel NURF complex that contains SMARC5 rather than SMARCA1, consistent with very low/non-expression of SMARCA1. They also determined that loss of function of this alternative NURF complex impacts MYC expression, consistent with decreased cell proliferation. Interestingly, targeting of the complex is not through the PHD2-Bromo domain in BPTF, but through other less well characterized domains. Mechanistically, decreased expression of Myc and other key regulators is associated with changes in accessibility of insulator regions that in turn impact promoter-enhancer interactions.

This study is well designed and well executed. All key findings are confirmed by orthologous approaches, and all results are judiciously interpreted. This work is impactful in several ways, including at least 2 therapeutic implications. First, bromodomain inhibitors are not likely to be efficacious in this setting, and second, SMARCA5 dependence provides a new therapeutic target.

Minor detail: on page, the test describing Fig 1D-E seems off relative to the data shown in those panels. Please recheck.

Referee #2:

GENERAL SUMMARY:

This manuscript is an interesting and potentially exciting work that identifies an alternative NURF complex as a new potential vulnerability of AML.

Strengths:
- authors uncover a protein complex that may represent a new target in MLL-rearranged AML
- authors employ a variety of modern techniques, ranging from CRISPR screens, CUT&RUN, to targeted protein degradation
- validation of portions of the work using a high-quality animal model
- refinement of certain functional elements to a more constrained portion of BPTF, yielding a surprising finding

Weaknesses:
- mechanistic chromatin work largely focuses on only one (prominent) cell line
- the majority of chromatin mechanism is assessed days after manipulation, with a smaller portion of the work performed using more rapid approaches
- a central claim that the new complex acts via altered chromatin insulator function has the potential to be masked by the potentially large-scale effects of cell differentiation, which the authors have not yet assessed
- a role of MYC has not been tested as rigorously in this setting as it has for other related chromatin remodelers such as SWI/SNF
- conceptual integration with related recent works in the literature, including SWI/SNF but potentially others could be

strengthened by highlighting shared and/or divergent mechanisms

SPECIFIC POINTS:

Given the successful advances in targeting MLL-rearranged AML with menin-MLL inhibitors, why did the authors choose specifically to target MLLr leukemias? Is the role of the new complex unique to MLLr AMLs or does BPTF/SMARCA5 have similar functions in non-MLLr AML settings? Given the lack of testing in a non-MLLr setting, I would recommend that authors either address this issue in a non-MLLr cell line as well, or consider changing their title to reflect MLLr AML rather than AML broadly.

Despite major similarities with the mechanism of action of SWI/SNF chromatin remodeling activity in AML, and despite recurrent hits to SWI/SNF subunits in their screen, authors do not mention it among the "several factors previously reported to sustain the proliferation of leukemic cells." This prominent ATP-dependent remodeling complex has recently been shown to be a strong and targetable dependency of AML by multiple labs (PMID 36662812, 34799403, 24285714), and as described below, acts via similar pathways. At a minimum, the distinction of the functional roles for the new NURF complex with those of SWI/SNF described in these earlier works should be more strongly identified and discussed. This is especially relevant, since SWI/SNF also acts by sustaining expression of MYC, and even more specifically, by modifying the chromatin landscape at the blood enhancer cluster (BENC), a particular focus of PMID 36662812 and the present manuscript. Are the mechanisms that the authors describe for the alternative NURF complex shared or distinct from those of SWI/SNF? This may help to highlight the shared or unique functions of these complexes.

Despite the prominent statement of the title, the authors have not strongly shown altered insulator function to be a primary effect rather than a secondary consequence. Since loss of MYC has been shown in multiple settings to be associated with differentiation in AML, an important unaddressed concern I have is that loss of BPTF and/or SMARCA5 may induce differentiation, which is furthermore a common therapeutic outcome in AML. If cells undergo a large-scale state change such as differentiation, it would be especially important for the authors to examine whether the alteration of insulator accessibility is a cause or consequence of differentiation. Fortunately the authors have generated a dTag system for SMARCA5, and they could employ it (rather than CRISPR KO) to examine more fast-acting effects on insulator function, for example via ATAC-seq. Authors should assess whether BPTF/SMARCA5 perturbations induce differentiation using flow cytometry for cell surface markers. If differentiation occurs, dTag studies would allow authors to assess whether the changes in chromatin accessibility at insulator sites occur prior to differentiation.

