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Transcriptional memory of cells-of-origin overrides β-catenin requirement of MLL cancer stem cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 August 2017

Thank you again for the submission of your manuscript (EMBOJ-2017-97994) to The EMBO Journal. We have carefully assessed your manuscript and the point-by-point response provided to the referee concerns that were raised during re-review at a different journal, and we have also discussed the work in our editorial team.

Based on our assessment, we decided that the following constructive requests from the previous referees are further reaching and not needed to be addressed for publication here:

>> Molecular mechanism of LSK-specific independence from beta-catenin.
>> Molecular mechanism of interaction and cooperation beta-catenin and Hoxa9/Prmt1.
>> Identification of Prmt1 targets and Prmt1's specific roles in LSK-derived CSCs.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in the EMBO Journal, pending the following minor revision:

• Please tone down claims on 'first experimental proof' as they can be easily misinterpreted.
• Please relativise statements regarding mechanistic insights, 'cross-talk' and 'networks' identified where applicable and point to the limitations of the findings regarding direct functional interactions.
• Please revise language editing of the manuscript.

Please also see below for some remaining formal formatting issues, which need to be adjusted at re-

submission.

We are therefore formally returning the manuscript to you for a final round of minor revision. Once we have received the revised version, we should then be able to swiftly proceed with formal acceptance and publication of the manuscript!

Authors' point-by-point response to the referees' concerns:

Reviewer #1:

While differential requirements for transformation of cells based on cell-of-origin is not a new concept, the specific findings here ostensibly contradict the results of a prior study (PUBMED ID 20339075), which revealed that both LSK and GMP cells require beta-catenin for leukemic transformation. However, the mechanistic basis for a differential requirement of GMPs versus LSK cells for beta-catenin or HOXA9 is not clear in this current manuscript nor is the discrepancy between the current study and prior study clarified.
Thank you for the comments. As pointed out by the reviewer, the current finding of b-catenin independent transformation in stark contrast to the prior study (Wang et al., 2010, Science, PUBMED ID 20339075) is extremely important for our understanding of cancer stem cell biology and treatment. Although this finding seems contradicting to the mentioned Science paper, our conclusions not only are compatible but also significantly advance our knowledge about b-catenin requirement and its molecular functions in MLL-CSC. When one examines the details of the exact works that had been performed in the indicated studies, it becomes evident that the conclusion of the Science paper was drawn by combining results from experiments with different settings. They showed that MLL-AF9 required b-catenin for GMP transformation and Meis1/Hoxa9 required b-catenin for HSC transformation, leading to their conclusion that b-catenin is required for both HSC- and GMP-derived leukemic stem cells. Although the authors actually have never tested the b-catenin requirement by the same fusion (i.e., MLL-fusion) in different cells of origins, it was written in the way that many people may interpret otherwise. This has been increasingly viewed as a dogma in the field. In contrast, the current study is the first to systematically study requirement of b-catenin in cell populations of different origins transformed by the **same MLL fusion**. Using this functional genomic approach in combined with global genomic and transcriptomic analysis, we clearly reveal the contrasting b-catenin requirement in MLL LSC from different cells-of-origin, in which self-renewal properties of leukemic stem cells derived from MLL-ENL transformed LSK cells are independent on b-catenin. Therefore the results here were retrieved by a purpose-built, systematic and robust experimental designs employed with sole purpose of discovering whether genetically identical populations of stem or progenitor cells can lead to phenotypically indistinguishable cancers with different cancer stem cell property – which we could prove, and explain on a detailed molecular level by global transcriptional analyses followed by functional validation of novel downstream targets (please see below).

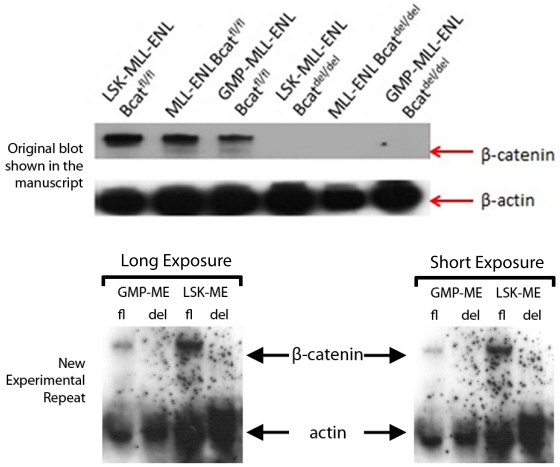
In addition, there are limitations to the molecular analyses performed here.
In order to further address this point, we now performed additional molecular analyses (summarized in new Figures 3 and 6, and their associated supplementary figures and tables) to delineate the molecular basis for a differential requirement of GMPs vs LSK cells for b-catenin or Hoxa9. While we and others have proposed important functions of b-catenin in AML stem cells (Yeung et al., Cancer Cell; Wang et al., 2010), very little is known about the molecular network and downstream targets of b-catenin responsible for its functions in AML. In contrast to epithelial tissues, here we reveal that majority of the known targets of b-catenin published in literature mostly identified in colon tissues or ES cells were not affected by b-catenin deletion in AML (Table S3C), suggesting that a very different and novel molecular network being regulated by b-catenin in the hematopoietic system. By performing detailed molecular analyses on primary MLL-fusion transformed hematopoietic cells expressing inducible endogenous level of b-catenin, here we showed for the first time that b-catenin regulates common downstream targets of Hoxa9 (new Figure 3A), which was expressed at a higher level in LSK-MLL-ENL cells and compensated for b-catenin function in mediating leukemic self-renewal. Global molecular analyses on LSK vs GMP derived MLL-ENL cells revealed significant up-regulation of stem cell transcriptional programs in LSK MLL-ENL (new Figure 3B), which expressed a higher level of Hoxa9 upon b-catenin deletion (new Figure 3C-

