A Cdc42-Borg4-Septin 7 axis regulates HSC polarity and function

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

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and I think all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance 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 changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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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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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.

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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. If no primary datasets have been deposited, please also state this in the respective section (e.g. 'No primary datasets have been generated and deposited'), see below.

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. This is now mandatory (like the COI statement). 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.

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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

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.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Referee #1:

Kandi et al present data elucidating the pathways affected by Cdc42 in hematopoietic cells. This is important as this group has previously demonstrated aging related phenotypes associated with increased Cdc42 expression and have elegantly demonstrated that mitigation of Cdc42 expression
can mediate age-associated phenotypes of aged HSCs and chemical inhibition with CASIN can also
drive mobilization of the LT-HSCs. The authors examine the expression of binders of Rho GTPases
proteins (borgs) and septins in stem cells and determine aging associated alterations in expression
of several borgs and septins that can be reset by inhibiting Cdc42 expression. The authors then
evaluate the effects of pan-hematopoietic loss of borg4 and septin 7 and report few phenotypes at
steady state, but striking phenotypes when marrow is transplanted, suggesting either implications
of this pathway for response to cycling, cell division, or perhaps homing. While the results suggest
that Cdc42 pathway involves septs and borgs, as reported in other tissues, several unresolved
questions remain from the presented data.

Major Concerns:
1) Did the loss of borg4 or septin 7 lead to changes in overall frequency of number of HSCs or just in
the frequency of polar HSCs- it appears in the FACS plots of Sept7-/- (Fig S4) HSC frequency may
be increased compared to WT.
2) The more pronounced phenotypes of the Sept7-/- and borg4-/- in hematopoietic cells are seen
after transplant / stress. However, these deletions of critical HSCs components would have also
been deleted in fetal liver HSCs which require robust cycling / stress during development. Perhaps
polarization of stem cells is required only in adult marrow, but given the robust cycling and
proliferation of fetal HSCs, arrays data supporting expression of cdc42, sept7, and borg4 in FL
HSCs, and the inference that this polarization pathway in critical during cell division, it would be
important to evaluate the potential of the fetal liver HSCs after the deletion of Borg4 and Sept7.
3) Could the authors posit why the Sept7 deletion led to such significantly different phenotypes
from the Borg4?
   a. In the Sept7 deleted transplants with very low donor chimerism, it is difficult to use the donor
derived frequencies of the B, T, and Myeloid cells to establish a lineage bias toward lymphoid cells
as it appears the donor HSCs were not robustly contributing to myeloid lineages. The increased
frequency of the lymphoid cells may be attributed to the turn-over rate of the differentiated cells vs
changes in the lineage potential of the transplanted cells (or perhaps due to increased CLPs in the
WBM transplant).
4) Analysis of public data sets for expression in borg4 and sept7 suggest robust expression in
downstream progenitors, with sept7 having more robust expression in lymphoid progenitors and
borg4 in myeloid progenitors. How have the authors ruled out that phenotypes during transplant
were not driven by changes in progenitor cells?
5) The authors state that the lack of septin 7 lead to impaired HSC expansion, however as
presented in figure 4N, they show a significantly increased HSC compartment in the sept7
knockout transplants. How do the authors resolve this potentially conflicting data?
6) For IF staining, how many cells were seeded and how? The total number of cells presented is
quite low (n>17). Understandably, there are not many young LT-HSCs, but if only 17 cells are
scored (figure 2) it would be important to know if only ~17 cells remained on the slide or if only 17
cells were stained.

Minor Concerns:
1) Could the authors clarify if the Septin7 knockout mice received from Hannover? From Fig S4
appear to be Floxed but please confirm in materials section?
2) Were there changes in the PB composition of knockout mice?
3) Was the H4K16ac polarity also altered in Sept7-/- and borg4-/-?
4) It would be informative to show "frequency of live bone marrow" and not just frequency of parent
gate.
Referee #2:

