nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: Nuclear corepressors NCOR1/NCOR2 regulate B cell development, maintain genomic integrity, and prevent transformation
Corresponding author name(s): Michael Farrar

Editorial Notes:

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A33516
Message: 23rd Mar 2022

Dear Mike,

Thank you for providing a point-by-point response to the referees' concerns voiced for your manuscript entitled, "Nuclear corepressors NCOR1/NCOR2 regulate B cell development, maintain genomic integrity, and prevent transformation" here at Nature Immunology. As noted in my previous message, while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the current manuscript for publication, but would be very interested in considering a revised version along the lines proposed in your response letter. Thus, we invite you to submit a substantially revised manuscript, however please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

Specifically, the revision should include new experiments to address:

(1) examine Ncor2 deletion efficiency
(2) reanalyze B cell precursor populations for Ki67 marker (noting that NI does not use the Hardy nomenclature - provide characteristic phenotypic markers instead for pre-pro-B, pro-B, etc) and provide absolute cell numbers
(3) confirm p-STAT5 abundance in Ncor1 and Ncor1/2 mutant cells as compared to WT

(can be Extended Data figure panel, just to rule out formal possibility of differential expression)
(4) examine more closely the chromatin accessibility of the Rag target sites within the Ig loci
(5) examine Rag activity & generation of possible genomic variants using whole-genome sequencing of CD19+ B cells as controls to the Ncor1 and Ncor1/2 mutants
(6) extend analysis of scATAC-seq datasets and compare Ncor1/2 targets to existing public ChIP-seq datasets for H3 histone marks (H3K27Ac & H3K27me3) and for STAT5 binding sites
(7) perform ChIP-qPCR of relevant EZH2 targets, Rag genes and STAT5 binding sites. Please include several target genes for each (would be good to check multiple EZH2/STAT5 target genes beyond p21 and Myc (which might be identified in the analysis addressed in point 5)
(8) examine NCOR1-deficient patient B-ALL samples for aberrant RAG1/RAG1-mediated cleavage and known STAT5 activity (transcriptomes, DNA damage)

Related points
expand on the potential role of Ncor1/2 regulation of CTCF binding sites (ie addressing referee #3 point 4g)

Editor concern related to response to referee #3 point 2 - are there unique Ncor1 or Ncor2 target sites?

Please include the additional textual clarifications as indicated in your response letter.

When you revise your manuscript, please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

* 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 will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. 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.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Nature Immunology 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.

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

Thank you for the opportunity to review your work.

Kind regards,

Laurie

Laurie A. Dempsey, Ph.D.
Senior Editor

Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Referee expertise:

Referee #1: B cell development

Referee #2: Rag-mediated DNA recombination

Referee #3: B cell leukemias & lymphomas

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The paper by Lee et al describes the characterization of normal and malignant B-lymphocyte development in mice carrying mutations in the Ncor-1 and -2 genes. The authors use a Cd79a CRE driver strain to cause B-lineage restricted inactivation of this gene. While the inactivation of any of the Ncor genes had rather modest effects on B-cell development, combined inactivation (DKO) caused a strong developmental block in early B-cell development. The authors analyze gene expression patterns, mainly using single cell RNA seq data, to obtain evidence for deregulated gene expression in mutated cells. This included deregulated expression of Rag genes in cycling cells prompting the authors to investigate a potential role of NCORs in malignant transformation in mouse models as well as human leukemias.

The report is interesting and contain substantial support for the idea that NCOR-1 and 2 has largely redundant, but essential functions in B-cell development. The authors use state of the art technology and combine experimental models and technologies in an elegant manner. However, the data are not presented in an optimal manner and the conclusions are not always strongly supported by the data. Furthermore, the paper displays some limitations in the experimental design. Hence, even though interesting, this report leaves the question about the role of NCOR proteins in normal and malignant development largely unresolved.

Specific comments:

1: Upon the generation of a new conditional mouse model it is of critical importance to resolve the efficiency of the deletion in different stages of development. A strong developmental block as observed in the DKO mice in this report, may cause a selection of cells carrying partial deletions downstream of the major developmental block. This could, for instance, explain the observed increase in marginal zone B-cells observed in Figure S1d. As far as I can understand, the authors only report a FACS analysis of NCOR2 protein levels in splenic T cells using a CD4 Cre driver (Figure S1B). This analysis only compares the levels to that of Wt cells making it difficult to understand how efficient the deletion is. Even though the strong phenotype in DKO mice supports efficient deletion, it is of importance to know that the cells in the periphery indeed carry the intended mutations.

2: While the phenotype of the DKO in Figure 1 a-d is clear, it is more complicated to fully understand the phenotypes of the partial KO (Hets). The data in Figure 1b and d are presented as percent lymphocytes in BM. As far as I understand this gate is based on the FSC/SSC gate in S1c. How this gate was defined is unclear and it certainly contain at least two populations. The data would be more informative if presented as absolute cell numbers. It is unclear to me what are the numbers in the FACS plots in Figure 1a c are. Figure 1 e, f suffers from similar problems.

3: Despite that the authors mainly use technologies that contain information at a single cell level they at time end up in somewhat circular arguments as a consequence of that data are analyzed using poorly defined populations of cells. In Figure 2C the authors report KI67 expression is measured in total CD19+CD43+ cells despite that Figure 1b shows changes in population composition. To be conclusive these data would need to be presented based on better defined populations.

4: From figure 2D the authors conclude that Ncor1/2 deficiency affects termination of light chain recombination "Thus, NCOR1/2-deficiency affects termination of light chain rearrangement." (Row 135). There is a clear shift in the K/L ratio, however, I cannot see that the authors present evidence for that this is a consequence of impaired termination of light chain recombination. How can the authors be sure of that this is not a consequence of differential accessibility of RAG target elements. As light chain recombination should be stopped upon functional rearrangement, would it not be expected to find k/l double positive cells in the DKO if the authors conclusion would be correct?

5: The data in figure 2f only show FACS plots without any proper quantification. It is unclear what the figures in the plots represents? Average of the two animals? The figure legend indicate that the data are from 2 animals of each genotype. This is an important experiment and even if I would agree with the authors that this is a partial rescue, the data should still be of proper quality to be presented in the scientific literature.

6: In figure 3G-J the authors aim to establish a link between loss of NCOR1/2 and STAT5 activity. They present support for this idea, however, a direct experiment measuring p-STAT5 in different populations using FACS would provide more direct evidence to this end.

7: The deregulated expression of Rag genes constitutes an important finding in this report. However, as far as I understand, the data in Figure 5a does not take the shifts in B-cell populations into regard making it rather uninformative. The expression of Rag1 is further analyzed in an elegant manner in Figure 5b and c. It is important to see that same analysis for Rag2 expression. As Rag2 has been suggested to be targeted for degradation during the cell cycle (Li Immunity 1996), it would be interesting to see if it is possible to detect changes in protein expression in DKO cells.

8: As far as I understand, the experiment in Figure 5f use tail DNA as control. To link NCOR to increased RAG activity. DNA from normal CD19+ cells should be included as control for a conclusive experiment.

9: As far as I understand, the survival plot in Figure 6a is based on combined data from homo and heterozygote Ncor1 mutant mice. In Figure S5, the data from heterozygote mice do not reach significance. Even though I would agree to that there appear to be increased tumor development I do not find it appropriate to mix data in this manner as the authors claim that heterozygote deletion is sufficient to cause increase leukemia

frequency. The presence of CD19 negative leukemia cells despite the use of a CD79 cre driver is also concerning. This especially in the light of that the control for the tumor experiment is Wt- caStat5b and not Ncor1fl/fl caStat5b+Cd79cre- mice as would be more appropriate.

Minor points

1: The phenotypes of the heterozygotes are rather complex. Can the differential effects of the different KO be explained by the expression of Ncor1/2 during development.

2: On row 156, the authors write" Similar to our flow cytometry
analysis (Figure 1c, 1d), the CD43- fractions showed reductions in immature and mature B cells in mice deficient in NCOR1 and NCOR1/2,". While this is true for the DKO mice the authors do not report any reductions in these populations in the Ncor1-/- mice in Figure 1D.

3: On row 159, The authors state " Notably, the frequency of pre-BCR-dependent stage pre-B cells was significantly reduced in the NCOR1/2 DKOs (Figure 3b, 3c).". What statistical analysis was performed for the authors reach this conclusion?

4: How was the data in figure 4D analyzed and how was the statistical analysis performed. 3 samples from two experiments. It appears as if all the control values are set to 1.

5: Row 296 looks strange. Should it be "often associated with B-cell transformation"?

Reviewer #2:
Remarks to the Author:
In this manuscript, the authors generated B lineage specific deletion of Ncor1, Ncor2, alone or together. The results uncovered a critical role of Ncor1/2 in early B cell development and B cell lymphomagenesis. The results complemented conditional deletion of Ncor1 (and sometime Ncor1) in hematopoietic stem cells (HSC) and T cells (CD4Cre) published before, identified both common and unique role of Ncor1/2 in B cell proliferation and differentiation in general.
Specific comments:
1) Figure 1 shows "percentage" of stage specific B cell populations among all B cells. Although CD79aCre is B cell specific, it would be helpful to show how B cells as whole affected by the Ncor1/2 deletion. This can be achieved by including 1) absolute B cell counts in bone marrow (preferred), or 2) the relative percentage of B cells (B220+) among all live gated bone marrow cells.

This is critical regarding the statement "while loss of NCOR1 resulted in an increase in pre-B cells but not immature or mature B cells.". This proportional increase of pre-B cells might be a true increase of pre-B cells or caused by depletion of mature B cell types.

2) The correlation between Ki67 and Ncor1 is very weak if any. Figure 2 seems to suggest that Ncor1 is expressed in both Ki67+ and Ki67- cells. The authors suggest Ncor1 levels correlate with proliferation. The immature and mature B cells are usually quiescent, not proliferative. Other proliferation markers (e.g., PCNA, Cyclin B, MCM2-7, etc.) beyond Ki67 might be helpful.