Does the dependency on the alternative NURF complex arise through sustaining MYC expression via BENC? Ectopic MYC expression would permit authors to directly evaluate if MYC expression is sufficient to rescue BPTF/SMARCA5 loss-of-function. If MYC expression is insufficient, this would provide strong evidence that indeed broader regulation of chromatin state is key. On the other hand, if forced MYC expression is sufficient, this would provide strong evidence of the relevant mechanism being the promotion of MYC expression.

The use of K562 cells for genomic annotation is not ideal. K562 are a chronic myelogenous leukemia (CML) cell line, which despite some similarities is a distinct type of cancer than AML. Many histone marks, CTCF, etc., are available for THP1 cells from GEO, ENCODE, and other resources; the use of ChromHMM on these available public ChIP datasets would provide a higher degree of rigor.

The authors' results strongly demonstrate that the PHD2 and BROMO domains of BPTF are not required, however they do not draw a conclusion for readers what this result proves or disproves from a mechanistic standpoint. If these domains are dispensable, then how does BPTF exert its function in AML? It would be helpful if authors state a more positive claim about how BPTF exerts its function for readers to understand how this system works.

Overall I believe the work is very interesting and with some additions, will be sound. If the authors can strongly address these points, I would support publication.

Minor/non-essential comments:

Some sentences could use some moderation, for example, in the introduction: "These fusions drive leukemia by dysregulation of the genes [...]" I believe might be better stated as "These fusions drive leukemia in part by dysregulation of the genes [...]"

"A large fraction of all active promoters and insulators demonstrates BPTF and SMARCA5 binding, while only a subset of enhancers (Expanded View Figure 4A)." -- This grammar here is confusing, and seemingly has a dangling incomplete phrase.

Response to Referees

New Figures:
Figure 1L,
Figure 4D, K, L.
Figure EV1D (addition of U937, OCI-AML2), F, G (addition of U937, OCI-AML2), J
Figure EV2A, B, K, L
Figure EV3C-J
Figure EV4B
Appendix Figure S2
Appendix Figure S3E

Changes to the old Figures:
Old Figure EV2, now Appendix Figure S1
Old Figure EV4B-F, now Appendix Figure S3A-D,F
Old Figure 4H,I, now Appendix Figure S3G,H
Old Figure 4C, now Figure EV3A

Referee #1:

Radzisheuskaya et al report the results of a CRISPRi screen to identify chromatin associated factors required for survival of MLL-rearranged AML cells. The authors discovered that BPTF depletion provides vulnerability to both THP-1 and MOLM-13 cells, which carry Mll-Af9 fusions. Moreover, the authors discover that in these cells, BPTF is part of a novel NURF complex that contains SMARC5 rather than SMARCA1, consistent with very low/non-expression of SMARCA1. They also determined that loss of function of this alternative NURF complex impacts MYC expression, consistent with decreased cell proliferation. Interestingly, targeting of the complex is not through the PHD2-Bromo domain in BPTF, but through other less well characterized domains. Mechanistically, decreased expression of Myc and other key regulators is associated with changes in accessibility of insulator regions that in turn impact promoter-enhancer interactions.

This study is well designed and well executed. All key findings are confirmed by orthologous approaches, and all results are judiciously interpreted. This work is impactful in several ways, including at least 2 therapeutic implications. First, bromodomain inhibitors are not likely to be efficacious in this setting, and second, SMARCA5 dependence provides a new therapeutic target.

Minor detail: on page, the test describing Fig 1D-E seems off relative to the data shown in those panels. Please recheck.