In Kandi et al., the authors examine the role of Cdc42, Borgs (Binder of RhGTPases), and Septins in regulating the polarity of HSCs, and thereby their functional potential, during aging. They show that during aging, mouse HSCs change expression and lose polarization of several Borg and Septin proteins. The polarization defect is partially restored by adding an inhibitor of Cdc42, CASIN. Conditional deletion of Borg4 or Septin7 in hematopoietic cells (via Vav-Cre) also leads to decreases in the polarity of several proteins involved in cytoskeletal remodeling and asymmetric/symmetric division. In Borg4 or Septin7 cKO mice, there are minor alterations in progenitor populations suggesting minor defects in lineage development, although no differences in mature lineages were noted. However, upon transplantation in a competitive setting, Borg4 and especially Sept7 cKO HSCs appear defective in engraftment and lineage reconstitution. Sept7 cKO HSCs appeared dysfunctional in differentiation at several stages, and a massive increase in lymphoid progenitors was noted along with a relative decrease in myeloid output. Sept7 cKO HSCs also had deficiencies in cell division and viability in vitro. Overall, this study suggests a role for cell polarity in regulating HSC differentiation function, which is disrupted in aging, and a mechanism by which Cdc42-GTP increases with age and inhibits polarization via Borg4 and Septin7.

In general, I enjoyed this study and found their basic premise supported by their data. They used a variety of different experiments from gene expression, imaging, proximity ligation assays, and transplantation to build a case that the Cdc42-Borg4-Septin7 axis plays an important role in the age related differentiation phenotype known for HSCs. Not all their data was clearly supportive, there were some inconsistencies in the data and how it was presented, and I didn’t agree with some of their interpretations, but overall I think this manuscript adds something novel to the literature and advances the field.

Major comments:

The decrease in donor chimerism in the Borg4 cKO in the blood (Figure 4H) appears different than the BM analysis (Fig 4 JK, L). It’s unclear how donor chimerism is being calculated in each case. For the blood, is it CD45.2 as a percentage of total CD45? For the BM, is it just within the CD45.2 cells? Both are labeled as "% of donor derived cells". The figure legends and methods sections don’t explain this. This is important as the data appears different, with the blood data suggesting a defect in Borg4 cKO HSC engraftment, and the BM suggesting no defect.

There’s also a disconnect between the peripheral blood data in the Sept7 cKO in 4I and the BM analysis in 4M to 4O. Why is there so little donor chimerism in the peripheral blood of the Sept7 KO, but a massive increase in CLP, B cells and T cells? Is data in 4M to 4O showing the distribution amongst donor-derived cells, whereas the peripheral blood it relative to total CD45 (including the competitor)? There isn’t much discussion for why the CLP population would be massively expanded yet the lymphoid population only mildly so. I think it may have to do with how the data is analyzed (CLP is plotted as a percentage of Lin- Sca1low ckitlow cells whereas B cells and T cells as a percentage of donor-derived cells).

The model implies that the loss of polarity contributes to the HSC aging phenotype, which is reduction in engraftment ability and a myeloid bias. So if one were to examine cKO mice that lose an important polarity protein, like Borg4 or Sept7, then this would lead to a premature aging phenotype. And yet the transplant data implies a lymphoid bias in the Borg4 and Sept7 cKO mice,
which have a defect in HSC polarity. This discrepancy isn't really explained in the discussion.

Minor comments:

Fig. 1A/1B - Gene expression qPCR data presented here clearly does not use biological replicates (or that's the most consistent gene expression patterns I've ever seen). I don't know the journal's policy on presenting error bars for technical replicates, but I would request that the authors clearly state in the figure legend that the replicates are technical replicates or remove the error bars.

On page 8 (of the pdf file), the authors state: "This strongly implies a strong feedback loop in which changes in the septin 7 localization and thus likely a disturbed septin network will in return influence the localization of Cdc42 as well as borg4." The changes in polarity in Figs 3C and F to me don't justify use of the word "strong", let alone twice in one sentence. I recommend toning this down.

Fig. 4A/4D - The bone marrow contains few T cells. The blood would be a better place to assess differences in lineage output, in my opinion.

Fig. 4B/4E. Either graph LSK as a percentage of total BM cells or LT-HSCs as a percentage of total BM cells. Fig 4B/E only shows LT-HSCs as a percentage of LSK, but total LSK could be decreasing in the KO and this wouldn't show that. Same with CLP. They are plotted as a percentage of the gate above them. It would be helpful to plot them as a percentage of total BM cells.

Fig. 4C/4F - CLP are also Flk2+. If Flk2 is in the stain, it would be good to gate CLP as Flk2+ IL7Ra+ (ckit low Sca1 low Lin-). In fact, if the lineage stain, Flk2 and IL7Ra stains are all good, you don't even need to gate ckit low and Sca1 low.