D). Consistently, GSEA revealed positive enrichment of Hoxa9 downstream targets in LSK MLL-ENL cells in contrast to its reduction in GMP MLL-ENL cells upon b-catenin (new Figure 3E), indicating a novel molecular link between two major self-renewal pathways mediated by b-catenin and Hoxa9 in regulating common downstream target for leukemic self-renewal. To further demonstrate their molecular complementation functions with relevant biological consequences, we showed that Hoxa9 indeed functional mimic b-catenin. Hoxa9 was also only required for GMP but not LSK-derived MLL-ENL cells (Figure 3F-G), which express high level of both Hoxa9 and b-catenin that can potentially compensate each other by activating common self-renewal pathways. To demonstrate this point, we generated the novel Hoxa9 and b-catenin conditional KO models where we performed detailed molecular and functional biology studies. Consistently, we showed that inactivation of both genes resulted in suppression of LSC maintenance transcriptional programs and abolishment of MLL-ENL mediated transformation in LSK cells (Figure 4, 5). In addition, we have then performed further in-depth transcriptional analysis to identify molecular targets that are synergistically regulated by b-catenin and Hoxa9 only when both proteins are inactivated in MLL-ENL LSK cells. Among them are Meis/Hoxa9 targets including Prmt1, which involves in epigenetic gene regulation. To gain further insights into the functional involvement of Prmt1 in b-catenin/Hoxa9 independent transformation by LSK-MLL-ENL, in this revision, we also performed new experimental and molecular studies to investigate the link between Prmt1 and Hoxa9/b-catenin by global transcriptomic analyses. We are able to show that suppression of Prmt1 leads to inhibition of common transcriptional programs including those involved in stem cell function and stemness mediated by b-catenin in Hoxa9$^{-/-}$ LSK MLL-ENL cells (new Figure 6). To further validate the functionality of this novel network in MLL leukemia, we demonstrate the ability of Prmt1 in replacing Hoxa9 or b-catenin function in MLL-ENL transformed LSK cells. In additional to the functional complementation data between Prmt1 and b-catenin, we now also provide new in vivo data where Prmt1 can replace the function of Hoxa9 in b-catenin KO LSK MLL-ENL cells (new Figure 7D-F), consistently with our hypothesis that Prmt1 is a key downstream mediator for Hoxa9/b-catenin functions. Together, this much-improved revised manuscript therefore not only reveals a previously unrecognized molecular network mediated by b-catenin and Hoxa9 in mediated leukemic self-renewal, but also has identified a novel common mediator, Prmt1 responsible for their essential function in MLL-ENL transformed LSK cells.

These and other issues are described below:
-The central argument that the authors have identified "contrasting" requirements of LSK and GMP cells for beta-catenin are hampered by the fact that Figures 1E and 1J shows distinct differences in survival in LSK cells transformed with MLL-ENL following beta-catenin deletion. It is possible that the lack of significant p-value in Figure 1E could be related to the number of mice included in each round of transplantation which is not provided in the figure or legend.
Thank you for the comments. As suggested by the reviewer, we have further increased the number of animals in Figure 1E (now Figure 1F), which was in contrast to Figure 1J (now Figure 1K) where the difference in survival was already statistically significant. As a result, we still obtain the same conclusion, and the difference in survival in Figure 1F remains statistically insignificant. Therefore these data consistently support our conclusion of a contrasting b-catenin requirement of LSK and GMP MLL-ENL cells. The numbers of animals used in the experiments were indeed provided in supplementary methodology in the original submission, but we have also added them to the figure legends for easy reference.

Related to this Figures 1B-C and 1F-G are taken from colony assays but have the authors checked beta-catenin deletion in leukemias at the time of animal death in Figures 1D-E?
The reviewer may have mistaken the results. Figure 1B-C (now Figure 1C-D) were taken from colony assays, but Figure 1F-G (now Figure 1G-H) were indeed from leukemia at the time of animal death as required by the reviewer. The results clearly indicate that b-catenin independent is not due to escape of the deletion, as confirmed by both PCR genotyping and Western blot.

Also, Figure 1C should be repeated with experimental and control mice on a single uninterrupted Western blot.
They were indeed done in the same blot but separated by other samples. In addition to providing image of the original blot below, we also repeated and showed the result of a new blot as references to the reviewer.



-The analyses performed to identify genetic alterations in leukemias derived from LSK or GMP cells are limited. First, it appears that the authors are attempting to identify somatic mutations in each form of leukemia but it is not stated that paired constitutional DNA was used for a control making the results of both RNA-seq and whole-genome sequencing analyses questionable.
In contrast to human, these are syngeneic C57/BL6 mice, which have been inbred and maintained in the same background for generations, so all the samples we are working with are close to genetically identical. Comparison with the C57/BL6 reference genome is usually regarded as the gold standard as there are rather little can be gained by sequencing the constitutional DNA. More importantly, both GMP and LSK were from the same mice, any constitutional changes due to the background will have also been taken out and considered during the analysis – this was a very important point for our experimental set-up, and for our aim of disentangling genetics and epigenetics. Therefore we do not think that sequencing of constitutional DNA will provide new additional insight or change to our conclusions.