Reviewer #3:
Remarks to the Author:
Lee et al explore the impact of NCOR1 and NCOR2 single or double knockout in early B-cell development. The question is of great interest given the intricate cross talk between signals and transcription factors dictating the various phenotypic transitions, as well as the potential vulnerability caused by RAG protein induced DNA damage during this process. Along these lines it is also intriguing that NCOR1 and NCOR2 mutations have been observed in B cell malignancies and yet nothing is known about how or whether these genetic lesions could contribute to malignant phenotypes. Herein, the authors first examine the impact of NCOR1 NCOR2 and double KOs in early development and find that they can mostly compensate for each other, but that the double KO results in severe reduction of these cell populations. Also of note the NCOR1 only knockout results in increased abundance of small PreB cells. NCOR1 knockout had subtle impact on light chain expression. A LISA analysis on DKO predicted EZH2 activation, and further analysis showed induction of p53 and DNA repair, and enhanced STAT5 and RAG expression and activity, with evidence for structural genomic lesions. NCOR1 mutations were shown to consist of many bona fide loss of function alleles and NCOR1 knockout cooperated with STAT5 constitutive activation to accelerate lymphomagenesis. RAG1/2 expression was generally higher in these patients and a univariate analysis suggested that low NCOR1 expression is linked to outcome.

Overall I found this manuscript to be topically of great interest and to provide some initial important insights into the biological impact of disrupting expression of NCOR1 and NCOR2, as well as confirming that NCOR1 could function as a tumor suppressor. On the other hand the mechanistic work is fairly superficial and purely observational, and the authors do not provide a clear mechanistic reason why NCOR1 contributes to leukemogenesis. There are fascinating implications (for example the increase in expression of EZH2 target genes and how might be linked to H3K27 acetylation. Do NCOR1 and NCOR2 have any direct impact on EZH2 regulated promoters or is the effect indirect through enhancer activation? The epigenetics aspect of the manuscript is underdeveloped and if pursued would allow at least one mechanistic aspect to be defined.

Major:

1) The authors should provide a clear sense of the epigenetic impact of NCOR1/2 single and double loss of function on epigenetic regulation. The ATACseq data are undeveloped and there is no chromatin analysis of any kind towards explaining corepressor loss of function regulation of the genome. These kinds of studies can be readily performed even in relatively small cell populations with methods such as Cut and Run. The question to address is whether the effects of corepressor loss are due to direct DNA damage perturbation as is claimed with HDAC3, or if this is indirectly caused by epigenetic reprogramming effects. Right now it is not clear exactly how especially NCOR1 loss functions as a tumor suppressor

2) The link between the B-cell development side and leukemogenesis is a bit difficult to reconcile. In part because the major focus of the first 2/3rds of the manuscript are focused on double KO of NCOR1/2, which seems to be qualitatively different in nature to NCOR1 single KO. For example, if induction of STAT5 activity is a relevant downstream

effect of NCOR1 mutation than why would there be cooperation to induce leukemias? It is possible that this effect is not generally applicable to NCOR1 KO alone, but this is not made clear. NCOR1 and NCOR2 are well known corepressor partners of BCL6, which was reported by the Muschen lab to play similar roles and by their group and earlier papers to compete with STATs for DNA binding. Is it possible that this mechanism could be relevant here? GCs and BCL6, STATs and BCL6?

3) The patient data could be stronger. It would be important to determine whether similar effects as observed in B-cells are occurring in the NCOR1 mutant patients (transcriptomes, DNA damage) vs all B-ALLs. It remains difficult to understand how the two parts of the story link together, and to assess clinical significance a multivariate analysis is required.

Here are suggestions organized by figure

Fig S1 and 1

I would like to see performance metrics for the NCOR1 flow cytometry. I would expect to see no signal in the NCOR1 KO cells and so would need additional controls for example related to Fig S1B. Also I would like to see flow side by side with QPCR and Western blots. NCOR1 and NCOR2 antibodies are notoriously fickle and non-specific. Suggest to show genotyping data in B-cells showing that NCOR1 and 2 are knocked out.

Suggest to show effect on germinal center B cells. Increase in MZ suggest there is deviation away from GCs as for example shown to be caused by forced NOTCH2 activation in mature B cells. Deviation from GCs would be consistent with impairing BCL6 function, which is highly dependent on NCOR1/2, and in which repression of P53 by BCL6 was shown to be important by the Dalla Favera group. Given the importance of BCL6 in lymphomas and also proposed by the Muschen group for ALL, this would be of importance to delineate in this case.

Fig 2

The correlation of NCOR1 and Ki67 is of interest but not highly convincing based on flow cytometry for reasons mentioned in relation to Fig 1. More in the way of controls would be helpful here. In addition to the above mentioned, the authors could correlate mRNA levels of these two genes. Given the distribution of cells in the correlation plot shown in 2A it would help the authors case if it were clear that theKi67 high cells where truly NCOR1 positive vs the apparently larger population of NCOR1 high cells with low Ki67 shown in the same plot

Fig 2B please also show mRNA (could even be from publicly available databases) – or even better by QPCR.

Fig 2C the result is clear, just not sure of the interpretation. NCOR1/2 DKO cells might fail to proliferate secondarily to effects on other cellular processes. I would just urge the authors to acknowledge this in the text since it seems implied that there is a direct link.

Fig 2F would benefit from summary plots showing reproducibility of these numbers

Fig S2 and 3

Were scATAC also done in replicates?

Please clarify what gender the specimens were from

3B: the color scheme does not have sufficient contrast to visualize where the various subpopulations distribute to in the UMAPs. Either use colors with greater contrast, or generate additional plots where only a few clusters are colored at a time leaving the others with grey, or can use contours to indicate the subpopulations.

3F: For all GSEAs shown here and later, the plots shown could be represented in a more rigorous manner. It would be recommended to show the histograms for differential gene expression, the normalized enrichment score for every single one of the GSEA plots. Also I was surprised that the numbers of genes in the gene sets were so sparse. For example the KEGG B cell receptor gene set used here has many more genes than shown. Same for the rest of the GSEAs shown in subsequent panels and figures.

S3 and 3J:
elaborate on the ATACs: How many of these were performed, how many cells per genotype, how did the QC look?

How well does the ATAC signal correlate with the gene expression profiles in each population?

S3B: not clear what this is showing. It is unlikely that just a handful of cells were profiled in the WT setting.

Can some insight be gained from the ATAC delineated populations regarding which stages of development are perturbed from the epigenetic standpoint, perhaps preceding where the RNA-seq places these findings?

Would be of interest to show pseudobulk analysis of key STAT target regulatory elements and perhaps superenhancers in the several subpopulations, especially for genes the authors believe to be critical mediators of the observed phenotype.

Figure 4
4D. It would be good to be consistent in how the abundance of the various genes are measured. For example, BIM should also be measured at the transcript level given that NCOR1/2 are transcriptional regulators, rather than only by flow.

4E is described as measuring “epigenetic” changes, but in fact all that is shown is flow cytometry for H3K27Ac. This is not evidence of epigenetic changes. The authors would need to a) perform acid extract western blots since these histone antibodies may not be highly specific and b) if they want to say there is an epigenetic consequence would need to perform Cut and Run (can be done in very small cell populations) to assess the genomic distribution and see if this is linked to differential accessibility or expression.

4F: The authors should determine whether the TFs captured by motif analysis are expressed in the various subpopulations of interest.

4G: what is the significance of the CTCF motif depletion?

Figure 5:

5C difficulty to interpret without proper statistics.

5E: the Y axis not annotated, so cannot tell if these are on similar scale. Also not clear what cell types this corresponds to.

5G: Please generate a pie chart or similar summarizing what types of structural lesions were present.
Also, If the authors wish to affirm that RAG1 targets overlap significantly with the DKO structural lesions then they need to perform appropriate statistical analysis, ideally taking into account covariates such as accessibility of these sites, etc.

Figure 6:

In addition to flow plots the authors should provide a sense of how robust this finding is by showing the data across multiple biological replicates.

Figure 7.

7B-C. The authors should show whether SVs and translocations are significantly more common in NCOR1 mutated patients vs the background of all B-ALLs.

7D. To claim there is a significant difference the authors must apply the appropriate statistics.

7E. The clinical data are not especially compelling. Does this relationship between NCOR expression survive a multivariate analysis controlling for known molecular and clinical biomarkers? Also, is there a link between outcome and NCOR1 mutations?

Suppl figure 6 does not seem to confirm that NCOR1 knockout causes the effects that are claimed. How does this relate to patients?

Is there an NCOR1 transcription signature in the B-cell populations tested in mice? And do RNA-seq profiles from patients with NCOR 1 mutations enrich for these signatures? Do NCOR1 low or mutated ALLs have a STAT5 signature?

Author Rebuttal to Initial comments

Response to Review

We were glad to hear that the reviewers felt our paper was interesting and appear to only have technical concerns. We believe we have addressed all the points raised by reviewers 1 and 2 and the vast majority of items requested by reviewer 3. We thank the reviewers for their invaluable comments that we believe has led to a significant improvement in this manuscript.

Specific responses to the reviewers' concerns are outlined in red text below; changes to the manuscript text are highlighted in yellow.

Reviewer #1

(Remarks to the Author)
The paper by Lee et al describes the characterization of normal and malignant B-lymphocyte development in mice carrying mutations in the Ncor-1 and -2 genes. The authors use a Cd79a CRE driver strain to cause B-lineage restricted inactivation of this gene. While the inactivation of any of the Ncor genes had rather modest effects on B-cell development, combined inactivation (DKO) caused a strong developmental block in early B-cell development. The authors analyze gene expression patterns, mainly using single cell RNA seq data, to obtain evidence for deregulated gene expression in mutated cells. This included deregulated expression of Rag genes in cycling cells prompting the authors to investigate a potential role of NCORs in malignant transformation in mouse models as well as human leukemias.