Fig. 4H - Hard to see which are squares and which are circles with the error bars there. Perhaps enlarge the symbols or make one open symbols to make it easier to distinguish.

Fig. 4J/4M - CD3 and B220 are switched between 4J and 4M.

Fig. 4B/4E and 4K/4N - one graph uses LMPPs, the other graph uses MPPs. In the results for 4E, it refers to LMPP but the bar graph is labeled MPP.

Fig. 4N - Why does the graph make it look like LT-HSC chimerism is increased in the Sept7 cKO when the blood data indicates a massive reduction in donor chimerism in the blood? It appears that this is also explained by the way the LT-HSC data is reported, as a percentage of total LSK cells, and not as a percentage of total BM cells.

Fig. 5A - Does this include only live cells or does it include the dying cells as well? It probably should only include live cells as that would focus on whether the division kinetics are slower. If it includes dead cells then the difference could easily be explained by the difference in dead cells as shown in 5B.

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Referee #3:

The manuscript "A Cdc42-Borg4-Septin 7 axis regulates HSCs polarity and function" by Kandi et al investigates the role Borg4 and Sept7 in hematopoietic function. Septins are emerging as novel
regulators of cytoskeletal remodeling with potential input in many cellular processes. However, they remain fairly uncharacterized and their role in physiological processes as well as their regulation and mechanisms of action are fairly unknown. Here the authors address this issue by investigating the role of septins in hematopoietic function. In addition, they explore the potential of Borg proteins (the only known upstream regulators of septins) and the regulation and function of septins in this system.

Using a vav-1 driven cre recombinase model, the authors specifically delete Borg4 and Sept7 in hematopoietic stem cells (HSCs), showing a reduced engraftment potential upon transplantation and skewed differentiation programs. Characterisation of HSC function and differentiation is very exhaustive, although very hard to follow. The authors show that deletion of Borg4 or Sept7 affects the proportions of different hematopoietic lineages at steady state, with more profound phenotypes after deletion of Sept7. Interestingly, phenotypes were more evident after stress (ie. BM transplantation), suggesting a particular role of this axis in this context. Although these findings are interesting, their relevance in health/disease is obscure. At this point, all the results are purely observational, without clear mechanistic explanation. It is not clear why sept7/borg4 function is only evident during transplantation, only affects certain levels of differentiation potential and not others, presents some characteristics of aged HSCs but not others, etc. Thus, most of the conclusions are purely speculative.

Furthermore, the mechanistic insights are very limited and it is not clear if (or how) borg4 and septins modulate HSC function. The rationale for investigating specifically Borg4 and Sept7 in HSC illustrated in the first part of the manuscript is also very arbitrary. The authors focus in gene expression analysis to define their primary candidates, but expression is not directly associated with function, and they did not confirm their qPCR results by alternative ways (thus, confirming that low levels of expression are not just a result of faulty primer design). Although the intricacies of investigating Borg proteins are acknowledged (lack of tools or specific antibodies), more robust results are required to convincingly determine the relevance of these factors (i.e. Borg4 and not others) in the process. This may include a loss-of-function genetic screen. For example, it is not clear why the specific requirement for Sept7 and not other septins, if they may be equally required for septin network assembly. If Sept2 and Sept6 do not relocalize after Cdc42 modulation, does it imply that this is a septin-network-independent function of Sept7? Or are other septins involved? Finally, their working model (Figure 6) is based in perturbations that are particularly noisy and that might have multiple undesired off-target effects (i.e. Cdc42 inhibition, young vs matured HSC). The validity of this model needs to be supported by additional, more robust perturbations including the modulation of the binding capacity of Borg4 towards Cdc42 and septins, using already described mutants. Given that Borg4 requires Cdc42 function for their correct positioning, it is not clear how interaction with Septin 7 is increased in low Cdc42-GTP conditions. For this assays, it is particularly important to analyse Cdc42 activity spatially. It is possible that polarity arises from concentrated activation of Cdc42 in certain areas (leading to the recruitment of Borg and septin in those regions), whereas high global Cdc42 activity leads to the disruption of Cdc42-GTP gradients and the inability to generate concentrated modules of Cdc42-GTP/Borg4/Sept7. There may be a competition between different Borgs for binding to Sept7, that is affected by Cdc42 modulation... Unfortunately, the authors did not exhaustively investigated alternative explanations.