Inter-stain genetic differences within strains of inbred mice occur and these differences are why constitutional DNA from the same individual mouse are required for rigorous mutational discovery (please review the actual results of PUBMED IDs 21917142 and 22724066 which identify large number of SNVs differing across exomes of mice of the same inbred strain). Moreover, the manuscript does not specify which C57BL/6 substrain was used in the experiments-- this itself is critical as distinct substrains of C57BL/6 mice have been identified to have different mutations with functional importance (see PUBMED IDs 23902802, 25341966, and 27210752 for just a few examples). Finally, the comparison of LSK to GMP cells from the mice would fail to reveal somatic mutations specific to hematopoietic cells and this point is not acknowledged.

If the authors do not feel that rigorous genetic analyses of the mouse tumors is central to this manuscript that may be understandable however their response to this question is not justified by the published literature and may not accurately reflect somatic mutations in LSK or GMP derived tumors.

Thank you for the positive comments. In regarding to the constitutional DNA control, we recognize that there are differences even within the same strains of inbred mice, we therefore have included genomic DNA of LSK/GMP within the hematopoietic lineage derived from the same mice for the analyses. We would like to point out that the analyses in the current study is not to identify somatic mutations specific in hematopoietic lineage (which can be shared between LSK and GMP), but any genomic/mutational differences between LSK and GMP in normal and transformed counterparts.

Descriptions of analyses for structural alterations, copy number changes, and fusions are also lacking. Although it is very likely that transcriptional differences alone underlie differing requirements for b-catenin in LSK versus GMP cells, revised genetic analyses should be performed if the authors want to definitively exclude the possible contribution of mutational differences in leukemias based on cell of origin.

In the current study, we have performed high-resolution SNP analyses on both RNA-seq and DNA-Seq results, which is a fairly standard and comprehensive approach to analyze the changes in transcribed and non-transcribed genomes. We see the more analysis we do, the more additional information we can get. However one will never be able to definitively exclude the contributions from potential genomic differences with the limitation of current sequencing technology and analysis pitfalls for every bioinformatics program. Nevertheless, we do appreciate the suggestion from the reviewer, and have now also performed additional CNV analyses, which consistently indicate that no significant difference can account for the differential b-catenin requirement (new Figure 2C-D, Supplemental table S1D.

-The AML prognostic data (Figure 2E and Table S2) does not appear to take into account genes recurrently mutated in AML which are well-established in predicting prognosis in AML. It is therefore unclear why the authors repeatedly refer to the gene expression prognosticator presented here as providing information beyond that prognostication utilizing mutational data.
Indeed, we have already taken into the consideration of the most recurrently mutated AML genes as indicated in the cytogenetic risk subgroups including chromosomal rearrangements and gene mutations in Table S2E (the list of the mutations included in each dataset are detailed in the cited original references). The transcriptional memory signature is still found by the Cox proportional hazards analysis to be an independent statistically significant survival marker, just as age or cytogenetic mutation is. Nevertheless, we appreciate that reports from different research/clinical groups will have slightly different criteria for combination of different cytogenetic/genetic risk grouping. For this, we now have used the term "cytogenetic/genetic risk" instead of genetic mutation in the relevant sections.

-The "transcriptional memory" signature seems to refer to those genes simply differentially expressed between LSK and GMP cells that are not targets of MLL-ENL.
These are the genes differentially expressed between LSK and GMP, which have still maintained the difference after transformation.

-A basis for the supposed "functional crosstalk" or "compensation" between beta-catenin, HoxA9, and PRMT1 are not provided. It is also not clearly justified to state that loss of Prmt1 "phenocopies" loss of beta-catenin loss simply on the basis of reduced colony numbers. The fact that combinations of genes may be required for leukemic transformation does not provide evidence that these factors are functionally interacting.
In order to further address this point, we now performed additional molecular analyses (summarized in new Figures 3 and 6, and their associated supplementary figures and tables) to delineate the molecular basis for a differential requirement of GMPs vs LSK cells for b-catenin or Hoxa9. While we and others have proposed important functions of b-catenin in AML stem cells (Yeung et al., Cancer Cell; Wang et al., 2010), very little is known about the molecular network and downstream targets of b-catenin responsible for its functions in AML. In contrast to epithelial tissues, here we reveal that majority of the known targets of b-catenin published in literature mostly identified in colon tissues or ES cells were not affected by b-catenin deletion in AML (Table S3C), suggesting that a very different and novel molecular network being regulated by b-catenin in the hematopoietic system. By performing detailed molecular analyses on primary MLL-fusion transformed hematopoietic cells expressing inducible endogenous level of b-catenin, here we showed for the first time that b-catenin regulates common downstream targets of Hoxa9 (new Figure 3A), which was expressed at a higher level in LSK-MLL-ENL cells and compensated for b-catenin function in mediating leukemic self-renewal. Global molecular analyses on LSK vs GMP derived MLL-ENL cells revealed significant up-regulation of stem cell transcriptional programs in LSK MLL-ENL (new Figure 3B), which expressed a higher level of Hoxa9 upon b-catenin deletion (new Figure 3C-D). Consistently, GSEA revealed positive enrichment of Hoxa9 downstream targets in LSK MLL-ENL cells in contrast to its reduction in GMP MLL-ENL cells upon b-catenin (new Figure 3E), indicating a novel molecular link between two major self-renewal pathways mediated by b-catenin and Hoxa9 in regulating common downstream target for leukemic self-renewal. To further demonstrate their molecular complementation functions with relevant biological consequences, we showed that Hoxa9 indeed functional mimic b-catenin. Hoxa9 was also only required for GMP but not LSK-derived MLL-ENL cells (Figure 3F-G), which express high level of both Hoxa9 and b-catenin that can potentially compensate each other by activating common self-renewal pathways. To demonstrate this point, we generated the novel Hoxa9 and b-catenin conditional KO models where we performed detailed molecular and functional biology studies. Consistently, we showed