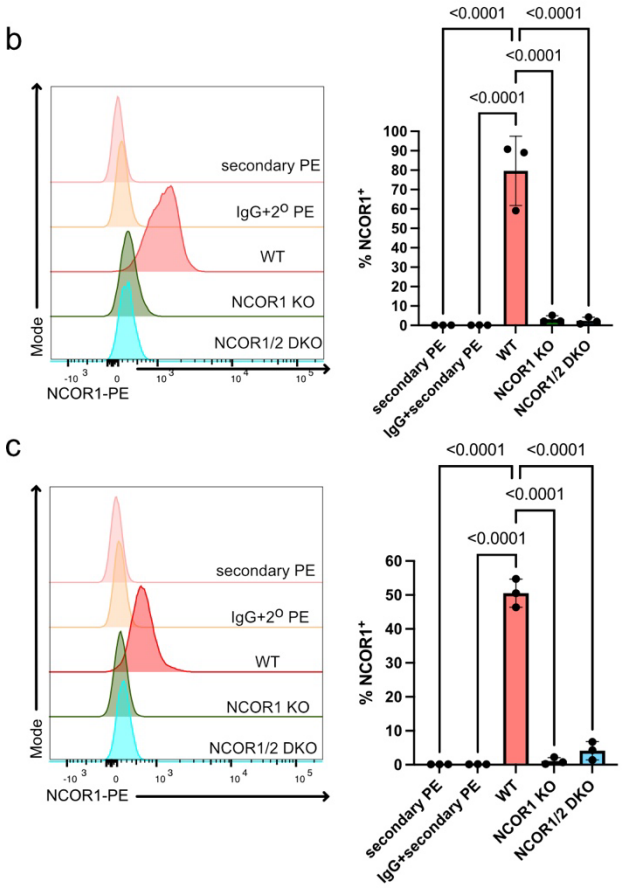
The report is interesting and contain substantial support for the idea that NCOR-1 and 2 has largely redundant, but essential functions in B-cell development. The authors use state of the art technology and combine experimental models and technologies in an elegant manner. However, the data are not presented in an optimal manner and the conclusions are not always strongly supported by the data. Furthermore, the paper displays some limitations in the experimental design. Hence, even though interesting, this report leaves the question about the role of NCOR proteins in normal and malignant development largely unresolved.

Specific comments:

1: Upon the generation of a new conditional mouse model it is of critical importance to resolve the efficiency of the deletion in different stages of development. A strong developmental block as observed in the DKO mice in this report, may cause a selection of cells carrying partial deletions downstream of the major developmental block. This could, for instance, explain the observed increase in marginal zone B-cells observed in Figure S1d. As far as I can understand, the authors only report a FACS analysis of NCOR2 protein levels in splenic T cells using a CD4 Cre driver (Figure S1B). This analysis only compares the levels to that of Wt cells making it difficult to understand how efficient the deletion is. Even though the strong phenotype in DKO mice supports efficient deletion, it is of importance to know that the cells in the periphery indeed carry the intended mutations.

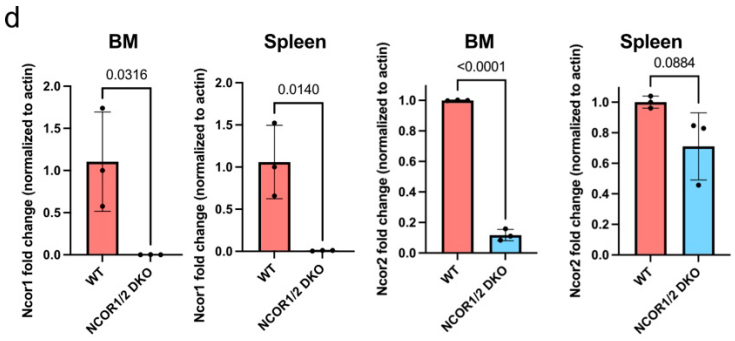
- We have now carried out a detailed evaluation of deletion of *Ncor1* and *Ncor2* in the bone marrow and spleen using the *Cd79a-Cre x Ncor1/2*$^{FL/FL}$ mice. Using appropriate

isotype controls with the NCOR1 antibody, we are able to confirm virtually complete deletion of NCOR1 protein in both the bone marrow and spleen and also confirm that the antibody is NCOR1-specific (reviewer 3 was concerned for antibody specificity).



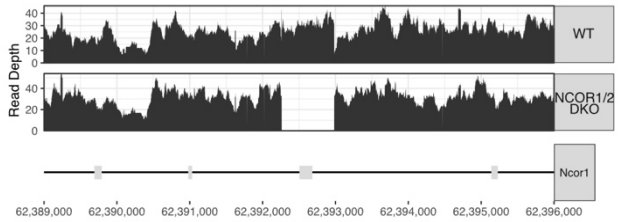
(Panel b is bone marrow; Panel c is spleen)

NCOR2 is more difficult to characterize as consistent with comments by reviewer 3, we have found that commercially available NCOR2 antibodies are not very specific. Thus, we have been unable to address NCOR2 protein deletion. However, using a qRT-PCR based approach we found that in the bone marrow of *Cd79a-Cre x Ncor1/2*$^{FL/FL}$ mice, *Ncor2* was virtually absent. In contrast, we found that in the spleen, while *Ncor2* expression was reduced it was not absent. This may be due to strong selective pressure for some NCOR1/2 protein in splenic B cells; since the *Ncor2* deletion approach works via a gene trap and not exon deletion (like *Ncor1*) it may be more likely to escape deletion. Since *Ncor2* is not completely deleted we have removed the previous description of splenic phenotypes including marginal zone B cells – this was only a small part of the initial manuscript.

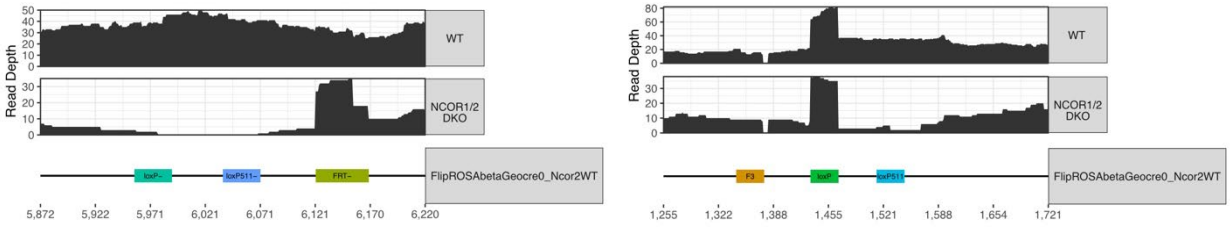


Finally, we confirmed knockout of Ncor1 and Ncor2 at the DNA level using our whole genome sequencing data. The Ncor1 gene region flanked by loxP sites had absence of reads in the NCOR DKOs. Similarly, we were able to confirm the inversion of the Ncor2 gene trap in the NCOR DKO B cells. Overall, Cre-mediated recombination in bone marrow B cells was robust for both *Ncor1* and *Ncor2*. These new findings are presented in supplementary figure 1.

NCOR1



NCOR2



2: While the phenotype of the DKO in Figure 1 a-d is clear, it is more complicated to fully understand the phenotypes of the partial KO (Hets). The data in Figure 1b and d are presented as percent lymphocytes in BM. As far as I understand this gate is based on the FSC/SSC gate in S1c. How this gate was defined is unclear and it certainly contain at least two populations. The data would be more informative if presented as absolute cell numbers. It is unclear to me

what are the numbers in the FACS plots in Figure 1a c are. Figure 1 e, f suffers from similar problems.

- We now present changes in absolute cell numbers in these figures as requested and clarified what the numbers in the FACS plots represent in the figure legends.

3: Despite that the authors mainly use technologies that contain information at a single cell level they at time end up in somewhat circular arguments as a consequence of that data are analyzed using poorly defined populations of cells. In Figure 2C the authors report KI67 expression is measured in total CD19+CD43+ cells despite that Figure 1b shows changes in population composition. To be conclusive these data would need to be presented based on better defined populations.

- To account for population composition differences seen in the NCOR-knockouts, we now show comparisons among the different knockouts for markers of interest (ex. Ki67, Bim) based on the Hardy fractions (as shown as in the phenotyping data in Figure 1). The only experiment/figure for which this was not possible to show was the pSTAT5 staining, as the methanol-based fixation approach required results in epitope destruction of certain surface markers and limits the use of possible fluorophores.

4: From figure 2D the authors conclude that Ncor1/2 deficiency affects termination of light chain recombination “Thus, NCOR1/2-deficiency affects termination of light chain rearrangement.” (Row 135). There is a clear shift in the K/L ratio, however, I cannot see that the authors present evidence for that this is a consequence of impaired termination of light chain recombination. How can the authors be sure of that this is not a consequence of differential accessibility of RAG target elements. As light chain recombination should be stopped upon functional rearrangement, would it not be expected to find k/l double positive cells in the DKO if the authors conclusion would be correct?

- The reviewer raises a good point. Clearly, kappa/lambda segregation is perturbed but we can’t be sure this has to do with termination versus altered accessibility. In analyzing our scATAC-Seq data, we observed that there was a significant decrease in accessibility of the kappa gene locus (Figure 4e). In contrast, the lambda locus was affected to a much smaller degree. Thus, the most straightforward interpretation of our data is that the altered kappa/lambda ratio is due to altered accessibility at kappa versus lambda loci. We have changed the text to reflect this new information.

5: The data in figure 2f only show FACS plots without any proper quantification. It is unclear what the figures in the plots represents? Average of the two animals? The figure legend indicate that the data are from 2 animals of each genotype. This is an important experiment and even if I would agree with the authors that this is a partial rescue, the data should still be of proper quality to be presented in the scientific literature.

- We have since generated and analyzed additional mice with the required genotype. Those results are quantified and presented in Figure 2f. They show a modest but statistically significant increase in the frequency of B cells in the bone marrow and the spleen in the presence of a rearranged functional BCR.

6: In figure 3G-J the authors aim to establish a link between loss of NCOR1/2 and STAT5 activity. They present support for this idea, however, a direct experiment measuring p-STAT5 in different populations using FACS would provide more direct evidence to this end.

- We have now done those experiments and show that pSTAT5 levels are in fact increased upon loss of NCOR1/2 (Figure 3i). This increase in pSTAT5 may be partially attributable to the increased IL7R expression that we found on NCOR DKO B cells (Figure 3j).

7: The deregulated expression of Rag genes constitutes an important finding in this report. However, as far as I understand, the data in Figure 5a does not take the shifts in B-cell populations into regard making it rather uninformative. The expression of Rag1 is further analyzed in an elegant manner in Figure 5b and c. It is important to see that same analysis for Rag2 expression. As Rag2 has been suggested to be targeted for degradation during the cell cycle (Li Immunity 1996), it would be interesting to see if it is possible to detect changes in protein expression in DKO cells.

