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Histone methyltransferase DOT1L controls state-specific identity during B cell differentiation

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1st Editorial Decision 30th Jun 2020

Dear Dr. Jacobs,

Thank you for transferring your manuscript from Review Commons to EMBO reports. I now went through your manuscript, the referee reports (attached again below), and your revision plan (point-by-point response). Both referees acknowledge the potential interest of the findings. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which you are willing to address during a major revision of the manuscript.

We thus would like to invite you to revise your manuscript for EMBO reports with the understanding that the referee concerns must be addressed in the revised manuscript and/or in a final detailed point-by-point response, as you indicated in your revision plan. Acceptance of your manuscript will depend on a positive outcome of a second round of review (using the same referees that have assessed the study before). It is our policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs

to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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9) Please add a conflict of interest statement (COI) to the manuscript after the author contributions.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO reports

Referee #1:

The manuscript titled Histone methyltransferase DOT1L controls state-specific identity during B cell differentiation by Aslam et al. addresses the role of the histone methyltransferase DOT1L in terminal B cell differentiation. Using mb1-Cre+/-;Dot1Lfl/fl mice to ablate DOT1L expression, the

authors showed that among mature B cells, GC B cells expressed the highest levels of DOT1L and were strongly reduced in DOT1L-deficient mice. Furthermore, GC B cell differentiation critically depended on DOT1L and upon in vitro activation, DOT1L-KO B cells did not proliferate and did not differentiate into GC B cells when challenged in vivo. Instead, DOT1L-KO B cells underwent accelerated partial plasma cell differentiation in vitro. Transcriptomic and epigenomic analyses revealed that DOT1L supported the repression of an anti-proliferative plasma cell differentiation program by maintaining expression of the H3K27 methyltransferase EZH2, the catalytic component of Polycomb Repressor Complex 2 (PRC2).

Major comments:

- Are the key conclusions convincing?

This is an elegant and clean study which provides convincing results by established interdisciplinary methodology: using the mb1-Cre+/-;Dot1Lfl/fl mouse line, the authors describe a B cell-compromised phenotype by conducting detailed in vivo and in vitro experiments. By combining transcriptomic and epigenomic approaches, they furthermore uncover the mechanism behind the observed phenotype.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
→ no

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Some interesting questions arise which may complement this study:

→ Is it possible to confirm DOT1L deletion directly on transcript or protein level?

→ The authors comment that reduced pre-B cells could be a result of impaired VDJ recombination in DOT1L-KO mice, but other causative factors cannot be excluded. Unfortunately, these factors are not addressed in further detail. It would be interesting to see whether the viability of DOT1L-KO cells is compromised in vivo; This is particularly interesting since the authors elegantly show that viability of the KO cells is compromised upon stimulation with LPS and IL-4 in vitro, (Fig S3d). Therefore, cell death (apoptosis) of B cell subsets in vivo should be addressed. This could be assessed by staining freshly isolated cells for cleaved caspase 3 or using Annexin V.

→ If impaired V(D)J recombination is indeed the cause for the reduced B cell pool, would B cell populations be restored if a pre-rearranged V(D)J fragments, such as B1-8 were expressed on DOT1L-deficient background?

→ In figure 3a-b the authors induce CSR using LPS, LPS and IL-4, or CD40 and IL-4 but show impaired proliferation only for the cells stimulated for CD40 and IL-4. Does stimulation with either LPS or LPS and IL-4 also result in impaired proliferation? Would there be any differences in frequency of switched cells among equally-proliferating populations when stimulated with either LPS or LPS and IL-4? It is important to assess proliferation upon combined LPS and IL-4 because this are the stimuli the authors use for RNA Seq analysis.

→ Would chemical DOT1L inhibition render similar results regarding GC B cells?