We appreciate the reviewer's positive feedback. We have made the necessary revisions to the text in question to improve its clarity. The updated version now reads as follows:

"Knock-down of BPTF in THP-1 dCas9 cells efficiently reduced BPTF expression (Figure 1D) and impacted cell proliferation, as measured in a competition assay with untransduced cells (Figure 1E)."

Referee #2:

GENERAL SUMMARY:

This manuscript is an interesting and potentially exciting work that identifies an alternative NURF complex as a new potential vulnerability of AML.

Strengths:
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- validation of portions of the work using a high-quality animal model
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- conceptual integration with related recent works in the literature, including SWI/SNF but potentially others could be strengthened by highlighting shared and/or divergent mechanisms

We appreciate the constructive feedback that helped us to improve our study. Please find our response to the raised questions below.

SPECIFIC POINTS:

Given the successful advances in targeting MLL-rearranged AML with menin-MLL inhibitors, why did the authors choose specifically to target MLLr leukemias? Is the role of the new complex unique to MLLr AMLs or does BPTF/SMARCA5 have similar functions in non-MLLr AML settings? Given the lack of testing in a non-MLLr setting, I would recommend that authors either address this issue in a non-MLLr cell line as well, or consider changing their title to reflect MLLr AML rather than AML broadly.

In the revised manuscript, we present evidence that the BPTF/SMARCA5-containing NURF complex has similar functions in MLLr and non-MLLr cell lines. In particular:

- We show that BPTF and SMARCA5 are essential in several MLLr and non-MLLr AML cell lines (new Figures EV1D and EV1G). In addition, Figure 1K represents Depmap portal data and highlights the dependency of most of the AML cell lines on SMARCA5 and BPTF (58/58 AML cell lines are sensitive to *BPTF* KO and 42/58 – to *SMARCA5* KO).

- We now demonstrate that BPTF and SMARCA5 proteins interact in non-MLLr cell lines SET2, OCI-AML2, and U937 (new Figure EV1F).

- Similarly, to our observations in THP-1 cells, we now show that BPTF and SMARCA5 co-bind most of the insulator regions in non-MLLr AML cell lines OCI-AML2 and U937 (new Figure EV3E-J).

Taken together, these results demonstrate that the BPTF/SMARCA5-containing NURF complex represents a vulnerability in AML cells and works by regulating the accessibility of the insulator regions.

Despite major similarities with the mechanism of action of SWI/SNF chromatin remodeling activity in AML, and despite recurrent hits to SWI/SNF subunits in their screen, authors do not mention it among the "several factors previously reported to sustain the proliferation of leukemic cells." This prominent ATP-dependent remodeling complex has recently been shown to be a strong and targetable dependency of AML by multiple labs (PMID 36662812, 34799403, 24285714), and as described below, acts via similar pathways. At a minimum, the distinction of the functional roles for the new NURF complex with those of SWI/SNF described in these earlier works should be more strongly identified and discussed. This is especially relevant, since SWI/SNF also acts by sustaining expression of MYC, and even more specifically, by modifying the chromatin landscape at the blood enhancer cluster (BENC), a particular focus of PMID 36662812 and the present manuscript. Are the mechanisms that the authors describe for the alternative NURF complex shared or distinct from those of SWI/SNF? This may help to highlight the shared or unique functions of these complexes.

In the revised manuscript, we remark the SWI/SNF complex subunits as hits on our CRISPRi screen. In addition, we discuss the similarities and differences in the function of the NURF and SWI/SNF complexes. This part of the revised manuscript reads as follows:

“Several previous reports demonstrated the importance of another chromatin remodeling complex, SWI/SNF, for the BENC enhancer function (Chambers et al., 2023) and MYC expression in AML cells (Rago et al., 2022; Shi et al., 2013). While both remodeling complexes (NURF and SWI/SNF) localize to the promoter, enhancer, and insulator regions (Barisic et al., 2019; Centore et al., 2020), their loss affects the accessibility of these regions differently. We found that NURF loss does not impact the accessibility of promoters and enhancers, while it reduces the accessibility of the insulator regions and impacts CTCF binding. Similar findings were observed upon Smarca5 KO in mouse ES cells (Barisic et al., 2019). SWI/SNF loss mainly affects the accessibility of enhancer regions, which leads to decreased binding of lineage-specific transcription factors and reduced target gene expression (Barisic et al., 2019; Centore et al., 2020; Chambers et al., 2023). In the AML context, the SWI/SNF complex mediates the enhancer function of BENC, enabling the binding of RUNX1, LMO2 and MEIS1 at the locus. SWI/SNF loss leads to a substantial reduction in MYC expression in AML cells, and the proliferation phenotype induced by SWI/SNF depletion can be rescued by MYC overexpression (Chambers et al., 2023; Rago et al., 2022; Shi et al., 2013). In turn, we found that the NURF complex has a global role in ensuring efficient TAD insulation in AML cells, including at the BENC-MYC locus. The NURF complex loss leads to a moderate (~30-40%) downregulation of MYC expression, consistent with TAD formation modulating transcriptional outputs rather than determining them (Misteli, 2020). We also found that MYC overexpression could not rescue the phenotype of NURF depletion in AML cells (Figures EV2K and EV2L), further supporting the role of the NURF complex as a global regulator of higher-order chromatin structure.”.

Despite the prominent statement of the title, the authors have not strongly shown altered insulator function to be a primary effect rather than a secondary consequence. Since loss of MYC has been shown in multiple settings to be associated with differentiation in AML, an important unaddressed concern I have is that loss of BPTF and/or SMARCA5 may induce differentiation, which is furthermore a common therapeutic outcome in AML. If cells undergo a large-scale state change such as differentiation, it would be especially important for the authors to examine whether the alteration of insulator accessibility is a cause or consequence of differentiation. Fortunately the authors have generated a dTag system for SMARCA5, and they could employ it (rather than CRISPR KO) to examine more fast-acting effects on insulator function, for example via ATAC-seq. Authors should assess whether BPTF/SMARCA5 perturbations induce differentiation using flow cytometry for cell surface markers. If differentiation occurs, dTag studies would allow authors to assess whether the

changes in chromatin accessibility at insulator sites occur prior to differentiation.

The revised manuscript provides evidence that the NURF complex directly regulates the accessibility of insulator regions. In particular,

- As suggested by the reviewer, we performed ATAC-seq in the SMARCA5-dTAG knock-in cell line after treatment with dTAG-V1 to reveal the earliest changes in chromatin accessibility upon SMARCA5 degradation. After only 4 hours of dTAG-V1 treatment, we observed a significant loss of chromatin accessibility at 212 genomic regions, with no gain in accessibility observed (new Figure 4K). 62% of the regions with reduced accessibility overlapped with BPTF binding sites and 72% - with the downregulated ATAC regions detected in the KO experiments (cluster 6, Figure 4L). Importantly, 50% of the significantly downregulated ATAC-seq peaks in the dTAG-V1-treated cells represented insulator regions (new Figure 4L). This provides strong evidence that the NURF complex remodels insulator regions in AML cells.

- As recommended, we also tested whether NURF loss induces the differentiation of AML cells. We determined the expression of the differentiation marker, CD11b after *BPTF* or *SMARCA5* KO in three AML cell lines: THP-1, SET2, and U937. These cell lines express CD11b (new Figure EV2A) and are known to exhibit significant CD11b upregulation during differentiation (Chanput et al., 2014; Fiskus et al., 2021; Skopek et al., 2023). *BPTF* and *SMARCA5* KO did not affect CD11b levels in THP-1 and SET2 cells but did induce differentiation in U937 cells (Figure EV2B). These findings indicate that while NURF complex KO induces strong proliferation defects in most of the AML cell lines (Figure 1K, EV1D,F), its effect on cell differentiation can vary depending on the cell type, and the observed changes in chromatin accessibility do not result from the cell state change.

- We also demonstrate that the function of the NURF complex depends on the catalytic activity of SMARCA5 (Figure 1L, EV1J).

Collectively, these observations provide strong evidence for the NURF-mediated nucleosome remodelling of the insulator regions.