- As the reviewer points out, the original Figure 5a showed bulk comparisons between WT, NCOR1 KO and NCOR1/2 DKO. So that the bulk comparison is not misleading, we have removed that figure. Instead, we now provide the expression and accessibility of *Rag1* and *Rag2* broken down by genotype within specific clusters. With respect to RAG protein expression, as far as we know, there are no good RAG antibodies for FACS and reporters such as RAG2-GFP can be problematic as GFP has a long half-life making interpretation difficult. It would also take a long time (probably > a year) to generate the mice (*RAG2-GFP x Cd79a-Cre x Ncor1*$^{FL/FL}$ *x Ncor2*$^{FL/FL}$). Our data clearly shows increased *Rag* transcription, which is what one would expect in the absence of a transcriptional repressor. Moreover, we also observed increased expression of *Foxo1*, an upstream activator of *Rag* transcription.

8: As far as I understand, the experiment in Figure 5f use tail DNA as control. To link NCOR to increased RAG activity. DNA from normal CD19+ cells should be included as control for a conclusive experiment.

- We have now repeated these studies using the more appropriate control of sorted $CD19^{+}B220^{+}$ WT B cells (n = 2) and $CD19^{+}B220^{+}$ NCOR DKO B cells (n = 3). These studies show the same general result that NCOR1/2 deficient B cells show an increase in structural variants compared to WT B cells (figure 5f).

9: As far as I understand, the survival plot in Figure 6a is based on combined data from homo and heterozygote Ncor1 mutant mice. In Figure S5, the data from heterozygote mice do not reach significance. Even though I would agree to that there appear to be increased tumor development I do not find it appropriate to mix data in this manner as the authors claim that heterozygote deletion is sufficient to cause increase leukemia frequency. The presence of CD19 negative leukemia cells despite the use of a CD79 cre driver is also concerning. This especially in the light of that the control for the tumor experiment is Wt- caStat5b and not Ncor1fl/fl caStat5b+Cd79cre- mice as would be more appropriate.

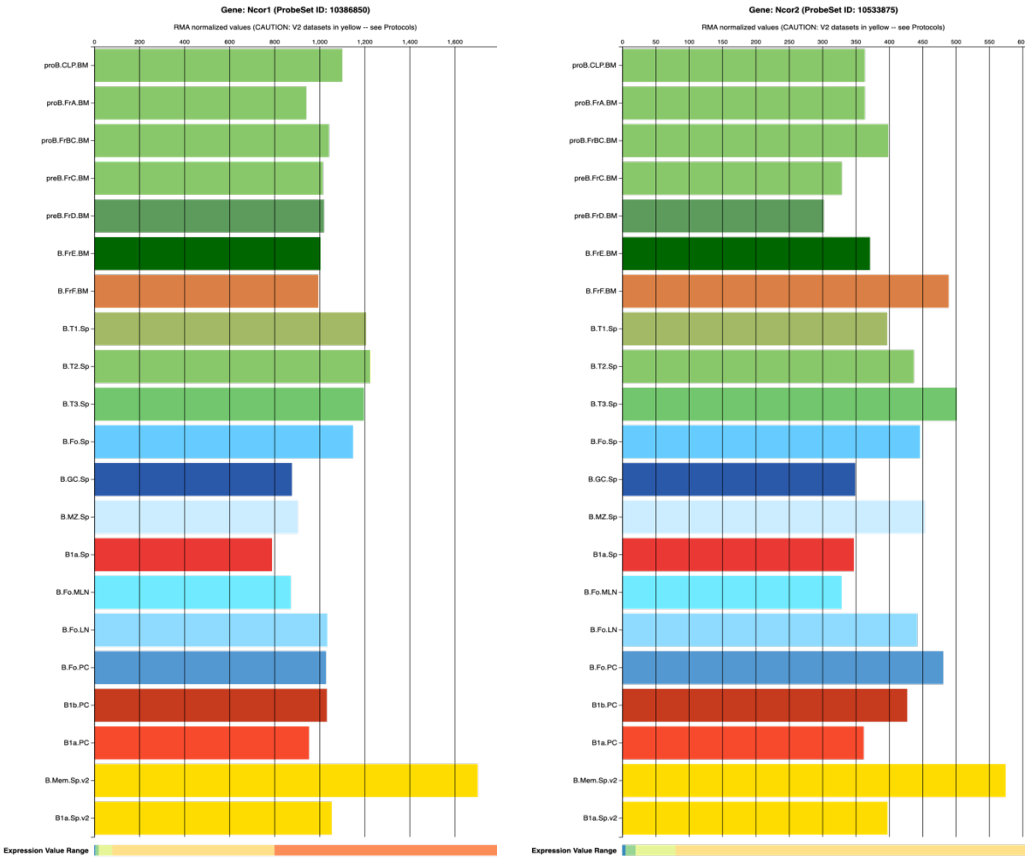
- We agree with the reviewer's comment and now show in Figure 6a the survival plot separated by genotype. A log-rank test for trend (p = 0.0066), which shows a significant decrease in survival as one goes from WT to NCOR1 het to NCOR1 KO, is statistically significant. As for *Cd79a-Cre*$^{-}$ x *Stat5b*-CA x *Ncor1*$^{fl/fl}$ mice, it requires a significant number of *Stat5b*-CA mice alone to detect increased incidence of leukemia. We did not have any CD79a-cre^{-} x Stat5b-CA x Ncor1fl/fl mice that came down with leukemia.

  Cd79a-Cre has been shown to result in gene deletion in a small fraction of cells that go on to become T cells (Hobeika et al PNAS 2006). We have observed this in our hands too. Thus, the presence of non-B cell leukemias is not completely unexpected. For [REDACTED].

Minor points

1: The phenotypes of the heterozygotes are rather complex. Can the differential effects of the different KO be explained by the expression of Ncor1/2 during development.

- We believe the phenotype can be explained by increased expression of NCOR1 versus NCOR2 in developing B cells (hence the stronger effect observed when deleting NCOR1 vs NCOR2 or in NCOR1KO/NCOR2 het vs NCOR1 het/NCOR2KO). This is consistent with data in the immgen database (data shown below), which shows much higher expression of NCOR1 versus NCOR2 in developing B cells. We have added a comment alluding to this in the revised manuscript.



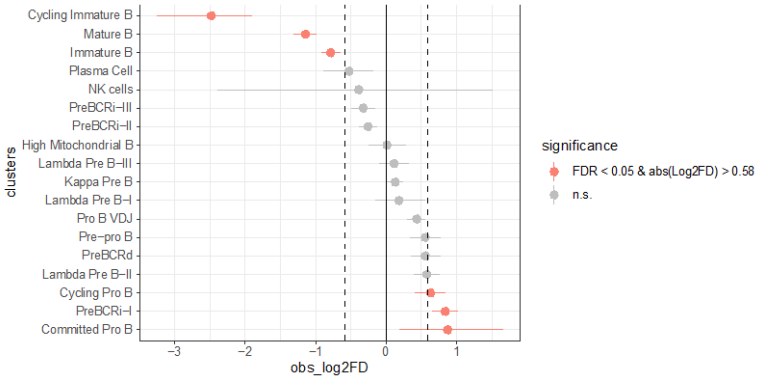
2: On row 156, the authors write" Similar to our flow cytometry analysis (Figure 1c, 1d), the CD43- fractions showed reductions in immature and mature B cells in mice deficient in NCOR1 and NCOR1/2,". While this is true for the DKO mice the authors do not report any reductions in these populations in the Ncor1-/- mice in Figure 1D.

- The text has been updated to indicate only statistically significant reductions in the NCOR1/2-deficient B cells.

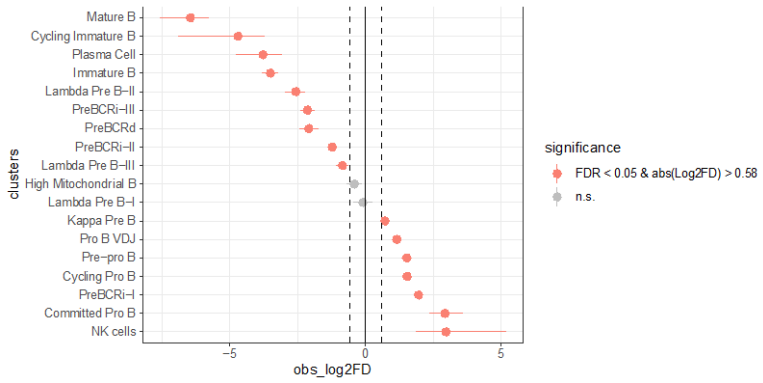
3: On row 159, The authors state ” Notably, the frequency of pre-BCR-dependent stage pre-B cells was significantly reduced in the NCOR1/2 DKOs (Figure 3b, 3c).”. What statistical analysis was performed for the authors reach this conclusion?

- To determine significant changes in the frequency of cells in each cluster between the different genotypes, we used a package called scProportiontest. In short, a permutation test is used to calculate the p-value for difference in the proportion between clusters and a confidence interval is generated via bootstrapping. Results from the comparison can be seen below. The proportions with significant differences are now reflected in the updated Figure 3c.

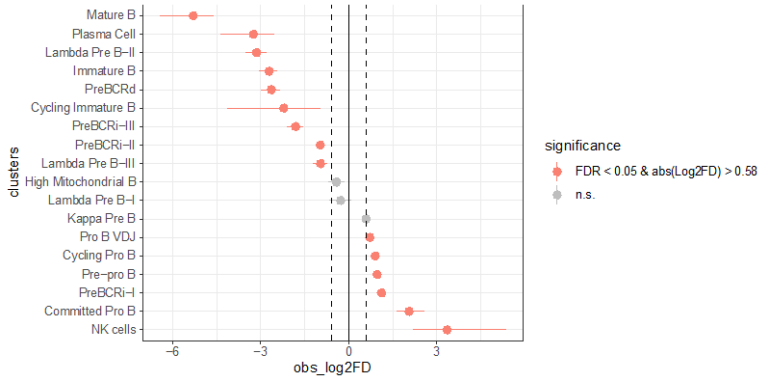
WT vs KO



WT vs DKO



KO vs DKO



4: How was the data in figure 4D analyzed and how was the statistical analysis performed. 3 samples from two experiments. It appears as if all the control values are set to 1.

- To avoid confusion, the figure was replotted with geometric MFI of BIM expression. A one-way ANOVA was performed to compute statistical significance. This updated figure can now be found in Supplementary Figure 4d.

5: Row 296 looks strange. Should it be ”often associated with B-cell transformation”?