→ Deletion of DOT1L at an early developmental stage seems to result in generation of a compromised B cell pool, which in turn might give rise to compromised GC. It would be interesting to study a germinal center specific deletion of DOT1L, for example by employing the GC-specific Cγ1-Cre (Casola et al, Proc Natl Acad Sci U S A. 2006 May 9;103(19):7396-401. Epub 2006 May 1) or conditional Cγ1-CreERT2 (Weber et al, Eur J Immunol 49 (1): 192-194) mouse lines. Alternatively, inducible Cre lines, which allow conditional deletion of loxP-flanked genes in mature B cell stages, such as hCD20-CreERT2 (Khalil et al, Science. 2012 Jun 1;336(6085):1178-81) or mb1-CreERT2 (Hobeika et al, EMBO J. 2015 Apr 1;34(7):925-39. doi: 10.15252/embj.201489732. Epub 2015 Jan 28) could be used.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.

→ some of the suggested experiment (e.g. the in vitro experiments) are definitely realistic and doable. Additional mouse experiments with different Cre-lines might be out of scope of this revision. However, the authors might still consider using e.g. GC-specific Cre-lines to complement this study.

- Are the data and the methods presented in such a way that they can be reproduced?

→ yes

- Are the experiments adequately replicated and statistical analysis adequate?

→ yes

Minor comments:

- Specific experimental issues that are easily addressable.

→ in vitro proliferation analysis of LPS+IL-4 stimulation

→ The sentence "The number of transcriptional regulators involved in B cell development affected by DOT1L ablation implies the existence of a complex regulatory network that warrants further investigations to untangle" is too complex and requires shortening, eg delete " to untangle"

- Are prior studies referenced appropriately?

→yes

- Are the text and figures clear and accurate?

→yes

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

To enhance the quality of the findings and the conclusion, the authors should have a look at this very recent study addressing the role of DOT1L in the ER stress response: DOT1L inhibition is

lethal for multiple myeloma due to perturbation of the endoplasmic reticulum stress pathway (Dafflon et al, Oncotarget, 2020, Vol. 11, (No. 11), pp: 956-968). Plasma cells rely on a very potent ER stress response (unfolded protein response; UPR). In the present study, the expression of the UPR genes is not addressed (e.g. XBP1, which is downstream of BLIMP1 and is one of the master regulators of plasma cell development). It is possible that, once DOT1L is deleted, the ER stress response is attenuated leading to the aberrant plasma cell and GC development. If possible, the authors should assess the UPR gene status (e.g. of the IRE1α and ATF4 branches) in their transcriptomic analysis.

Significance:

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.

Since some lymphomas and leukaemias display DOT1L dependency it is of paramount importance to understand the molecular mechanisms of DOT1L in B cell development and differentiation

My field of expertise is general B cell immunology, B cell development and differentiation and in CLL; I have a lot of experience with the Cre/loxP mouse models in general and with mb1-Cre in particular.

Referee #2:

This paper describes the impact of Dot1L inactivation, an epigenetic writer that catalyzes methylation of H3K79, on B cell development and differentiation. One specific interest in Dot1L function comes from its role in mixed-lineage leukemia, for which it constitutes a druggable target.

B-cell specific deletion of Dot1L (through mb1-Cre) results in a global reduction in mature B cells (and a complete absence of marginal zone B cells), but the authors focused their study on the very impressive impact on the germinal center B cell reaction, which was completely abolished.

In vitro B cell activation documented a premature plasma cell differentiation, together with a proliferation defect. By RNA-seq and H3K79me CHIP-seq approaches, they identified convincing targets, Myc (a target of obvious relevance for lymphomagenesis), Bach2, and Ezh2.

The study is well-performed, well-controlled, and I have no major criticisms to make.

Inactivation of Dot1L appears to have a broad impact on several major transcriptional regulators, as discussed by the authors. Nevertheless, the similarity that they highlight between Dot1L and Ezh2 inactivation immediately raises the question whether restoring Ezh2 expression in in vitro activated B cells might - at least partially - restore the proliferative capacity and the differentiation profile of Dot1L KO B cells. Such complementation assay, whatever the result, would enlarge the discussion on the role of Dot1L for terminal B cell differentiation.