that inactivation of both genes resulted in suppression of LSC maintenance transcriptional programs and abolishment of MLL-ENL mediated transformation in LSK cells (Figure 4, 5). In addition, we have then performed further in-depth transcriptional analysis to identify molecular targets that are synergistically regulated by b-catenin and Hoxa9 only when both proteins are inactivated in MLL-ENL LSK cells. Among them are Meis/Hoxa9 targets including Prmt1, which involves in epigenetic gene regulation. To gain further insights into the functional involvement of Prmt1 in b-catenin/Hoxa9 independent transformation by LSK-MLL-ENL, in this revision, we also performed new experimental and molecular studies to investigate the link between Prmt1 and Hoxa9/b-catenin by global transcriptomic analyses. We are able to show that suppression of Prmt1 leads to inhibition of common transcriptional programs including those involved in stem cell function and stemness mediated by b-catenin in Hoxa9$^{-/-}$ LSK MLL-ENL cells (new Figure 6). To further validate the functionality of this novel network in MLL leukemia, we demonstrate the ability of Prmt1 in replacing Hoxa9 or b-catenin function in MLL-ENL transformed LSK cells. In additional to the functional complementation data between Prmt1 and b-catenin, we now also provide new in vivo data where Prmt1 can replace the function of Hoxa9 in b-catenin KO LSK MLL-ENL cells (new Figure 7D-F), consistently with our hypothesis that Prmt1 is a key downstream mediator for Hoxa9/b-catenin functions. Together, this much-improved revised manuscript therefore not only reveals a previously unrecognized molecular network mediated by b-catenin and Hoxa9 in mediated leukemic self-renewal, but also has established Prmt1 as a novel common mediator responsible for their essential function in MLL-ENL transformed LSK cells.

Also, what is the evidence that the requirement for PRMT1 is specific to leukemia, regardless of origin, versus other hematopoietic cells?
Indeed, we did not specifically make the claim that Prmt1 requirement is specific to leukemia versus other hematopoietic cells in this paper. However, the therapeutic potentials of targeting PRMT1 for leukemia suppression has been specifically demonstrated in our Cancer Cell paper (Cheung et al., 2016), which was also cited in the discussion section of the manuscript.

-The authors refer to PRMT1 as an epigenetic modifying enzyme but there are a number of well-described non-histone substrates of PRMT1 (some which have established relevance in hematopoietic cells such as RUNX1 and RBM15) so this description should be modified.
Prmt1 has been viewed as an epigenetic modifying enzyme for the fact that it regulates gene expression in part by modifying histone methylation status. Like all other epigenetic modifying enzymes, Prmt1 can also certainly have other functions and substrates. We simply refer to the most commonly known characteristic of Prmt1. As suggested by the reviewer in the next point, we also provide global H4R3 methylation data to support one of its functions in mediating this critical epigenetic modification in new Supplementary Figure 6.

More importantly, the only evidence for PRMT1 downregulation is qRT-PCR data without evidence of effects of PRMT1 knockdown on global arginine methylation.
As suggested by the reviewer, we now also provide global H4R3 methylation data to support one of its functions in mediating this critical epigenetic modification in new Supplementary Figure 6.

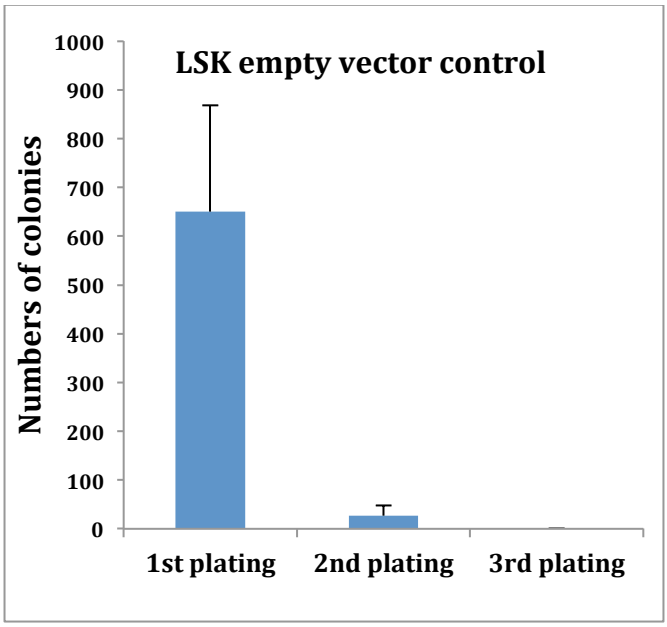
-The Discussion needs to be heavily revised as it contains a number of statements that are more consistent with opinions of the authors than careful assessment of prior work. For example, there are several sentences (see the last 2 sentences on page 11) which imply that prior work elucidating molecular basis for distinct cell of origin of AML are somehow less enlightening than the current study. It is simply not helpful to include these statements.
It has been suggested by readers of our manuscript that we put the work into context, acknowledge the strengths and limitations of previous publications, and clearly show how the current study provides the additional insights to the current knowledge. Nevertheless, we have taken the reviewer's view on board and have amended the discussion to avoid such a feeling.