- IL7R expression is associated with B cell markers as originally stated, but can clearly also be expressed by non-B cells. As the reviewer describes, increased IL7R expression can be associated with B cell transformation as well. The original sentence was removed

and we now simply state that IL7R expression is increased on *Stat5b-CA x Cd79a-Cre x Ncor1*$^{FL/FL}$ leukemias relative to *Stat5b-CA x Cd79a-Cre x Ncor1*$^{FL/+}$ leukemias or WT B cells.

Reviewer #2

(Remarks to the Author)
In this manuscript, the authors generated B lineage specific deletion of Ncor1, Ncor2, alone or together. The results uncovered a critical role of Ncor1/2 in early B cell development and B cell lymphomagenesis. The results complemented conditional deletion of Ncor1 (and sometime Ncor1) in hematopoietic stem cells (HSC) and T cells (CD4Cre) published before, identified both common and unique role of Ncor1/2 in B cell proliferation and differentiation in general.
Specific comments:
1) Figure 1 shows "percentage" of stage specific B cell populations among all B cells. Although CD79aCre is B cell specific, it would be helpful to show how B cells as whole affected by the Ncor1/2 deletion. This can be achieved by including 1) absolute B cell counts in bone marrow (preferred), or 2) the relative percentage of B cells (B220+) among all live gated bone marrow cells.

This is critical regarding the statement "while loss of NCOR1 resulted in an increase in pre-B cells but not immature or mature B cells.". This proportional increase of pre-B cells might be a true increase of pre-B cells or caused by depletion of mature B cell types.

- As also suggested by reviewer 1 comment #2, we now show absolute B cell counts. The difference in WT vs NCOR1 KO in the small pre-B cells was not statistically significant when plotted as absolute cell count.

2) The correlation between Ki67 and Ncor1 is very weak if any. Figure 2 seems to suggest that Ncor1 is expressed in both Ki67+ and Ki67- cells. The authors suggest Ncor1 levels correlate with proliferation. The immature and mature B cells are usually quiescent, not proliferative. Other proliferation markers (e.g., PCNA, Cyclin B, MCM2-7, etc.) beyond Ki67 might be helpful.

- While validating the NCOR1 deletion status from our NCOR1 KO mice, we noticed that the isotype and NCOR1 antibody were binding to the anti-IgM-APC antibody bound to cells that were highly expressing IgM. The anti-IgM-APC antibody used at the time was a $F(ab)_2$ fragment goat anti-mouse IgM. This antibody was solid-phase adsorbed to minimize cross reactivity with human, bovine, and horse serum proteins, but exhibited cross-reactivity with the polyclonal IgG rabbit antibodies used to stain NCOR1. Therefore, we reanalyzed WT mice, using a different anti-IgM-APC antibody that did not

cross-react with the NCOR1 antibody. This showed that in fact the immature and mature B cells had low NCOR1 expression, as suggested by their usually quiescent nature. Overall, the positive correlation between NCOR1 and Ki67 was strengthened (r^2 = 0.2837), and NCOR1 expression by B cell developmental stage showed that the pro B ($B220^+CD43^+CD24^+BP1^-$) and pro-B/pre-B ($B220^+CD43^+CD24^+BP1^+$) cell stages, which harbor the majority of proliferating cells, had the highest NCOR1 expressions, whereas the pre-pro B, small pre-B, immature B, and mature B cells had lower NCOR1 expressions. These updated results are now available in Figure 2a and 2b.

Reviewer #3

(Remarks to the Author)
Lee et al explore the impact of NCOR1 and NCOR2 single or double knockout in early B-cell development. The question is of great interest given the intricate cross talk between signals and transcription factors dictating the various phenotypic transitions, as well as the potential vulnerability caused by RAG protein induced DNA damage during this process. Along these lines it is also intriguing that NCOR1 and NCOR2 mutations have been observed in B cell malignancies and yet nothing is known about how or whether these genetic lesions could contribute to malignant phenotypes. Herein, the authors first examine the impact of NCOR1 NCOR2 and double KOs in early development and find that they can mostly compensate for each other, but that the double KO results in severe reduction of these cell populations. Also of note the NCOR1 only knockout results in increased abundance of small PreB cells. NCOR1 knockout had subtle impact on light chain expression. A LISA analysis on DKO predicted EZH2 activation, and further analysis showed induction of p53 and DNA repair, and enhanced STAT5 and RAG expression and activity, with evidence for structural genomic lesions. NCOR1 mutations were shown to consist of many bona fide loss of function alleles and NCOR1 knockout cooperated with STAT5 constitutive activation to accelerate lymphomagenesis. RAG1/2 expression was generally higher in these patients and a univariate analysis suggested that low NCOR1 expression is linked to outcome.

Overall I found this manuscript to be topically of great interest and to provide some initial important insights into the biological impact of disrupting expression of NCOR1 and NCOR2, as well as confirming that NCOR1 could function as a tumor suppressor. On the other hand the mechanistic work is fairly superficial and purely observational, and the authors do not provide a clear mechanistic reason why NCOR1 contributes to leukemogenesis. There are fascinating implications (for example the increase in expression of EZH2 target genes and how might be linked to H3K27 acetylation. Do NCOR1 and NCOR2 have any direct impact on EZH2 regulated promoters or is the effect indirect through enhancer activation? The epigenetics aspect of the manuscript is underdeveloped and if pursued would allow at least one mechanistic aspect to be defined.

Major:

1) The authors should provide a clear sense of the epigenetic impact of NCOR1/2 single and double loss of function on epigenetic regulation. The ATACseq data are undeveloped and there is no chromatin analysis of any kind towards explaining corepressor loss of function regulation of the genome. These kinds of studies can be readily performed even in relatively small cell populations with methods such as Cut and Run. The question to address is whether the effects of corepressor loss are due to direct DNA damage perturbation as is claimed with HDAC3, or if this is indirectly caused by epigenetic reprogramming effects. Right now it is not clear exactly how especially NCOR1 loss functions as a tumor suppressor

- Our initial attempt at assessing the epigenetic impact of NCOR deletion was trying Cut&Run. However, Cut&Run unfortunately failed in our hands. ChIP-qPCR attempts have also been difficult due to getting insufficient number of cells to work with. Finally, Cut&Run and ChIPseq/ChIP-qPCR are methods that require using surface marker-based cell sorting of different stages. Due to the limitations of surface marker-based population delineation, cell sorting using surface markers is going to contain some heterogeneous populations (ex. cycling pro B/pro B VDJ/cycling pre-B cells). Single-cell Cut&Run and single-cell Cut&Tag approaches have recently been published and would be great technologies to use in the future that could overcome these inherent limitations. At the moment though they are still quite challenging and expensive. Therefore, we believed that scATAC-seq was a surface marker bias-free approach that was going to provide the best way to characterize the epigenetic landscape upon Ncor1 or Ncor1/2 perturbation in multiple B cell developmental stages.

  We agree with the points raised by the reviewer that the data from the scATAC-seq was underdeveloped at the time. Using the suggestions from the reviewer, we focused on further harnessing our scATAC-seq data and have strengthened existing findings and gained new insights, including:

  1. Light chain recombination defects that can be attributed to decreased kappa light chain accessibility in the NCOR DKO B cells (Figure 4e)
  2. Overlap of STAT5 and H3K27Ac ChIP-seq sites with differentially accessible peaks in WT, NCOR1 KO and NCOR DKO B cells. These new results show that the more accessible peaks in the NCOR-knockouts primarily overlap with H3K27Ac sites and in many cases with STAT5 binding sites (Figure 4f).
  3. Aberrant KLF2 expression and accessibility, resulting in increased expression and accessibility of KLF2 target genes, Cd69 and Sell (L-selectin; CD62L). NCOR-knockout B cells also have increased KLF2 motif enrichment (Figure 4g). We were able to confirm the increased frequency of CD62L+ protein expression in the NCOR DKO B cells (Supplementary Figure 5).

4. In addition to now showing increased expression and accessibility of *Rag1* and *Rag2*, we also were able to identify increased expression and accessibility of the upstream regulator *Foxo1* (Figure 5a, 5b, Supplementary Figure 3). We identified motif enrichment for FOXO1 in the NCOR-knockout B cells (Figure 4g). This is also consistent with the STAT5 findings, as FOXO1 has been shown to upregulate IL7R[1]. Consistent with that concept, we found that the NCOR DKO B cells have increased IL7R expression which is shown in new Figure 3.
5. Strengthened the STAT5 aspect of the story, as we now show STAT5 target genes also display increased accessibility in the NCOR-knockout B cells (Supplementary Figure 3 and Supplementary Table 3).
6. All scATAC-seq data comparisons done at a cluster level to detect differences and make comparisons according to B cell development stage (previous analyses were done at a bulk level).

2) The link between the B-cell development side and leukemogenesis is a bit difficult to reconcile. In part because the major focus of the first 2/3rds of the manuscript are focused on double KO of NCOR1/2, which seems to be qualitatively different in nature to NCOR1 single KO. For example, if induction of STAT5 activity is a relevant downstream effect of NCOR1 mutation than why would there be cooperation to induce leukemias? It is possible that this effect is not generally applicable to NCOR1 KO alone, but this is not made clear. NCOR1 and NCOR2 are well known corepressor partners of BCL6, which was reported by the Muschen lab to play similar roles and by their group and earlier papers to compete with STATs for DNA binding. Is it possible that this mechanism could be relevant here? GCs and BCL6, STATs and BCL6?

- The reviewer raises an important question about the biological/leukemogenesis relevance of our studies (how do our findings from Ncor1 KO, NCOR1/2 DKO developing B cells extend to human B-ALLs with bona fide loss of function *NCOR1* or *NCOR2* mutations?). It is clear from our knockout phenotyping data that *Ncor1*-knockout alone have mild defects, while *the Ncor1/2*-double knockout B cells have strong defects in proliferating and differentiation stages, suggesting functional redundancy between *Ncor1* and *Ncor2*. However, despite the phenotypic functional redundancy, we also find stepwise changes in gene expression and genomic accessibility related to preBCR-signaling (ex. *Nrgn*), STAT5-target genes (ex. *Pim1*), recombination (ex. *Rag*, *Xrcc6/Top1*), and transcription factor motifs (ex. CTCF), where the effect becomes more profound upon deleting more *Ncor* genes. This suggests non-redundancies at the transcriptional/epigenetic level. Putting this together, the gene dosage of *Ncor1* and *Ncor2* appears to fine-tune its targets transcriptionally and epigenetically. Therefore, in leukemias, the loss of NCOR1 or NCOR2 function alone may be the “sweet spot” that drives alterations in these pathways but prevents the strong selective pressure against having defects in both *Ncor1* and *Ncor2* as that could be synthetically lethal. This is

further evidenced in human B-ALLs by the seemingly mutually exclusive nature of *NCOR1* and *NCOR2* mutations and the absence of mutations in the *HDAC3* gene in human B-ALLs. (https://pecan.stjude.cloud/proteinpaint/HDAC3).