Point that would benefit from additional data:

-fig.S4a-e: the basal Ig levels is an important issue, and, considering the inherent variability of such data, there are clearly too few mice studied (4 in each group). Moreover, an analysis at two different ages would be interesting (young mice 3-4 weeks compared to older ones) to look for a possible

age-compensatory process.

Minor points:

-the B1a cell defect is intriguing, but the sole description of splenic B1a cells (with the phenotype CD19+B220low) is somewhat unsatisfactory. I would advise either to remove it or (better) to perform B1a/B1b analysis in the peritoneal cavity. B1 cell reduction is not even mentioned in the discussion.

-page 5: "unchanged between DOTL1-proficient and -deficient T cells" should be corrected since DOTL1 is not deleted in T cells in these mice.

-page 6: arguing that the switch machinery is functional because transcription of its components are normal is not convincing, since CSR depends on many factors like switch region accessibility. But data contained in fig.3e are convincing.

-page 9, last line: ref 85 does not exist, I guess it is 65.

-the presence of GC B cells in Peyer's patches is intriguing and might possibly deserve a comment. Was it described for Ezh2 (I'm not aware of such data)?
Significance (Required) -A large body of work has addressed the contribution of transcription factors and regulatory networks to the different commitment steps of the lymphocyte lineage. Studies on epigenetic regulation of such differentiation process have emerged more recently, and one specific interest in such study is the recurrence of epigenetic deregulations involved in oncogenic processes, which has led to the development of "epidrugs" to specifically target them. One specific interest of this study is its focus on a gene with major histone writer function for which relatively little is known so far.

-Immunologists will be obviously interested in such a study, but developmental biologists might be as well.

-my expertise: B cell immune response (both cellular and molecular), and there are no parts of the paper that fall out of my filed of expertise.

Amsterdam, November 3rd 2020

From: Dr. Heinz Jacobs
The Netherlands Cancer Institute
Immunology
Plesmanlaan 121
Amsterdam, North Holland 1066 CX
Netherlands

To: Dr. Achim Breitling
Editor EMBO reports

Point by point reply EMBOR-2020-51184V1 [RC-2020-00232]

Reviewer #1

Summary:

The manuscript titled Histone methyltransferase DOT1L controls state-specific identity during B cell differentiation by Aslam et al. addresses the role of the histone methyltransferase DOT1L in terminal B cell differentiation. Using mb1-Cre+/-;Dot1Lfl/fl mice to ablate DOT1L expression, the authors showed that among mature B cells, GC B cells expressed the highest levels of DOT1L and were strongly reduced in DOT1L-deficient mice. Furthermore, GC B cell differentiation critically depended on DOT1L and upon in vitro activation, DOT1L-KO B cells did not proliferate and did not differentiate into GC B cells when challenged in vivo. Instead, DOT1L-KO B cells underwent accelerated partial plasma cell differentiation in vitro. Transcriptomic and epigenomic analyses revealed that DOT1L supported the repression of an anti-proliferative plasma cell differentiation program by maintaining expression of the H3K27 methyltransferase EZH2, the catalytic component of Polycomb Repressor Complex 2 (PRC2).
The authors thank the reviewer for this precise summary.

Major comments:

- Are the key conclusions convincing?
This is an elegant and clean study which provides convincing results by established interdisciplinary methodology: using the mb1-Cre+/-;Dot1Lfl/fl mouse line, the authors describe a B cell-compromised phenotype by conducting detailed in vivo and in vitro experiments. By combining transcriptomic and epigenomic approaches, they furthermore uncover the mechanism behind the observed phenotype.
Thanks for appreciating this study.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
→ no

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Some interesting questions arise which may complement this study:

→ Is it possible to confirm DOT1L deletion directly on transcript or protein level?
In our hands, and also in other studies (Sabra et al, 2013 PMID: 23750013; Ngyuen et al, 2011 PMID: 21289070), none of the commercially available antibodies (nor one that we made ourselves) detects endogenous DOT1L protein levels in mouse cells. However, the Cre-mediated deletion of exon 2 that we use here leads to disruption of the conserved catalytic domain of DOT1L and introduces a frameshift. Therefore no functional DOT1L protein is expected to be present after Cre-mediated recombination (as confirmed by the loss of H3K79me2 in this study and other studies (e.g. Vlaming et al, 2019, PMID: 31304633; Jo et al, 2011, PMID: 21398221; Bovio et al, 2019, PMID: 30302725). At the transcript level, Dot1L mRNA is detectable in WT and KO by RNA-seq and as we now also show by RT-PCR (Fig. EV1A). To confirm deletion of exon 2 at the transcript level as suggested by the reviewer, we performed RT-PCR using cDNA from sorted KO B cells and confirmed the deletion of exon 2 by sanger sequencing. Both RT-PCR and sanger sequencing analysis clearly indicated effective deletion of exon 2 in Dot1L transcripts specifically from KO B cells. Furthermore, sequence analysis showed that deletion of exon 2 renders the Dot1L transcript out of frame and generates multiple translational stop codons. We explained this point in more detail in our revised manuscript (please see figure EV1A, Appendix; Figure S1 and the results section page 5).

→ The authors comment that reduced pre-B cells could be a result of impaired VDJ recombination in DOT1L-KO mice, but other causative factors cannot be excluded. Unfortunately, these factors are not addressed in further detail. It would be interesting to see whether the viability of DOT1L-KO cells is compromised in vivo; This is particularly interesting since the authors elegantly show that viability of the KO cells is compromised upon stimulation with LPS and IL-4 in vitro, (Fig S3d). Therefore, cell death (apoptosis) of B cell subsets in vivo should be addressed. This could be assessed by staining freshly isolated cells for cleaved caspase 3 or using Annexin V.
The reviewer raises a valid point, that we addressed by performing Annexin-V staining on freshly isolated bone marrow and spleen cells from WT and KO mice. This ex-vivo analysis revealed that compared to WT, KO B cells did not show an increased frequency of early or late apoptotic cells (see figure EV1C-I). Furthermore, to avoid any confounding issue associated with ex-vivo processing that might influence the results of Annexin-V staining, as a complementary approach we performed IHC using cleaved caspase-3 staining on spleen sections from WT and KO mice to determine the presence of apoptotic cells in situ. In line with the findings from Annexin-V staining, the in-situ results also led us to conclude that in vivo, the viability of Dot1L-KO cells is not compromised (see figure EV1J). These results are now described in the manuscript on page 6.

→ If impaired V(D)J recombination is indeed the cause for the reduced B cell pool, would B cell populations be restored if a pre-rearranged V(D)J fragments, such as B1-8 were expressed on DOT1L-deficient background?

This is indeed an interesting point. However, addressing this question requires combining multiple alleles, which takes 6 month of breeding (2 generations) and additional time for experimentation. In addition, fully understanding the contribution of DOT1L in V(D)J recombination will require a combination of sophisticated approaches, including chromosome-conformation analyses. Since the focus of our paper is on the role of DOT1L in B cell differentiation, we consider that understanding the role of DOT1L in VDJ recombination represents an independent project that is beyond the scope of the current manuscript.

→ In figure 3a-b the authors induce CSR using LPS, LPS and IL-4, or CD40 and IL-4 but show impaired proliferation only for the cells stimulated for CD40 and IL-4. Does stimulation with either LPS or LPS and IL-4 also result in impaired proliferation? Would there be any differences in frequency of switched cells among equally-proliferating populations when stimulated with either LPS or LPS and IL-4? It is important to assess proliferation upon combined LPS and IL-4 because this are the stimuli the authors use for RNA Seq analysis.

We entirely agree with the reviewer and performed these requested experiments. Interestingly, the proliferation data generated either from LPS or LPS + IL-4 revealed that intrinsically Dot1L-KO B cells are capable of proliferating like WT. Subsequent analyses of their switching potential revealed that despite their proliferation proficiency, DOT1L KO B cells showed reduced class switch recombination. These data are described and discussed in the revised manuscript (please see figure EV3B-E and result section page 7).