In addition, the sentences in the Discussion and Abstract about current development of targeted therapies being "almost solely based on specific mutations" seems to ignore vast ongoing efforts to target cell-surface molecules, the immune system, and a number of enzymatic processes which are not clearly linked to specific mutations. There are numerous examples of ongoing clinical trials with such agents in AML (and these may actually outnumber mutation-specific targeted therapy trials) and the accuracy of this statement is questionable. There are also a number of claims are made

which need to be curtailed (such as the claims about providing "the first experimental proof" need to be made more carefully).
Thanks for the suggestions. We have now modified the text in the discussion and abstract accordingly.

The current study has major limitations in terms of molecular analysis and providing prognostic information as noted above.
Responded above already.

-Most of the colony replating assay data in the manuscript only show a limited number of replatings (3 or 4 replatings). It would be much more informative to show more rounds of replating than this given that even normal LSK cells may replate for 3-4 replatings.
In contrast to transformed cells, normal LSK cells can never give rise to 3rd round colony in our assay (So et al., Cancer Cell, 2003b). As an additional reference to the reviewer, below were the replating results of normal LSKs.



-The use of acronym "RTTA" should be revised. It is not clear why the authors need to use an acronym for the commonly used practice of retroviral overexpression of a cDNA (especially using an acronym which more commonly used to describe an entirely unrelated system).
This acronym has been commonly used for this experimental procedure for over a decade including in established methodology text book and many primary research articles (e.g. PMID:16473277, PMID: 17613435, PMID:17891136, PMID:19202074, PMID: 19277591, PMID:19965660, PMID:21156284, PMID:21624810, PMID:23951311, PMID:25510432, PMID:26766589).

Reviewer #2:

This is an intriguing study, which sheds new light on how cell-of-origin may influence the molecular biology of leukemia stem cells. The genetics and functional readouts convincingly demonstrate the differential role of beta-catenin in LSK vs. GMP derived disease, and also nicely demonstrate the key role of HoxA9 in LSK-derived leukemia. The subsequent identification of Prmt1 is a common downstream target is exciting and has potential for future targeting strategies. Finally, the correlation with AML prognostic outcome supports the concept that the underlying biology is relevant to human disease.

Many thanks to this reviewer for the positive comments.

Addressing a few relatively minor points would further strengthen the manuscript:

1) It's not immediately clear why LSK vs. GMP signatures from an MLL-based disease would be broadly predictive of outcome for all types of AML. It would help to have further discuss of this point.
We are also very encouraged by this result. We believe the broadly predictive power can be due to the explicit subtraction of specific MLL signatures by comparing MLL-ENL LSK vs MLL-ENL GMP, and these results suggest that the cells-of-origin is important in AML with many different genetic drivers. We have modified the manuscript to further discuss this point as suggested.

2) The analysis of Prmt1 is somewhat superficial and/or less clearly described. The text of the results sections seems to imply that Prmt1 should lie at a common juncture downstream of beta-catenin and HoxA9, however the only analyses shown are from HoxA9-/- derived leukemic cells. Is there some reason Prmt1 KD was not studied in cells WT for HoxA9? In other words, if Prmt1 is a key common downstream target, wouldn't one would predict that it's loss would phenocopy loss of HoxA9?
Thank you for the constructive advice. In this revision, we also performed new molecular and functional biology analyses to further demonstrate the link between Prmt1 and Hoxa9/b-catenin (new Figure 6 and 7). Specific to the reviewer's comment, 1) our new RNA-seq data comparing the effects of Prmt1 KD vs b-catenin KO in Hoxa9-/- LSK-MLL-ENL cells reveal Prmt1 and b-catenin co-regulate common transcriptional programs including those involved in stem cell functions in LSK-MLL-ENL cells (new Figure 6); 2) a similar functional complementation assay using b-catenin$^{-/-}$ derived leukemic cells WT for Hoxa9 was also performed as suggested by the reviewer. As a result, it is indeed the case that the loss of Prmt1 phenocopies loss of Hoxa9 in LSK-MLL-ENL cells (new Figure 7D-F).

3) As a general point, the presentation of the data figures is extremely dense and hard to follow. The manuscript would benefit substantially by clearer presentation.
In addition to manuscript text, we have now also substantially modified the presentation of data figures to further improve the ease of reading.

4) This is a semantic point, but near the top of page 12 the authors claim "…we provide the first experimental proof that cells-of-origin transcriptional memory can govern molecular pathways available for CSC self-renewal, and predict AML patient survival beyond DNA mutations." This could be interpreted as claiming the first data showing transcriptional signatures can predict AML outcome, which it is not. Suggest simply rephrasing to clarify the point that the present data is the the first cell-of-origin transcriptional data that predicts AML outcome.
Thanks for the suggestion and we have modified the sentence accordingly.

Reviewer #3:

While the findings are interesting and somehow novel, the main message of the manuscript is too diffuse. The manuscript focuses on too many concepts and the main findings appear diluted. The paper offers an exciting insight on the role of B-catenin in AML, and the different B-catenin requirements on AMLs wit distinct cell-of-origin. However, the idea that distinct hematopoietic populations lead to different degrees of disease aggressiveness has been previously been shown and, as it is, it doesn't add much more (George et al, Nat Comm 2016). A more in-depth study of the molecular crosstalk between b-catenin, hoxa9 and prmt1 is warranted.

We thank the reviewer for her/his positive comments on the "exciting insight on the role of B-catenin in AML and the different B-catenin requirements on AML with distinct cell-of-origin". While our comprehensive manuscript has addressed a number of important areas in stem cell biology, they all converge to support the concept and the underlying novel molecular network, in which cancer cells-of-origin govern utilization of self-renewal pathways in phenotypically and genetically indistinguishable leukemia, a defining feature of cancer stem cells. The study by George et al., Nat Comm 2016 reported distinct hematopoietic populations leading to different degrees of

disease aggressiveness, but did not examine the impact on self-renewal. Importantly, their report also did not have any functional validation study in identifying molecular pathways that are responsible for the phenotypes. Therefore, our work goes far beyond the finding by George et al. We would also like to take this opportunity to reiterate the main findings of our manuscript.