Borg and septins are relatively novel and thus fairly uncharacterized factors related to Cdc42 function and cytoskeletal regulation. However, their pathobiological role and regulation is still not well understood. Overall, this manuscript provides evidence of a possible new biological role for Borgs and septins in hematopoietic cells. However, it provides very limited advances in the function and regulation of Borgs and septins, or the underlying molecular mechanisms affecting HSCs.
Other comments:
- Use alternative sources/material to confirm expression data (e.g. Human Cell Atlas).
- Expression does not determine relevance. Perform genetic screen - is there any other Borg modulation affecting Sept7 phenotype?
- Explain why polar for Sept7 and not the rest, if septin filaments are oligoheteromers. Is this a septin-network-independent function of Sept7? Or are other septins involved? Which?
- If Borg are Cdc42 effectors, why reduction of Cdc42 activity is associated with redistribution? What is determining the localization of borg/sept in Cdc42-GTP low cells leading to polarity?
- Expression of candidate factors after CASIN treatment is not relevant unless you show mechanism controlling their expression.
- Experiments in Fig 2 are over interpreted - if there is no Cdc42 activity, how can borg reach septins? It implies that septin regulation is independent of Cdc42. Analysis of effect of Cdc42-binding and septin-binding mutants of Borg4 is required. Interaction between Cdc42 and Borg4, and Borg4 and Sept7 needs to be shown.
- Analysis of polarization are mainly phenotypic and therefore prone to observational bias. In order to add rigour, additional readouts are required, as well as a quantitative ways to illustrate significant differences between the experimental points. Independent readouts of polarity (not dependent on the main effector under study i.e. Cdc42) are required. This is particularly important since candidate perturbations (Borg4/Sept7) seem to affect Cdc42 localization, landing on a chicken and egg scenario.
- The link between polarity and function is not clear.
- PLA analysis is particularly hard to interpret, as it involves what appears clusters of cells. Why there is PLA mainly occurring in the perinuclear region? Why is it not polarised?
- Show data not shown.
Point-to-Point response to the comments of the reviewers
EMBOR-2021-52931-T: A Cdc42-Borg4-Septin7 axis regulates HSC polarity and function by Kandi et al.

Referee #1:
Kandi et al present data elucidating the pathways affected by Cdc42 in hematopoietic cells. This is important as this group has previously demonstrated aging related phenotypes associated with increased Cdc42 expression and have elegantly demonstrated that mitigation of Cdc42 expression can mediate age-associated phenotypes of aged HSCs and chemical inhibition with CASIN can also drive mobilization of the LT-HSCs. The authors examine the expression of binders of Rho GTPases proteins (borgs) and septins in stem cells and determine aging associated alterations in expression of several borgs and septins that can be reset by inhibiting Cdc42 expression. The authors then evaluate the effects of pan-hematopoietic loss of borg4 and septin 7 and report few phenotypes at steady state, but striking phenotypes when marrow is transplanted, suggesting either implications of this pathway for response to cycling, cell division, or perhaps homing. While the results suggest that Cdc42 pathway involves septs and borgs, as reported in other tissues, several unresolved questions remain from the presented data.

1) Did the loss of borg4 or septin 7 lead to changes in overall frequency of number of HSCs or just in the frequency of polar HSCs- it appears in the FACS plots of Sept7/-/- (Fig S4) HSC frequency may be increased compared to WT.

Response: In case of the deletion of septin7, there is no increase in the number or frequency of HSC at steady state but upon transplantation (Figure 4 E&N). Upon deletion of Borg4, the number or the frequency of HSC was similar to controls in both steady state and upon transplantation (Figure4 B&K).

2) The more pronounced phenotypes of the Sept7/-/- and borg4/-/- in hematopoietic cells are seen after transplant / stress. However, these deletions of critical HSCs components would have also been deleted in fetal liver HSCs which require robust cycling / stress during development. Perhaps polarization of stem cells is required only in adult marrow, but given the robust cycling and proliferation of fetal HSCs, arrays data supporting expression of cdc42, sept7, and borg4 in FL HSCs, and the inference that this polarization pathway in critical during cell division, it would be important to evaluate the potential of the fetal liver HSCs after the deletion of Borg4 and Sept7.