→ Would chemical DOT1L inhibition render similar results regarding GC B cells?

Thank you for the suggestion. Systemic application of DOT1L inhibitors in vivo will also affect other cell types, including CD4 T cells, and more specifically also Tfh that do contribute to the formation of GC and depend on DOT1L (Kwesi-Maliepaard et al 2020 PNAS, PMID: 32764145, Scheer et al, 2019 PMID: 30604761, and Scheer et al https://doi.org/10.1101/821348). Current DOT1L inhibitors also have poor pharmacokinetic properties which therefore require continuous infusion (Shortt et al, 2017, PMID: 28228643). Together, these factors complicate in vivo studies and make experiments very expensive, limiting the possibilities for studies on DOT1L inhibition to study GC specific effects in vivo. As an alternative approach, we performed in vitro assays to study GC B cell associated processes by exposing activated B cells to a specific DOT1L inhibitor, Pinometostat. Interestingly, the findings made in DOT1L-inhibited B cells closely recapitulate the phenotypes of Dot1L-KO B cells regarding proliferation, CSR, and plasma blast formation. These findings imply that the catalytic activity of DOT1L is involved in the phenotypic changes that we observed in the Dot1L-KO mouse model and that the phenotypic changes can occur in mature B cells independent of events earlier in the B cell lineage (this is also relevant for the next point). Therefore, these independent chemical inhibition studies further substantiate our findings made by genetic ablation of DOT1L early in the B cell lineage (please see figure EV3M-R, figure EV4F-I, figure EV 5 and result section page 7-8).

→ Deletion of DOT1L at an early developmental stage seems to result in generation of a compromised B cell pool, which in turn might give rise to compromised GC. It would be

interesting to study a germinal center specific deletion of DOT1L, for example by employing the GC-specific Cγ1-Cre (Casola et al, Proc Natl Acad Sci U S A. 2006 May 9;103(19):7396-401. Epub 2006 May 1) or conditional Cγ1-CreERT2 (Weber et al, Eur J Immunol 49 (1): 192-194) mouse lines. Alternatively, inducible Cre lines, which allow conditional deletion of LoxP-flanked genes in mature B cell stages, such as hCD20-CreERT2 (Khalil et al, Science. 2012 Jun 1;336(6085):1178-81) or mb1-CreERT2 (Hobeika et al, EMBO J. 2015 Apr 1;34(7):925-39. doi: 10.15252/embj.201489732. Epub 2015 Jan 28) could be used.

Confirming the findings of our mouse model in future studies with independent alternative models will indeed be important. Addressing this question by genetic models requires import of new Cre-driver mice and again complex breeding (2 generations) along with time consuming experimentation. However, we believe that the new findings provided by chemical inhibition of DOT1L in vitro (as mentioned above) not only provided the evidence that loss of DOT1L activity in mature B cells will result in compromised GC reaction but also as correctly pointed out by the reviewer excludes any potential indirect effect associated with the early ablation of Dot1L during B cell ontogeny. These results further support the findings of our mouse model with an independent approach as explained in detail (please see figure EV3M-R, figure EV4F-I, figure EV 5 and result section page 7-8).

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.

→ some of the suggested experiment (e.g. the in vitro experiments) are definitely realistic and doable.

Additional mouse experiments with different Cre-lines might be out of scope of this revision. However, the authors might still consider using e.g. GC-specific Cre-lines to complement this study.

As explained above, given the considerable amount of time and complex breeding plans as well as extensive experimentation we also consider this out of the scope, as suggested by this reviewer. In addition, because of COVID-19 restrictions, we were not allowed to establish new mouse lines which further limited us in performing the suggested in vivo experiments within a reasonable time frame. Yet, we feel that the new in vitro data provided by the chemical inhibition of DOT1L has nicely complemented our data regarding critical role of DOT1L in determining the fate of GC B cells.

- Are the data and the methods presented in such a way that they can be reproduced?

→ yes

- Are the experiments adequately replicated and statistical analysis adequate?