1. **This is the first demonstration of differential b-catenin requirement in mediating self-renewal for both initiation and maintenance of phenotypically and genetically indistinguishable MLL-leukemias from different cells-of-origin.** This dependence is governed by cells-of-origin transcriptional memory, which can predict patient survival and is characterized by high level of Hoxa9 expression in LSK-originated leukemia.
2. **The is also the first study showing that the transcription memory gene, Hoxa9, is differentially required for stem cells- vs progenitors-derived MLL leukemia.** Similar to b-catenin, Hoxa9 is only required for GMP- but not LSK-derived leukemia. These findings not only further strengthen the concept and functional significance of the newly identified transcriptional memory, but also suggest a functional link between b-catenin and Hoxa9 in mediating a common leukemic self-renewal pathway.
3. **The study also demonstrates for the first time the transcriptional network co-regulated by b-catenin and Hoxa9 in mediating self-renewal of LSK-derived MLL leukemia.** While b-catenin and Hoxa9 do not necessarily directly regulate each other, this report discovers their regulation of common transcriptional programs critical for leukemic self-renewal. Using a novel b-catenin/Hoxa9 cKO mouse in combination with transformation assay and global expression analyses, we are able to demonstrate important functional crosstalk between b-catenin and Hoxa9 at both transcriptional and cellular transformation levels in mediating self-renewal of LSK-derived MLL leukemia.
4. **This study also identifies Prmt1 as a novel and key downstream targets of b-catenin and Hoxa9 for mediating leukemic self-renewal in LSK-derived MLL leukemia.** To gain further novel insights into the molecular pathway mediated by b-catenin and Hoxa9 in LSK-derived leukemia, we identify that Prmt1 as a key component of the Hoxa9/Meis1 geneset is a critical downstream target co-regulated by b-catenin and Hoxa9 in LSK-derived MLL leukemic cells. Loss of Prmt1 molecularly and biologically phenocopies the loss of b-catenin in Hoxa9-/- LSK-ME (or vice versa). These findings reveal a novel molecular network mediated by Hoxa9/Prmt1 in mediating leukemic self-renewal that allows b-catenin independent transformation in LSK-ME cells.

Memory transcriptional signatures are not very clearly described. What are the key features of the novel signatures? which class of genes and biological functions are important in these signatures? We are happy to provide these additional information requested by the reviewer. Please refer to figure S2F-I. Briefly, while various classes of protein are well represented in both signatures, we observe a slight over-presentation of nucleic acid binding proteins observed in GMP's one. More importantly, both signatures strongly associate with AML in gene ontology analysis. In addition, LSK's signature also significantly associates with hematopoietic phenotypes in mouse, consistent with its role in hematopoietic malignancy. A heatmap with a few key names would be more informative. We indeed have already done this by highlighting the key ones in the MA-plot in Figure 2E, where we not only show the fold changes but also the expression level. The full list of transcriptional memory genes can also indeed be found in the supplementary table 2B. We feel that copying the complete long list of gene names from supplementary table to main figure does not add much to the manuscript but make the figure difficult to read. At the end, why are LSKs different than GMPs when it comes to b-catenin dependence? As discussed in the manuscript, it is at least in part because of the expression level of Hoxa9. Not only is the Hoxa9 expression level higher in b-catenin wildtype LSK-ME compared to GMP-ME (Figure 2E,F) but also in b-catenin deleted LSK-ME compared to GMP-ME (Figure 3,C, D, E). To further demonstrate the function of Hoxa9 in allowing b-catenin independent transformation in LSK-derived MLL leukemia, we show that inactivation of Hoxa9 results in loss of leukemic self-renewal transcriptional program (Figure 5A-B) and transformation ability (Figure 4A-B, E-G) in otherwise transformation competent b-catenin deficient LSK-ME.

Regarding b-catenin requirement and molecular memory: why MLL GMP cells are different than normal GMP cells when it comes to b-catenin requirement? It is because normal GMP, in contrast to transformed GMP, does not have to have unlimited self-renewal property, which is a hallmark of cancer cells. And, why is there a transcriptional memory in this context when normal

HSC/LSK/GMP cells do not even require b-catenin to function. In general, b-catenin is required for self-renewal of stem cells (e.g., embryonic stem cells; colon stem cells; various cancer stem cells) (Holland et al., 2013, Current Opinion in Cell Biology) but not normal progenitors, which do not have self-renewal property. In this report, we also show that HSC has a distinctive transcriptional program using other molecules such as Hoxa9 to mediate self-renewal in the absence of b-catenin. As shown in the manuscript, this transcriptional network remains active after transformation and therefore transformed HSC can bypass b-catenin requirement.