Response: Indeed, the role of polarity in fetal liver hematopoiesis and thus also the role of septin 7 and borg 4 for fetal liver hematopoiesis has not been addressed yet. Which leaves the question open whether polarity is only important/relevant for adult HSCs and hematopoiesis. These are indeed very interesting questions. While we regard information on the role of polarity in fetal hematopoiesis and thus the role of Sept7 and Borg4 in fetal hematopoiesis beyond the current scope of the manuscript, we still performed additional experiments to have a preliminary look at the connection of polarity and function of fetal liver HSCs. To this end, we determined polarity and potential upon transplantation of HSCs (Lin, c-Kit+, Sca-1+, CD150+ cells) from borg4-/- and control fetal livers. While there is a strong trend for a difference in the polar distribution of Cdc42-GTP, this is not the case for septin 7 (Figure 1 for reviewers, below)[Figures for referees not shown.], and the frequency of HSCs polar for Cdc42GTP overall was only slightly lower than for adult HSCs (Figures 1,3; Figure EV1 N-O). Chimerism support by borg4-/- cells at 4 weeks after transplant was also similar in comparison to chimerism supported by controls, which is distinct from results obtained from transplants of adult borg4-/- HSCs in which they show reduced contribution even at 4 weeks (Figure 4H). This preliminary data indeed support distinct roles for polarity for fetal liver compared to adult HSCs, and thus distinct roles for the regulators of polarity in
developmental of hematopoiesis, which we plan to address in a subsequent publication.

3) Could the authors posit why the Sept7 deletion led to such significantly different phenotypes from the deletion of Borg4?

Response: This in interesting question we also asked ourselves multiple times. In general, loss of a regulator of a network (like Borg4) might have less effect on the overall network compared to the loss of a central component of core of the filament (septin 7). Next, septin 7 is the only member of it family, and thus likely compensation does not play a role. In case of the deletion of Borg4 other Borgs like Borg 2 (Figure EV3) might be able to compensate the loss of Borg4. The frequency of cells polar for septin2 or septin6 is similar in borg4fl/fl or borg4∆/∆ LT-HSCs (Figure EV4 G), which supports such a hypothesis.

In the Sept7 deleted transplants with very low donor chimerism, it is difficult to use the donor derived frequencies of the B, T, and Myeloid cells to establish a lineage bias toward lymphoid cells as it appears the donor HSCs were not robustly contributing to myeloid lineages. The increased frequency of the lymphoid cells may be attributed to the turn-over rate of the differentiated cells vs changes in the lineage potential of the transplanted cells (or perhaps due to increased CLPs in the WBM transplant).
Response: We indeed observed very low chimerism in animals transplanted with Sept7−/− HSCs. We are though confident that we can reliably measure lineage contribution in animals that show at least 0.1% overall chimerism, like in xenotransplants (Amoah et al, 2021). In addition, in case donor cells do not robustly contribute to chimerism, they will in our experience contribute at least transiently to the myeloid lineage, but usually not to the lymphoid lineage. Our data does not reveal the reason for the elevated relative frequency of lymphoid B-cells in BM, but as the reviewer stated, one likely possibility is the elevated relative increase in the frequency of CLPs in bone marrow.

4) Analysis of public data sets for expression in borg4 and sept7 suggest robust expression in downstream progenitors, with sept7 having more robust expression in lymphoid progenitors and borg4 in myeloid progenitors. How have the authors ruled out that phenotypes during transplant were not driven by changes in progenitor cells?

Response: We now provide in addition to our data also data from public data sets (blood spot, https://servers.binf.ku.dk/bloodspot/) on the level of expression of septins and borgs in hematopoiesis (Figures EV2 and EV3). The public data sets also include expression of the genes in downstream progenitors and mature hematopoietic cells using public data set. i) data from the public data sets are in general consistent with our own expression data of septins and borgs in HSCs. ii) In this curated data set, the expression of septin 7 is indeed higher in both myeloid and lymphoid progenitors compared to HSCs, and indeed lower in lymphoid progenitors for borg4. iii) most surprisingly tough is the heterogeneity in the expression patterns among both septins and borgs. This implies a complicated regulatory network, and likely multiple types of function of the individual proteins outside the core “borg-septin-filament-polarity” system. In general, it can indeed not be ruled out, though in any transplant experiment with sorted HSCs, that the phenotype associated with the lack of a gene, although the lack is already present in HSCs, will only manifest upon differentiation. All progenitor cells though are derived from transplanted HSCs, so the initial deletion in HSCs contributes to the overall phenotype.

5) The authors state that the lack of septin 7 lead to impaired HSC expansion, however as presented in figure 4N, they show a significantly increased HSC compartment in the sept7 knockout transplants. How do the authors resolve this potentially conflicting data?