Does the dependency on the alternative NURF complex arise through sustaining MYC expression via BENC? Ectopic MYC expression would permit authors to directly evaluate if MYC expression is sufficient to rescue BPTF/SMARCA5 loss-of-function. If MYC expression is insufficient, this would provide strong evidence that indeed broader regulation of chromatin state is key. On the other hand, if forced MYC expression is sufficient, this would provide strong evidence of the relevant mechanism being the promotion of MYC expression.

We have performed the suggested experiment and found that MYC overexpression doesn't rescue the phenotype of *BPTF* or *SMARCA5* KO (new Figures EV2K and EV2L). Since the observed changes in chromatin accessibility after the NURF loss are substantial (Figures EV3C and EV3D), and 27% of all TAD boundaries are lost upon the SMARCA5/BPTF depletion, we believe that the NURF complex has a global role in regulating higher-order chromatin organization in AML cells. Facilitating MYC expression is only a part of this mechanism.

The use of K562 cells for genomic annotation is not ideal. K562 are a chronic myelogenous leukemia (CML) cell line, which despite some similarities is a distinct type of cancer than AML. Many histone marks, CTCF, etc., are available for THP1 cells from GEO, ENCODE, and other resources; the use of ChromHMM on these available public ChIP datasets would provide a higher degree of rigor.

As suggested, we used publicly available histone modifications data (H3K4me3, H3K27ac, H3K4me1, H3K27me3 and H3K9me3) and our BPTF, SMARCA5 and CTCF Cut&Run data to build a 12-state ChromHMM model for THP-1 cells (new Figure 4D, new Figure EV3D). Due to the limited histone modification data available for THP-1 cells, our model did not efficiently resolve transcribed regions and heterochromatin categories. However, it allowed us to further confirm that BPTF and SMARCA5 binding is mostly confined to insulators and active promoters in THP-1 cells (states 5, 6 and 7) (new Figure 4D, E). To be thorough, we kept both ChromHMM analyses (K562 and THP1) in the manuscript.

The authors' results strongly demonstrate that the PHD2 and BROMO domains of BPTF are not required, however they do not draw a conclusion for readers what this result proves or disproves from a mechanistic standpoint. If these domains are dispensable, then how does BPTF exert its function in AML? It would be helpful if authors state a more positive claim about how BPTF exerts its function for readers to understand how this system works.

We have revised the manuscript by modifying the language in the sections related to the PHD2-BROMO domain functions. We have emphasized the positive aspect of our findings, specifically that the absence of these two domains leads to a decrease in BPTF chromatin binding. Although this alone does not impact AML cell growth, it does indicate that these domains represent a component of the multi-modal mechanism for BPTF chromatin recognition. We have also added a discussion on various potential mechanisms of BPTF recruitment to chromatin. This section now reads as follows:

“Interestingly, we found that while PHD2-BROMO domains contribute to the efficient NURF binding, they do not determine it, as BPTF lacking these domains can bind chromatin and sustain the proliferation of leukemic cells. Similar findings were reported for BRD4, where chemical inhibition of its BROMO domain was shown to reduce chromatin localization but was insufficient to confer a phenotype comparable to the degradation of the protein (Winter et al., 2017; Zheng et al., 2023). This demonstrates that a combination of mechanisms determines the recruitment of the multidomain chromatin regulators. In the case of BPTF, chromatin binding by the PHD1 domain could represent an additional mode of chromatin recognition. Importantly, it has been demonstrated that BPTF can directly bind to DNA in vitro (Jordan-Sciutto et al., 1999). This binding activity is primarily found in the first 400 amino acids of the protein, which include the DDT and WHIM domains (Jordan-Sciutto et al., 1999). While these domains are known to mediate the interaction with the ISWI ATPases (Aravind & Iyer, 2012; Dong et al., 2013; Eberharter et al., 2004; Sharif et al., 2021), they were also proposed to have DNA binding functions in other proteins (Aravind & Iyer, 2012; Doerks et al., 2001). To deepen our understanding of how the NURF complex is directed to chromatin, it will be important to analyze the functional significance of the different segments of the N-terminal BPTF region. This will also help identify the most effective strategy for targeting BPTF with small molecules in the future.”