Despite the tons of RNA-Seq data, it is unclear how (or if) b-catenin and Hoxa9 functionally cooperate at the molecular level. The paper fails to really identify direct (or indirect) connections between these nodes (and later with prmt1) in the system. What is a b-catenin target, or a Hoxa9 target? How do they connect at the molecular level? With due respects, we disagree with reviewer on this point. We indeed have performed extensive characterization work in identifying downstream targets of b-catenin, Hoxa9 and subsequently Prmt1 in the manuscript. B-catenin targets are shown in Figure 3A, 5A, B,, 6A-Dand table 4A, 5A, Hoxa9 targets in Figure 5A, B and table 4A, Prmt1 targets in Figure 6A-D and table 5A. From there, we further demonstrate that they share significantly common targets (Figure 6A), which are involved in various stem cell functions and differentiation processes (Figure 6D and table 5B). However we don't attempt to distinguish if they are either direct or indirect downstream targets, which are outside the scope of the current studies and the results also would not change our conclusions. Is the Hox locus in these cells controlled by b-catenin/TCF1 factors or viceversa? We don't think the Hox locus in these cells is controlled by b-catenin/TCF1 factors or vice versa. Loss of Hoxa9 in LSK-ME does not change b-catenin expression, and the expression of Hoxa9 varies modestly upon b-catenin deletion. Importantly, the level of Hoxa9 expression is still significantly higher in b-catenin deleted LSK-ME than in b-catenin deleted GMP-ME (Figure 3 C, D, E).This is also consistent with the absence of known Hox or TCF1 binding motifs in these loci.

How is Hoxa9 regulated in controls vs catenin deleted? If Hoxa9 is already elevated in transformed LSK, how does it contribution to the leukemic phenotype changes upon catenin depletion? As mentioned above, Hoxa9 expression only varies modestly upon b-catenin deletion in LSK-ME, and it remains significantly higher in b-catenin deleted LSK-ME than b-catenin deleted GMP-ME (Figure 3C, D, E), consistent with the idea that it helps to overcome b-catenin deletion as a part of the transcriptional memory genes. We would also like to point out that Hoxa9 and b-catenin unlikely directly regulate each other, but expression of some common downstream target genes such as Prmt1. Can Hoxa9 overexpression rescue and increases GMPs self-renewal and leukemogenic potential? We believe that over-expression approach is less physiological and importantly will not be able to address the role of Hoxa9 in transformed LSK cells. Therefore we opted for a more physiologically relevant approach using genetic knockout to demonstrate the critical functions of Hoxa9 in maintaining leukemic self-renewal in the absence of b-catenin. Also instead of addressing if Hoxa9 is required for b-catenin independent transformation in LSK leukemia, it brings up a different question if over-expression supra-physiological level of Hoxa9 is sufficient to support transformation in GMP leukemia in the absence of b-catenin, which can be further complicated by the possibility that some other transcriptional memory genes may also be required. In line with our own findings and the literatures (including George et al., 2016 mentioned by the reviewer), we choose to focus on LSK-leukemia as it is more resistant to treatment compared to GMP-MLL-ENL leukemia, and is clinically more important.

Data for the LSK-ME Hoxa9 -/- presented in Figure 3F and 4A are not consistent. Plating 3 is going up in 4A, but not in 3F. What is the most common scenario? We would like to point out that these results were obtained using independent experimental approaches with slightly different genetic backgrounds, one with Hoxa9 KO only whereas the other is Hoxa9 KO and b-catenin floxed. Under these slightly different experimental conditions, although the colony numbers varied between experiments, we reached the same conclusion, in which LSK-ME can continuously give raise to third round colony and even induce leukemia in vivo in both conditions (Figure 3G, 4F, G).

Along the paper, many GSEA plots are shown but very little gene names or biological functions. Labelled heatmaps would benefit the understanding of the pathways that are altered and how they are potentially connected.

Again, we could provide labelled heatmaps with all gene names. But we have indeed already provided the full list of gene names in the supplementary tables (Supplementary table 2B, 3A, 4A, 5A), and putting them again in the main figures will not add any new information but make the figures too busy to read. More importantly, individual gene name usually has very limited

biological meaning. People in the field in general use GSEA analysis to shed light into biological functions (see recent publications from Vu et al, *Nature Genetics* (2017), Lin et al, *Cancer Cell* (2016)), which is also what we have carried out for this and our previous studies (Esposito et al., Nature Medicine (2015), Cheung et al., Cancer Cell (2016)).

The logic to get to Prmt1 is difficult to follow. What is the expected direction of regulation? What is the involvement in self-renewal? Are they both (b-catenin and prmt1) repressing or activating the gene?
This may be related to the previous points brought by this reviewer as all these are actually already shown in the manuscript. Briefly, in order to gain further insights into the molecular pathways mediated by b-catenin and Hoxa9 in supporting LSK leukemic self-renewal, we sought out to determine the genes that are significantly deregulated only upon both b-catenin and Hoxa9 deletion when leukemic self-renewal is compromised. Prmt1 is one of the synergistically downregulated genes upon deletion of both Hoxa9 and b-catenin in transformed LSK, and is also one of the seven genes in the Hoxa9/Meis1 targets_UP geneset that is commonly suppressed when leukemic self-renewal is lost in LSK-ME when moving from single to compound b-catenin and Hoxa9 KO (Figure 5C), suggesting a critical function of Prmt1 in mediating leukemic self-renewal in LSK-ME.

Both b-catenin and Prmt1 share significant number of similar transcriptional targets, up to 20% of b-catenin targets are also regulated by Prmt1 in the same directions including both repressing and activating gene expression (Figure 6A). GSEA analysis further showed that there are very significant number of gene sets which are shared between Prmt1 and b-catenin in the Hoxa9 ko background (Figure 6C, D). On the other hand, suppression of Prmt1 as a result of a) deletion of both b-catenin and Hoxa9 (Figure 5A-C, 4F-G), or b) its knockdown in the single Hoxa9 or b-catenin knockouts (Figure 6A-D, 7A-F) results in loss of leukemic self-renewal transcriptional program and suppression of MLL leukemic cells. Nevertheless, we can describe this logic better in the text.