→ yes

Minor comments:

- Specific experimental issues that are easily addressable.

→ in vitro proliferation analysis of LPS+IL-4 stimulation

We entirely agree, the data has been added and explained in detail in the revised manuscript (please see figure EV3 C and E and result section page 7).

→ The sentence "The number of transcriptional regulators involved in B cell development affected by DOT1L ablation implies the existence of a complex regulatory network that warrants further investigations to untangle" is too complex and requires shortening, eg delete " to untangle"
We have shortened this sentence according to the reviewer's suggestion.

- Are prior studies referenced appropriately?
→yes

- Are the text and figures clear and accurate?
→yes

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

To enhance the quality of the findings and the conclusion, the authors should have a look at this very recent study addressing the role of DOT1L in the ER stress response: DOT1L inhibition is lethal for multiple myeloma due to perturbation of the endoplasmic reticulum stress pathway (Dafflon et al, Oncotarget, 2020, Vol. 11, (No. 11), pp: 956-968). Plasma cells rely on a very potent ER stress response (unfolded protein response; UPR). In the present study, the expression of the UPR genes is not addressed (e.g. XBP1, which is downstream of BLIMP1 and is one of the master regulators of plasma cell development). It is possible that, once DOT1L is deleted, the ER stress response is attenuated leading to the aberrant plasma cell and GC development. If possible, the authors should assess the UPR gene status (e.g. of the IRE1α and ATF4 branches) in their transcriptomic analysis.
We thank the reviewer for bringing this to our attention. Our new analyses of RNA-Seq data from activated B cells regarding transcription of UPR genes revealed that UPR genes are not expressed differentially between Dot1L-deficient and -proficient B cells. The data including gene set enrichment analysis and a comprehensive table (Appendix; Table EV2) mentioning the expression level of the individual genes involved in UPR has been added and discussed in the revised version. We now also refer to the Dafflon et al publication (please see figure EV6G and H and result section page 8-9).

Reviewer #1

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.
Since some lymphomas and leukaemias display DOT1L dependency it is of paramount importance to understand the molecular mechanisms of DOT1L in B cell development and differentiation.
My field of expertise is general B cell immunology, B cell development and differentiation and in CLL; I have a lot of experience with the Cre/loxP mouse models in general and with mb1-Cre in particular.

Reviewer #2

This paper describes the impact of Dot1L inactivation, an epigenetic writer that catalyzes methylation of H3K79, on B cell development and differentiation. One specific interest in Dot1L function comes from its role in mixed-lineage leukemia, for which it constitutes a druggable target.
B-cell specific deletion of Dot1L (through mb1-Cre) results in a global reduction in mature B cells (and a complete absence of marginal zone B cells), but the authors focused their study on the very impressive impact on the germinal center B cell reaction, which was completely abolished.
In vitro B cell activation documented a premature plasma cell differentiation, together with a proliferation defect. By RNA-seq and H3K79me CHIP-seq approaches, they identified convincing targets, Myc (a target of obvious relevance for lymphomagenesis), Bach2, and Ezh2.
The study is well-performed, well-controlled, and I have no major criticisms to make.
Thanks for this positive response.