Finally, the reviewer wondered why if NCOR mutations enhance STAT5 signaling, it would cooperate with a STAT5 gain-of-function allele to induce transformation. However, we believe this is entirely consistent with our previous findings. Our previous studies evaluating cooperating mutations with STAT5b-CA revealed additional mutations within the JAK/STAT5 pathway that results in further activation of STAT5[2]. In those studies, it was clear that one needs a higher threshold of STAT5 signaling than achieved with our GOF STAT5 allele alone. Thus, aberrant expression of the FOXO1/Il7R axis likely contributes to leukemogenesis, even on a *Stat5b*-CA background.

3) The patient data could be stronger. It would be important to determine whether similar effects as observed in B-cells are occurring in the NCOR1 mutant patients (transcriptomes, DNA damage) vs all B-ALLs. It remains difficult to understand how the two parts of the story link together, and to assess clinical significance a multivariate analysis is required.

- All human B-ALL data presented in this paper were derived from publicly available databases (St Jude Protein Paint & PRECOG). These public databases limit access to some of the raw data, making some of the analyses suggested by the reviewer more difficult. However, we have been able to further analyze the human data and now demonstrate that FOXO1, IL7R, RAG1 and RAG2 show increased expression in leukemias with NCOR mutations relative to B-ALLs with intact NCORs. We also show increased structural variants in NCOR mutated leukemias versus non NCOR-mutated leukemias. A multivariate analysis is difficult, as the frequency of NCOR1 or NCOR2 mutations in human B-ALL patients, is quite low (~3%). This leads to small sample size that results in insufficient statistical power to make multivariate comparisons.

Here are suggestions organized by figure

Fig S1 and 1

I would like to see performance metrics for the NCOR1 flow cytometry. I would expect to see no signal in the NCOR1 KO cells and so would need additional controls for example related to Fig S1B. Also I would like to see flow side by side with QPCR and Western blots. NCOR1 and NCOR2 antibodies are notoriously fickle and non-specific. Suggest to show genotyping data in

B-cells showing that NCOR1 and 2 are knocked out.

- We now show more performance metrics data for the NCOR1 flow cytometry. As data shown in the response to reviewer 1 comment #1, we have found that the NCOR1 KO or NCOR1/2 DKO B cells when stained with the NCOR1 antibody drop to the levels of the isotype controls (Supplementary Figure 1b, 1c). This confirmed both clean deletion of NCOR1 at the protein level in both NCOR1 KO and NCOR1/2 DKO B cells but also confirmed specificity of the NCOR1 antibody used. In addition to NCOR1 protein knockout validation, qPCR results also suggested very clean deletion of NCOR1 in both the bone marrow and splenic B cells (Supplementary Figure 1d). Finally, we used our whole genome sequencing data from our NCOR1/2 DKO B cells and found that the *Ncor1* gene region flanked by the *loxP* sites had essentially undetectable reads, confirming deletion at the genetic level.

  For NCOR2 protein, the reviewer was correct that the NCOR2 antibodies we tried were non-specific, so we were not able to validate deletion at the protein level. However, using qPCR we found that *Ncor2* had a 90% decrease in expression in bone marrow B cells, suggesting good knockout efficiency. However, in the spleen, there was only a partial decrease in *Ncor2* expression, suggesting the presence of cells that haven't knocked out *Ncor2* completely. This may be due to strong selective pressure for some NCOR1/2 in splenic B cells; since the *Ncor2* deletion approach works via a gene trap and not exon deletion (like *Ncor1*) it may be more susceptible to escaping deletion. At a genetic level, in accordance with the decreased *Ncor2* expression in bone marrow B cells, we were able to confirm the inversion of the *Ncor2* gene trap, suggesting efficient Cre-mediated recombination.

Suggest to show effect on germinal center B cells. Increase in MZ suggest there is deviation away from GCs as for example shown to be caused by forced NOTCH2 activation in mature B cells. Deviation from GCs would be consistent with impairing BCL6 function, which is highly dependent on NCOR1/2, and in which repression of P53 by BCL6 was shown to be important by the Dalla Favera group. Given the importance of BCL6 in lymphomas and also proposed by the Muschen group for ALL, this would be of importance to delineate in this case.

- Given the presence of *Ncor2* non-deleters in the periphery, we have removed the splenic phenotyping data. Likewise, assessing germinal center B cells may not accurately reflect the true effect of *Ncor1/2*-deficient B cells, given the issues with *Ncor2* deletion.

Fig 2

The correlation of NCOR1 and Ki67 is of interest but not highly convincing based on flow cytometry for reasons mentioned in relation to Fig 1. More in the way of controls would be

helpful here. In addition to the above mentioned, the authors could correlate mRNA levels of these two genes. Given the distribution of cells in the correlation plot shown in 2A it would help the authors case if it were clear that theKi67 high cells where truly NCOR1 positive vs the apparently larger population of NCOR1 high cells with low Ki67 shown in the same plot

We hope that the new data demonstrating the specificity of the NCOR1 antibody will provide more confidence to the reviewer of its expression relationship with Ki67. Also, as noted in the response to reviewer 2, comment #2, we noticed while validating our NCOR1 KO deletion status, that the isotype and NCOR1 antibody were binding to the $F(ab)_2$ anti-IgM antibody bound to cells expressing high amounts of IgM. As part of this revision, we reanalyzed wildtype mice with a different anti-IgM antibody that was not cross-reactive, and were able to further strengthen the positive correlation between NCOR1 and KI67 (Figure 2a, 2b).

Fig 2B please also show mRNA (could even be from publicly available databases) – or even better by QPCR.

- As shown in the response to reviewer 1, minor comment 1, *Ncor1* expression is much higher than *Ncor2* expression at the RNA level (immgen database). Furthermore, *Ncor1* expression in the developing B cells appear to be constant throughout B cell developmental stages. On the other hand, *Ncor2* expression is highest in the mature B cells, followed by the pro-B/pre-B proliferating cells.

Fig 2C the result is clear, just not sure of the interpretation. NCOR1/2 DKO cells might fail to proliferate secondarily to effects on other cellular processes. I would just urge the authors to acknowledge this in the text since it seems implied that there is a direct link.

- Per reviewer's suggestion, the text has been updated as following:

  "Among the pre-pro- B, pro- B and pr- B/pre-B cells, Ki67 expression was lower in the NCOR1/2 DKO progenitor B cells (Figure 2c), suggesting that the ability of cells to proliferate may be directly impaired in the absence of nuclear corepressors or secondarily due to the effects on other biological processes."

Fig 2F would benefit from summary plots showing reproducibility of these numbers

- Summary plots with replicates have now been added to Figure 2f

Fig S2 and 3

Were scATAC also done in replicates?

- scATAC-seq was performed once for each genotype in three separate library captures.

Please clarify what gender the specimens were from

- The wildtype mouse was a male, while the NCOR1 KO and NCOR1/2 DKO mice were females. To address this source of sex bias we did the following. First, to ensure that we would deal with potential bias across all chromosomes, we used publicly available RNA-Seq data from IMMGEN to determine differentially expressed genes for male vs female B-cells. In our reanalysis of samples, we found that the only genes that were differentially expressed (FDR<= 0.05, no fold change threshold) between male and female mouse B-cells were on ChrX and ChrY (Xist, Eif2s3y, Ddx3y, Uty, and Kdm5d). This result meant that we didn't need to remove any autosomal genes from our analysis and we only needed to address the sex chromosomes. In our next step, we eliminated all ChrX and ChrY peaks from our scATAC-Seq data prior to our differential accessibility testing, to adjust for the sex bias in the experiment.
-

3B: the color scheme does not have sufficient contrast to visualize where the various subpopulations distribute to in the UMAPs. Either use colors with greater contrast, or generate additional plots where only a few clusters are colored at a time leaving the others with grey, or can use contours to indicate the subpopulations.

- The color scheme has been changed to provide better contrast and identification of clusters. If reviewers find it still difficult to differentiate, we can change colors again or utilize better labeling of the clusters.

3F: For all GSEAs shown here and later, the plots shown could be represented in a more rigorous manner. It would be recommended to show the histograms for differential gene expression, the normalized enrichment score for every single one of the GSEA plots. Also I was surprised that the numbers of genes in the gene sets were so sparse. For example the KEGG B cell receptor gene set used here has many more genes than shown. Same for the rest of the GSEAs shown in subsequent panels and figures.

- As part of deriving differentially expressed genes, we only retrieved genes that at least had a $\log_2$ fold change of 0.25 and that had a p-value < 0.05. This gene list was originally used to derive the GSEAs, which resulted in the sparsity in number of genes. We have now updated all GSEAs shown in the figures as requested using all genes. We now include NES scores for all GSEA plots and show tick marks for all genes in the gene sets.

S3 and 3J:
elaborate on the ATACs: How many of these were performed, how many cells per genotype, how did the QC look?

- scATAC-seq was performed once for each genotype in three separate library captures. We repeated the capture for the wildtype population since the initial capture yielded few cells. We are using only the second high quality Wildtype data set in this revised manuscript along with the original KO and DKO scATAC-seq libraries which were high quality. We used standard Signac QC thresholds for the data: peak_region_fragments > 3000 & < 20000, pct_reads_in_peaks > 15, blacklist_ratio <0.05, nucleosome_signal < 4, & TSS.enrichment > 2. After this QC filtering we had 8574 DKO cells, 6008 KO cells, and 8852 WT cells.

How well does the ATAC signal correlate with the gene expression profiles in each population?