Overall I believe the work is very interesting and with some additions, will be sound. If the authors can strongly address these points, I would support publication.

Minor/non-essential comments:

Some sentences could use some moderation, for example, in the introduction: "These fusions drive leukemia by dysregulation of the genes [...]" I believe might be better stated as "These fusions drive leukemia in part by dysregulation of the genes [...]"

We have corrected this sentence in the revised version of the manuscript, as suggested.

"A large fraction of all active promoters and insulators demonstrates BPTF and SMARCA5

binding, while only a subset of enhancers (Expanded View Figure 4A)." -- This grammar here is confusing, and seemingly has a dangling incomplete phrase.

The sentence was corrected as follows:
“BPTF and SMARCA5 co-bind a significant portion of active promoters and insulators in AML cells (Figure EV3B). However, only a small subset of enhancers demonstrates NURF complex binding (Figure EV3B).”

Dear Dr Radzisheuskaya, dear Dr Helin,

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that their previous concerns have been addressed and now recommend publication of the manuscript. There remain only a few mainly editorial points that have to be addressed before I can extend formal acceptance of the manuscript:

1. Please rename "Conflict of interest" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest). Please also add the following disclaimer: "Kristian Helin is a member of the Advisory Editorial Board of The EMBO Journal. This has no bearing on the editorial consideration of this article for publication."
2. Please make sure that the order of the sections in the manuscript is as follows: abstract, introduction, results, discussion, materials & methods, data availability section, acknowledgments, disclosure statement and competing interests, references, main figure legends, tables, expanded figure legends.
3. CRediT has replaced the traditional author contributions section because it offers a systematic, machine-readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our online submission system to add specific details on the author's contribution. More information is available in our guide to authors.
4. Please rename callout Appendix Figure 2 to to Appendix Figure S2
5. Please upload Appendix Table S1-S9 individually and name the files Dataset EV1-EV9 with the appropriate callouts in the manuscript text.
6. Please ad page numbers to the Appendix Figures Table of Contents file
7. Synopsis:
Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 3-4 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the image size is rather small, and that text needs to be readable at the final size. Please send us this information together with the revised manuscript.
8. Our data editors have flagged the following issues in figure legends that need correcting:
- Please note that in the legend of figure EV2i, value for * is given as *p<0,05 instead of *p<0.05. "
- "Please indicate the statistical test used for data analysis in the legends of figures 1k; EV1h"
- "1. Please note that information related to n is missing in the legend of figures 1k; 2d-e; 4k; EV1h, i; EV5h.
- Please note that n=2 in figure EV2i. Please either ad a third biological replicate or remove the statistical test.
- Please note that the error bars are not defined in the legend of figures EV2j; EV5g."
9. Please define clearly what biological, independent, and technical replicate exactly means and label each experiment appropriately in the figure legends. There are several instances (e.g., figure 3) where the initial statement claims independent or biological replicates and the 'data Information' section at the end of the figure legend seems to suggest that these are technical replicates and that there were independent replicates that are not shown.
10. Please provide source data for the independent replicates that are not shown in the figures (examples are all the competition experiments such as figure 1E/F/K/L and 2D/F or 6B and several EV figures)
11. Please do not use statistical analysis for experiments with only 2 replicates such as figure EV2I.

Please let me know if you have any questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best regards,

Cornelius

_____
Cornelius Schneider, PhD
Editor
The EMBO Journal
c.schneider@embojournal.org

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 (7th Jan 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

I remain impressed with the impact and quality of the findings in this paper. The authors have carefully addressed the issues raised by the other reviewers, and the paper is even stronger now.

Referee #2:

The authors have substantially improved the manuscript and have addressed my concerns. I believe the manuscript is ready for publication.

All editorial and formatting issues were resolved by the authors.

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The data shown in figures should satisfy the following conditions:
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Each figure caption should contain the following information, for each panel where they are relevant:
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