Inactivation of Dot1L appears to have a broad impact on several major transcriptional regulators, as discussed by the authors. Nevertheless, the similarity that they highlight between Dot1L and Ezh2 inactivation immediately raises the question whether restoring Ezh2 expression in in vitro activated B cells might - at least partially - restore the proliferative capacity and the differentiation profile of Dot1L KO B cells. Such complementation assay, whatever the result, would enlarge the discussion on the role of Dot1L for terminal B cell differentiation.
DOT1L affects several major transcriptional regulators. One of these factors is EZH2. Addressing whether the defect in Dot1L-KO cells can be restored by restoring EZH2 is an important question, but also a difficult one to answer. In vivo experiments require establishment of a new transgenic system, in which EZH2 levels are restored via an ectopic allele. In vitro, this will depend on viral transduction to overexpress EZH2, which is hard to accomplish as DOT1L KO B cells die upon activation, a prerequisite for efficient transduction. Furthermore, EZH2 acts in a dose-dependent manner (Sarris 2013, PMID 23503463; Piunti 2016, PMID 27257261); therefore, we expect that extensive optimization may be needed to restore EZH2 expression back to wild-type levels in order to prevent non-physiological effects. Finally, we expect that restoring EZH2 levels may not be sufficient given the deregulation of the other key transcriptional regulators in the KO setting. Taking these issues together, we consider this a technically very challenging approach that may not give a conclusive answer. Therefore, we propose that this issue is best addressed in independent future studies. We made some adjustments in the text to emphasize that EZH2 regulation, or more specifically regulation of a subset of the targets of EZH2, is one node in a larger network affected by DOT1L (please see the abstract and the discussion on pages 13-14).

Point that would benefit from additional data:

-fig.S4a-e: the basal Ig levels is an important issue, and, considering the inherent variability of such data, there are clearly too few mice studied (4 in each group). Moreover, an analysis

at two different ages would be interesting (young mice 3-4 weeks compared to older ones) to look for a possible age-compensatory process.
To address this important point, we determined and added basal Ig titers data for IgM, IgA, IgG1, IgG2b and IgG3 from additional mice from WT and KO (now a total of 8 mice per genotype). These data are now provided in figure EV4A which further strengthened our conclusions. Regarding the analysis of basal Ig titers to determine any age-related compensation we realized that it requires first a large cohort of mice to draw any definitive conclusion and second the sera should be analyzed from the same mice at different time points (age) rather than comparing them across mice with different age groups. Due to the COVID-19 crisis our in-house regulations did not permit large expansions of our mouse cohorts. Considering the number of age- and gender-matched mice and the time to establish aged matched cohorts (~ 30 weeks), we could not address age related effects on CSR.

Minor points:

-the B1a cell defect is intriguing, but the sole description of splenic B1a cells (with the phenotype CD19+B220low) is somewhat unsatisfactory. I would advise either to remove it or (better) to perform B1a/B1b analysis in the peritoneal cavity. B1 cell reduction is not even mentioned in the discussion.
We agree with this suggestion and removed this part as it is not the focus of this report.

-page 5: "unchanged between DOTL1-proficient and -deficient T cells" should be corrected since DOTL1 is not deleted in T cells in these mice.
Thank you, it has been corrected in the revised version.

-page 6: arguing that the switch machinery is functional because transcription of its components are normal is not convincing, since CSR depends on many factors like switch region accessibility. But data contained in fig.3e are convincing.
CSR is indeed a multifactorial process affected by the involvement of many cis and trans acting factors. In addition, association of DOT1L with DNA damage response and induction of apoptosis led us to suggest that actively switching cells may become prone to die in the absence of DOT1L. For further clarification, we rephrased this part in the revised version of the manuscript (please see the result section page 7).

-page 9, last line: ref 85 does not exist, I guess it is 65.
Thanks for pointing this out to us, it has been corrected.

-the presence of GC B cells in Peyer's patches is intriguing and might possibly deserve a comment. Was it described for Ezh2 (I'm not aware of such data)?
We looked into the relevant literature and found that GC B cells in Peyer's patches were also drastically reduced in absence of Ezh2 (Caganova et al., 2013). We have commented on this in the discussion section (please see discussion section page 13-14).

Reviewer #2 (Significance (Required)):

-A large body of work has addressed the contribution of transcription factors and regulatory networks to the different commitment steps of the lymphocyte lineage. Studies on

epigenetic regulation of such differentiation process have emerged more recently, and one specific interest in such study is the recurrence of epigenetic deregulations involved in oncogenic processes, which has led to the development of "epidrugs" to specifically target them. One specific interest of this study is its focus on a gene with major histone writer function for which relatively little is known so far

-Immunologists will be obviously interested in such a study, but developmental biologists might be as well.

-my expertise: B cell immune response (both cellular and molecular), and there are no parts of the paper that fall out of my filed of expertise.