- This suggestion was very helpful in strengthening existing findings but also revealing new insights. We compared the overlap between genes that were both increased in expression and accessibility amongst the different stages of B cells. This led to an emerging of key biological processes. For example, we found STAT5 target genes (*Socs2*, *Ccnd2*, *Pim1*, *Mcl1*, *Gimap6*, and *Rhoh*) were both upregulated in expression and accessibility (Supplementary Figure 3). In addition, we found increased expression and accessibility in V(D)J recombination genes (*Foxo1*, *Rag1*, and *Dntt*), DNA damage and repair genes (*Shisa5*, *Asf1a*, *Btg1*, *Btg2*, and *Apobec3*), and cellular localization and egress genes (*Klf2* and *Cd69*) (Supplementary Figure 3). We were able to identify that the KLF2-target gene CD62L (also increased in accessibility) was expressed at a higher frequency in NCOR DKO B cells (Supplementary Figure 6).

S3B: not clear what this is showing. It is unlikely that just a handful of cells were profiled in the WT setting.

- We agree that our initial scATAC-Seq data for the WT sample had relatively few cells. We substituted the previous wildtype scATAC-seq dataset with a new one that returned

much higher numbers of cells (~8,800 cells) that we used to reanalyze our scATAC-Seq studies. The new UMAP plots for distribution of cells in each cluster can be seen in Figure 4a.

Can some insight be gained from the ATAC delineated populations regarding which stages of development are perturbed from the epigenetic standpoint, perhaps preceding where the RNA-seq places these findings?

- We now show scATAC-seq at a cluster level and compared across the three genotypes to determine epigenetic perturbations instead of bulk cells. As in the response to the comment regarding correlation between ATAC and RNA, this led to analyses that identified changes in accessibility of STAT5 and FOXO1 target genes, DNA damage genes, and cellular localization and egress transcription factor genes (KLF2). In addition, we used the stages defined from scATAC-seq to compare the differentially accessible peaks in NCOR deleted B cells with STAT5 and H3K27Ac ChIP-seq data. This allowed us to demonstrate that changes in chromatin accessibility overlapped strongly with previously identified H3K27Ac sites in developing B cells. We were not able to identify epigenetic perturbations that preceded the changes in RNA-seq.

Would be of interest to show pseudobulk analysis of key STAT target regulatory elements and perhaps superenhancers in the several subpopulations, especially for genes the authors believe to be critical mediators of the observed phenotype.

- This relates to the comment above – H3K27Ac sites and STAT5 binding sites overlap significantly with superenhancers in B cells (see Katerndahl et al, Nature Immunology 2017). We show that there is significant overlap between sites with altered chromatin accessibility upon NCOR1/2 deletion and these two markers that correlate with superenhancers.

Figure 4
4D. It would be good to be consistent in how the abundance of the various genes are measured. For example, BIM should also be measured at the transcript level given that NCOR1/2 are transcriptional regulators, rather than only by flow.

- We have now added in the expression and accessibility data for the *Bim* (*Bcl2l11*) gene. While *Bim* RNA expression is difficult to ascertain, likely due to single-cell transcript drop out issues, we do find the accessibility of the *Bim* gene is increased in the NCOR-knockout B cells (Supplementary Figure 4e).

4E is described as measuring “epigenetic” changes, but in fact all that is shown is flow cytometry for H3K27Ac. This is not evidence of epigenetic changes. The authors would need to

a) perform acid extract western blots since these histone antibodies may not be highly specific and b) if they want to say there is an epigenetic consequence would need to perform Cut and Run (can be done in very small cell populations) to assess the genomic distribution and see if this is linked to differential accessibility or expression.

- We have removed this H3K27Ac flow cytometry figure. As mentioned in response to major comment #1, our attempt with Cut&Run was unfortunately unsuccessful. Furthermore, traditional ChIP-seq/ChIP-qPCR has been difficult due to the inability of obtaining sufficient cell numbers to work with for the different stages of B cell development. Ultimately, the cell input for these ChIP studies requires surface marker-based cell sorting, but we were concerned that this may result in the analysis of heterogeneous populations. Instead, we used our scATAC-seq data to compare with existing STAT5 and H3K27Ac ChIP-seq data to ascertain how much of the differentially accessible peaks overlapped with these sites. This is shown in figure 4f.

4F: The authors should determine whether the TFs captured by motif analysis are expressed in the various subpopulations of interest.

- Key transcription factor motifs that were the main focus of this paper (STAT5, KLF2, FOXO1) are shown for their expression in the different stages. This is now summarized in supplementary figure 3 and shown in supplemental tables 1 and 3.

4G: what is the significance of the CTCF motif depletion?

- CTCF function has important implications in V(D)J locus contraction. For example, the Igh locus undergoes loop contraction, bringing the distal V_H segments closer to the DJ_H segments for recombination. CTCF is important for mediating this locus contraction as reduction in CTCF binding decreases locus contraction[5], resulting in increased proximal V_H usage. Similarly, HDAC3-knockout B cells have been shown to have increased proximal V_H usage[6]. Therefore, our scATAC-seq CTCF motif depletion finding in the NCOR-knockouts suggests impaired locus contraction as a possible mechanism contributing to recombination defects.

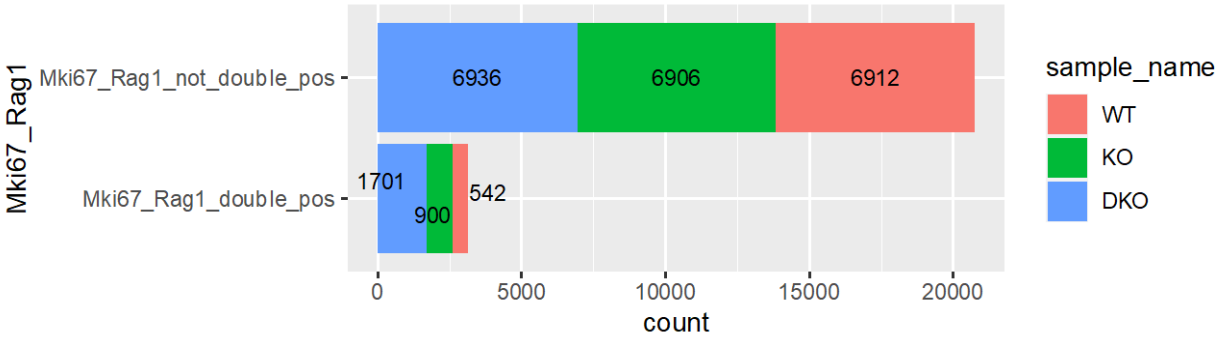
  In addition, CTCF also serves as impediments to long range RAG scanning within chromatin loops. Recent studies by the groups of Dr. Fred Alt and Dr. Meinrad Busslinger created inversions of the V_H locus, rendering the CTCF-binding elements in the reverse orientation[7,8]. This led to the generation of 2.4Mb+ chromatin loops and off-target RAG recombination outside the V_H locus due to unimpeded long-range RAG scanning. Therefore, in our studies, aberrant Rag1/2 expression and the depletion of

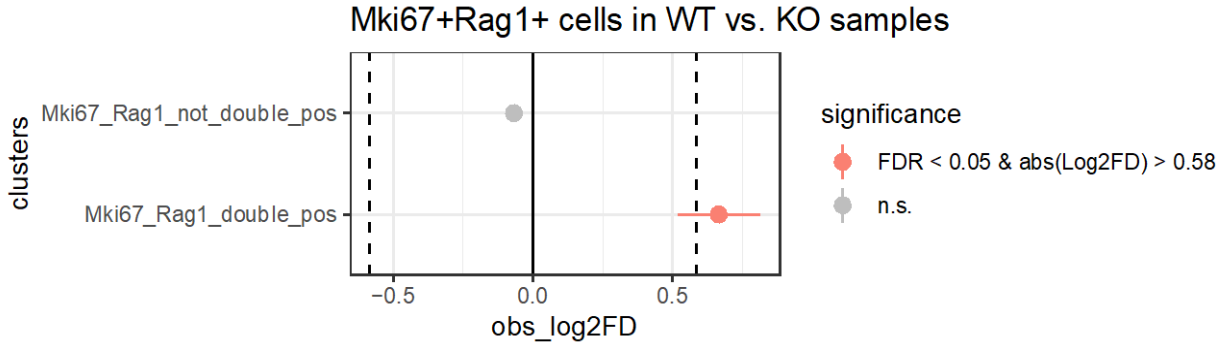
CTCF motifs in NCOR-knockouts could indicate long range RAG scanning across large chromatin loops, increasing the chances of potential off-target RAG recombination. These points are included in the discussion.

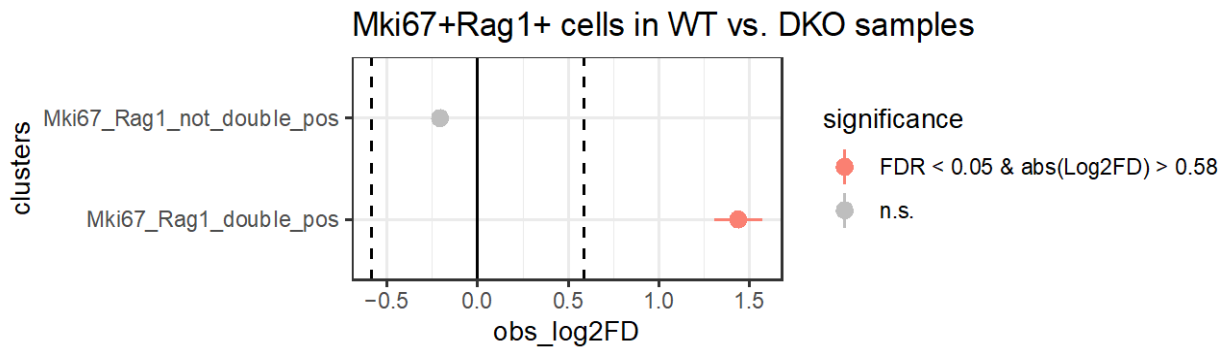
Figure 5:

5C difficulty to interpret without proper statistics.

- This figure has now been moved to Figure 5d and we now provide statistical analysis to determine the frequency differences of *Mki67*$^{+}$*Rag1*$^{+}$ expressing cells in the different genotypes. Similar to the statistical analysis to determine frequency differences of certain clusters between the genotypes (related to reviewer 1, minor comment #3), we performed a permutation test to derive the p-value for difference in the proportion of cells expressing both *Mki67* and *Rag1* in the NCOR1 KO and NCOR1/2 DKO compared to the wildtype B cells. Results demonstrated a statistically significant increase in the proportion of *Mki67*$^{+}$*Rag1*$^{+}$ B cells in the NCOR1 KO and NCOR1/2 DKO compared to wildtype B cells (shown below). These statistics are now represented as asterisks in figure 5d and corresponding figure legends.