Although potentially interesting, Figure 5D shows that more genes are upregulated upon KD of Prmt1 or KO of Catenin, however the authors claim "we observed a higher percentage of overlapping targets for differentially down-regulated genes upon their individual activation". Can the authors comment on why there are more genes showing a higher expression upon loss of prmt1 or b-catenin in Hoxa9-/- leukemias. Indeed there are more genes being upregulated than downregulated upon Prmt1 or b-catenin loss in the Hoxa9 ko background. Some of the gene upregulation can be secondary events, for an example, a result of suppression of a master transcriptional suppressor upon Prmt1 or b-catenin loss. We have amended and commented this in the manuscript as suggested by the reviewer. Also, with this data there is no evidence of a functional overlap (unless they do a rescue experiment). In regard to the comments that there is no evidence of a functional overlap, we respectfully disagree with the reviewer. We provide evidence for a functional overlap on two levels, molecularly and biologically. On the molecular level, we provide GSEA analysis showing largely overlapping transcriptional functions in regulating common gene sets (Figure 6C, D) in the Hoxa9 ko background when Prmt1 in knocked-down or b-catenin is knocked-out. On the biological level, Prmt1 knockdown (Figure 7C) and b-catenin knockout (Figure 4F, G) in Hoxa9 knockout cells both impair MLL-leukemia. Thus, instead of expressing a supra-physiological level of candidate protein in clinically less important GMP population, which may also require other transcriptional memory genes to overcome b-catenin independence, we opted for a more physiologically relevant knock down approach in LSK-derived leukemia, and have successfully demonstrated their functional complementation.
Figure 6D. Do the authors have data for a longer survival time? We believe the reviewer refers to old Figure 6C and we have now provided a longer survival time (Figure 7C). We now provide observation time of 300 days and show that no additional mouse transplanted with shPrmt1 Hoxa9$^{-/-}$ LSK-MLL-ENL developed disease. Thus the conclusion that Prmt1 mediates b-catenin function in LSK derived MLL CSCs, which had also been drawn from the previous data with much shorter observation times, remains unchanged.

Although the prmt1 finding is exciting, it is not investigated deep enough. What are the targets at the chromatin level? Are those the same as Catenin or Hoxa 9? How do the biological functions overlap?
It is an interesting question, however the technological limitation of available ChIP-grade Hoxa9/b-catenin/Prmt1 antibodies prohibit any meaningful chromatin studies. More importantly, accessing

chromatin binding and modification alone do not always directly relates to change in RNA or gene expression, which are regulated by complicated crosstalk among DNA methylation, chromatin modification (a combination of various histone modifications in addition to transcriptional factor binding), chromatin accessibility, enhancer interaction and microRNA/ncRNA regulation. Dissecting this epigenetic network will be out of the scope of the current studies. In order to better address the question on biological function overlap, we have indeed provided the more functionally relevant RNA data showing that Prmt1, b-catenin and Hoxa9 regulate significantly overlapping targets and similar biological processes including leukemic transformation and self-renewal (Figure, 5A, B, F, 6A, C, D).

Have the authors investigated the levels of prmt1 in leukemias of GMP origin?

Since the focus is why and how LSK-derived leukemia is independent on b-catenin or Hoxa9, we did not examine the levels of Prmt1 in GMP origin leukemia. Moreover, the function of Prmt1 in GMP derived leukemia can also be different, and depends on if other components within the pathways remain intact in GMP originated leukemic cells.

What is the role of Prmt1 in the hematopoietic system? Is it specific to leukemia? and if so, which are those targets?

Nobody has characterized the role of Prmt1 in normal hematopoiesis, it will be an extremely interesting subject for a future study. Its specificity to leukemia has already been reported in the previous works by others and us, and there are also evidence suggesting its involvement in solid tumors (Cheung et al., NCB, 2007, Cheung and So, FEBS letters, 2011; Shai et al., Blood 2012; Cheung et al., Cancer Cell, 2016; Blanc and Richard, Mol. Cell, 2017). Also as demonstrated in our 2016 Cancer Cell paper, there seems to have an enough therapeutic window to target cancer cells while sparing normal cells using an early stage Prmt small molecule inhibitor.

The overall idea and the findings described here add little therapeutic or human relevance.

We respectively disagree. If HSC-derived MLL leukemia is not dependent on b-catenin, then the current clinical trial using targeted therapy based on b-catenin inhibitor will not be effective in this group of patients and it will have significant therapeutic implications. In addition, transcriptional memory signature can robustly predict human AML patient survival, which further demonstrates the therapeutic relevance of our finding for risk stratification of patient receiving current standard chemotherapies.

Minor comments

Some figures are missing or mislabeled (e.g 1K, 2C-D) – We are not sure exactly what he/she refers to. We have checked again but all information are presented there correctly. We cannot find missing or mislabel ones. Nevertheless, we will add additional labels to make them even clearer.

The manuscript would benefit from editing. The rationale and flow are is difficult to follow and the main message gets too diluted. We have slightly modified the manuscript to further emphasize the main points of this study and to improve the flow.

2nd Editorial Decision 22 August 2017

Thank you for sending us the revised version of your manuscript (EMBOJ-2017-97994R). We have carefully assessed your manuscript and found most concerns regarding claims and wording adequately addressed.

As mentioned, we are thus pleased to inform you that your manuscript has been accepted in principle for publication in the EMBO Journal, pending minor revision of remaining formatting issues, which need to be adjusted in a re-submitted version.

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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.

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The data shown in figures should satisfy the following conditions:

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

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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  - definition of 'center values' as median or average;
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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.

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