1st Revision - Editorial Decision 19th Nov 2020

Dear Dr. Jacobs,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please provide individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

- We can accommodate up to 8 main figures and 5 EV figures. Presently, there are 6 EV figures. Thus, please re-arrange this accordingly.

- Please name the 'Methods' section 'Materials and Methods'.

- Please add a conflict-of-interest statement to the final version of the manuscript, next to the acknowledgements and the author contributions.

- Please remove the referee tokens from the data availability section of final version of the paper and assure that the deposited data will be public upon publication of the paper.

- In Fig. 6F some of the plots are marked by black boxes, some are not. Is this to highlight something? Or should there be boxes for all plots. Please check.

- In the legends it is stated several times that source data for a figure is available online. But no related source data files have been uploaded. Please check and upload the SD as one pdf file per figure.

- Please add a TOC (table of contents) to the Appendix file with page numbers. Please name the figures and tables Appendix Figure Sx, Appendix Table Sx and use this as callout in the manuscript text.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. It seems, you already addressed these, but please check again. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors adequately addressed all our concerns.

Referee #2:

I previously reviewed this paper for Review Commons, and formulated a few comments. Feasible experiments have been performed (and do not change the message), while those more complex or lengthy have been rebutted with reasonable and sound arguments. I have therefore no criticisms for this revised version.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 8th Dec 2020

Dr. Heinz Jacobs
The Netherlands Cancer Institute
Immunology
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Amsterdam, North Holland 1066 CX
Netherlands

Dear Dr. Jacobs,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Achim Breiling
Editor
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Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51184V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	As the effect sizes were unknown, we used published, standard sample sizes for our initial analyses. As the effect sizes were enormous, the statistical power of our data was in retrospect very high.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample sizes of our experiments involving mice were based on published data using similar settings (standard sample sizes).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	We standardized our assays to omit any potential confounders. This included the maintennace of our mouse cohort in a stable environment, i.e. individually ventilated cages (IVC) using our in house breeding facility w/o allocation. Biological and technical replicates were usded to minimize confounding issues and determine the robustness and reproducibility of our results.
For animal studies, include a statement about randomization even if no randomization was used.	As our analyses required the comparison of transgenic and non-transgenic mice, selection was made on the basis of the genotype and age-matched mice. Littermates were used whenever available with mixed genders. Randomization was not done.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Experiments were repeated and analysed independently by different investigators. Blinding was not applied.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done. Actually, as the phenotypes were very prominent, we retrospectively could genotype the mice based on their phenotype, independent of any prior indication of the genotype.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical tests applied were based on established methods for each assay, considering the distribution of the data.
Is there an estimate of variation within each group of data?	No, we did not estimate the variations within each group.

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Is the variance similar between the groups that are being statistically compared?	The statistical methods used to compare the different groups take into account the variance.

C- Reagents

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used in this study.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mb1-Cre+/-;Dot1Lfl/fl mice were derived by crossing the Dot1Ltm1a(KOMP)Wtsi line - generated by the Wellcome Trust Sanger Institute (WTSI) and obtained from the KOMP Repository (www.komp.org) - with the MB1-Cre strain kindly provided by M. Reth(Hobeika, Thiemann et al., 2006). Mice from this newly created Mb1-Cre+/-;Dot1L strain were maintained under specific pathogen free (SPF) conditions at the animal laboratory facility of the Netherlands Cancer Institute (NKI; Amsterdam, Netherlands).Mice used for experiments were between 6-8 weeks old and of both genders unless stated otherwise.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were approved by the Animal Ethics Committee of the Netherlands Cancer Institute (NKI) and performed in accordance with institutional, national, and European guidelines for animal care and use.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All experiments were approved by the Animal Ethics Committee of the Netherlands Cancer Institute (NKI) and performed in accordance with institutional, national, and European guidelines for animal care and use.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N.A.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N.A.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N.A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N.A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N.A.
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The accession number is provided in the 'Data Availability' section of M & M.
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