5E: the Y axis not annotated, so cannot tell if these are on similar scale. Also not clear what cell types this corresponds to.

- The coverage plot represents immature B cells. The y-axis is identical for all three plots (range 0-160). This information has been added into the main text and figure legend. This figure has now been moved to Fig. 5C

5G: Please generate a pie chart or similar summarizing what types of structural lesions were present.
Also, If the authors wish to affirm that RAG1 targets overlap significantly with the DKO structural lesions then they need to perform appropriate statistical analysis, ideally taking into account covariates such as accessibility of these sites, etc.

- A summary figure of the different structural variant subtypes is now shown in Figure 5f and demonstrates increased number of structural variants present in the NCOR DKO cells. As RAG can bind and scan along the chromatin to ultimately mediate cleavage, RAG binding overlap with structural lesions may be difficult to interpret. Instead, we looked at deletions that were found specifically in the DKO samples and found cryptic heptamer sequences present within the deleted regions of some of these genes (Figure 5h). These cryptic heptamer sequences are associated with off-target RAG recombination. Furthermore, possible RAG-independent mechanisms of genomic instability are presented in the discussion:

  “For example, previous work in mouse embryonic fibroblasts deficient in HDAC3 demonstrated increased DNA damage via increased H4K5 acetylation. The deletion of *Hdac3* also led to insufficient DNA repair in fibroblasts”.

Figure 6:

In addition to flow plots the authors should provide a sense of how robust this finding is by showing the data across multiple biological replicates.

- A summary figure of the leukemia phenotypes is now shown in Figure 6.

Figure 7.

7B-C. The authors should show whether SVs and translocations are significantly more common in NCOR1 mutated patients vs the background of all B-ALLs.

- We show that the NCOR1 or NCOR2 defective B-ALL patients have increased number of structural variants compared to NCOR-wildtype B-ALL patients (Figure 7e).

7D. To claim there is a significant difference the authors must apply the appropriate statistics.

- We now show the FPKM expression differences in genes *FOXO1*, *RAG1*, *RAG2*, and *IL7R*, which demonstrated higher expression of these genes in the *NCOR1* or *NCOR2*-defective human B-ALLs, compared to the wildtype *NCOR* samples. Two-tailed unpaired t-test was performed to demonstrate statistically significant differences.

7E. The clinical data are not especially compelling. Does this relationship between NCOR expression survive a multivariate analysis controlling for known molecular and clinical biomarkers? Also, is there a link between outcome and NCOR1 mutations?

- The survival analysis was obtained from St. Jude and Precog public databases, which only show univariate correlations between *NCOR1* expression and survival. Thus, it is possible that the worse outcomes are due to association of *NCOR* expression with other covariates. We now mention this caveat about our survival data in the text. Finally, while no direct link between *NCOR1* mutations and outcome has been established in B-ALLs, *NCOR1* alterations in GC Diffuse Large B cell Lymphomas have been shown to associate with poorer prognosis[9].

Suppl figure 6 does not seem to confirm that NCOR1 knockout causes the effects that are claimed. How does this relate to patients?

- The updated figure now shows the differences in expression and accessibility of *Btg1* and *Erg* by genotype in different stages of development. This information is pertinent as *Btg1* and *Erg* deletions are mediated by RAG and frequently co-occur in ETV6-RUNX1

and DUX4 B-ALL subtypes, respectively. *Ncor1* or *Ncor2* mutations were most commonly found in these two subtypes of human leukemia (Figure 7c). As *Rag* primarily binds to accessible regions in the genome, this increased accessibility of *Btg1* and *Erg* upon reduced NCOR expression or function could make them more likely to be targeted by RAG-mediated recombination.

Is there an NCOR1 transcription signature in the B-cell populations tested in mice? And do RNA-seq profiles from patients with NCOR 1 mutations enrich for these signatures? Do NCOR1 low or mutated ALLs have a STAT5 signature?

- To our knowledge, there is no known NCOR1 transcription signature of B cell populations tested in mice or humans. As for the STAT5 signature in NCOR mutated mice, this is difficult to tease apart as some subtypes with NCOR mutations already have strong STAT5 signatures (Ph+ or Ph-like). While we did not have the data to determine if an increased STAT5 signature is present, we were able to find that *IL7R* expression was increased in the NCOR-defective B-ALLs. This increased IL7R expression may contribute to increased STAT5 signaling and possible therapy escape mechanisms in B-ALL[1].

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Decision Letter, first revision:

Subject: Your manuscript, NI-A33516A

Message: Our ref: NI-A33516A

30th Aug 2022

Dear Dr. Farrar,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Nuclear corepressors NCOR1/NCOR2 regulate B cell development, maintain genomic integrity, and prevent transformation" (NI-A33516A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-portfolio/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external

peer review of your manuscript entitled "Nuclear corepressors NCOR1/NCOR2 regulate B cell development, maintain genomic integrity, and prevent transformation". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Immunology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

Best regards,

Elle Morris
Senior Editorial Assistant
Nature Immunology
Phone: 212 726 9207
Fax: 212 696 9752
E-mail: immunology@us.nature.com

On behalf of

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Reviewer #1:
Remarks to the Author:
The manuscript is improved and the majority of the control experiments I suggested has been performed to a conclusive end. This allows for a reader to better validate the presented data. The authors has also modified the text to better align with the experimental results presented in the paper.

The manuscript clearly show that combined deletion of Ncor1 and Ncor2 result in a dramatic impairment of B-cell development and that NCOR proteins are linked to leukemia . The authors has used a large set of complementary technical approaches to characterize B-cell development in these animals finding a substantial number of discrepancies that may explain the role of these repressors in B-cell development and leukemia. However, I still cannot see that the data presented allows for a conclusive understanding of the roles of NCOR proteins in either normal or malignant B-cell development.

Minor comments

Figure S1g and Figure legend indicate that the cells gated are B220+CD43+ but text (row 108) only mention CD43. To avoid any confusion it could be indicated that the cells are B220+ .

In the survival plots in figure 6a, the survival of control animals (caSTAT5b) are indicated for up to about 300 days while a significant part of the leukemias from Ncor1 het mice are detected at a later time point. I find it unsuitable to present survival data from timepoints beyond the termination of the control group.

Reviewer #2:
Remarks to the Author:
The revision has addressed many of my previous concerns. The primary characterization of the DKO is important and provides new information on the physiological functions of these two putative tumor suppressor genes. The additional control experiments clarified the model. While the deregulated RAG expression and increased chromatin accessibility are supported by the data, RAG protein levels and function are also subjected to tight regulation at the post-translational levels. It is not entirely clear whether RAG overactivation is the major course of the malignant transformation. We would encourage the authors to discuss other possibilities beyond RAG alone.

Reviewer #3:
Remarks to the Author:
The authors have sufficiently addressed the prior comments, I have no further questions for them.

Author Rebuttal, first revision:

Response to Review

We were glad to hear that the reviewers and editors were largely satisfied with our revisions and the manuscript has been accepted in principle. Our response to the final questions raised by the reviewers is outlined in red text below.

Reviewer #1:
Remarks to the Author:
The manuscript is improved and the majority of the control experiments I suggested has been performed to a conclusive end. This allows for a reader to better validate the presented data. The authors has also modified the text to better align with the experimental results presented in the paper.

The manuscript clearly show that combined deletion of Ncor1 and Ncor2 result in a dramatic impairment of B-cell development and that NCOR proteins are linked to leukemia . The authors has used a large set of complementary technical approaches to characterize B-cell development in these animals finding a substantial number of discrepancies that may explain the role of these repressors in B-cell development and leukemia. However, I still cannot see that the data presented allows for a conclusive understanding of the roles of NCOR proteins in either normal or malignant B-cell development.

We are happy that the reviewer was largely happy with our revisions. We are not exactly sure what the reviewer is asking for in the last sentence above. We believe that we have presented several mechanisms that account for the effects of NCOR1/2 on B cell development and transformation. It is unlikely of course that we have comprehensively addressed every possible mechanism by which NCORs affect B cells.

Minor comments

Figure S1g and Figure legend indicate that the cells gated are B220+CD43+ but text (row 108) only mention CD43. To avoid any confusion it could be indicated that the cells are B220+ .

We have fixed this issue so that the text and figure legend now match.

In the survival plots in figure 6a, the survival of control animals (caSTAT5b) are indicated for up to about 300 days while a significant part of the leukemias from Ncor1 het mice are detected at a later time point. I find it unsuitable to present survival data from timepoints beyond the termination of the control group.

We thank the author for this suggestion. We have redone this figure to censor all mice on the Stat5b-CA x Ncor1 het curve at 300 days. The results remain the same – p-value is slightly larger but still significant.

Reviewer #2:
Remarks to the Author:
The revision has addressed many of my previous concerns. The primary characterization of the DKO is important and provides new information on the physiological functions of these two putative tumor suppressor genes. The additional control experiments clarified the model. While the deregulated RAG expression and increased chromatin accessibility are supported by the data, RAG protein levels and function are also subjected to tight regulation at the post-translational levels. It is not entirely clear whether RAG overactivation is the major course of the malignant transformation. We would encourage the authors to discuss other possibilities beyond RAG alone.

We agree that mechanisms other than just alterations in *Rag* gene expression may also underlie the effects of NCOR1/2 deletion. We have discussed some of these in this manuscript, including effects on STAT5 activation, the p53 pathway, and alterations in CTCF locus accessibility. While we would be happy to discuss this in more detail, the editors have already asked us to cut ~1200 words from the existing manuscript which constrains our ability to add further discussion.

Reviewer #3:
Remarks to the Author:
The authors have sufficiently addressed the prior comments, I have no further questions for them.

We are glad that we were able to satisfactorily address this reviewer's concerns.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A33516B
Message: In reply please quote: NI-A33516B

Dear Mike,

I am delighted to accept your manuscript entitled "Nuclear corepressors NCOR1/NCOR2 regulate B cell development, maintain genomic integrity, and prevent transformation" for publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link

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Kind regards,

Laurie

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X