Response: Thank you for pointing out a potentially conflicting interpretation of our data. The overall chimerism supported by septin 7−/− HSCs is low (Figure 4I), so the overall function of HSCs is indeed impaired. The data in Figure 4N shows the relative frequency of HSCs among LSK progenitor cells, which is significantly increased for HSCs derived from septin 7−/− HSCs. The data in Figure 5 shows proliferation as stated in line 295 (header of that paragraph). Proliferation is not always equal to expansion or maintenance of potential. That is very reminiscent of the phenotype of aged HSCs upon transplantation: aka lower overall function upon transplantation and thus lower chimerism supported by aged HSCs, though higher relative contribution to the HSCs compartment (Florian et al, 2012; Sacma et al, 2019). We attributed this relative increase of aged HSCs upon transplantation to the tendency of aged HSCs to undergo symmetric divisions while losing their potential (Florian et al, 2018). We therefore corrected HSCs expansion to HSCs proliferation in lines 295 and 804 in the manuscript to resolve the potentially conflicting interpretation.

6) For IF staining, how many cells were seeded and how? The total number of cells presented is quite low (n>17). Understandably, there are not many young LT-HSCs, but if only 17 cells are scored (figure 2) it would be important to know if only ~17 cells remained on the slide or if only 17 cells were stained.

Response: For IF staining, 1000-2000 LT-HSCs were seeded on fibronectin coated coverslips. Coverslips are then incubated for 2hr at low oxygen incubator. After a standard IF staining protocol, we usually retain 50-70% of cells on the coverslip for further analyses. In proximity ligation assay (PLA), the number of cell remaining on the coverslip was, due to
more washing steps, more in the range of 30%. The cells for the PLA analysis were chosen randomly from these cells. We measured the volume of the interaction points on the confocal microscopy (LSM) in 3D, which takes a very long time and a great effort. So we focused on an initially smaller target number of cells (about 20) compared to our usual 50 cells to start our analyses. Statistics were already clear with this number of cells, so that no additional analyses were conducted for the PLA assay.

Minor Concerns:

1) Could the authors clarify if the Septin7 knockout mice received from Hannover? From Fig S4 appear to be Floxed but please confirm in materials section?
Response: The septin7 mice were indeed obtained from Hannover, we state that again in the Materials and Methods section.

2) Were there changes in the PB composition of knockout mice?
Response: We analyzed PB from both borg4 and septin7 knockout mice with a blood cell counter and also by flow cytometry. We observed, similar to the steady state data from bone marrow, no significant difference in the cell composition of PB, which further supports or steady state phenotype of both knockout strains (no change in BM or PB). This data is now included in Figure EV6 A&B of the revised manuscript.

3) Was the H4K16ac polarity also altered in Sept7-/- and borg4-/-?
Response: We determined the frequency of cells polar for H4K16ac in both Sept7-/- and borg4-/- LT-HSCs. We observed a significant decrease in the frequency of LT-HSCs polar for the distribution of H4K16ac in septin 7-/- LT-HSCs where there was no significant difference in the case of borg4-/- LT-HSCs. In general, loss of a regulator of a network (like Borg4) might have less effect on the overall network compared to the loss of a central component of core of the filament (septin 7). Next, septin 7 is the only member of its family, and thus likely compensation does not play a role. In case of the deletion of borg4 other borgs like borg2 (Figure EV3) might be able to compensate the loss of borg4. We have included the results on H4K16ac polarity in the revised manuscript in Figure EV4 E& F and H&I.

4) It would be informative to show “frequency of live bone marrow” and not just frequency of parent gate.
Response: We do follow a standard analyses scheme for gating of different populations and reporting them as frequencies. This is in general valid our case in which there is no change in the overall number of PB or BM cells. As per suggestion of the reviewer, we plotted the absolute number of populations among BM cells now in Figure EV6, which provides results that are in line with the data that reports frequency.
In Kandi et al., the authors examine the role of Cdc42, Borgs (Binder of RhoGTPases), and Septins in regulating the polarity of HSCs, and thereby their functional potential, during aging. They show that during aging, mouse HSCs change expression and lose polarization of several Borg and Septin proteins. The polarization defect is partially restored by adding an inhibitor of Cdc42, CASIN. Conditional deletion of Borg4 or Septin7 in hematopoietic cells (via Vav-Cre) also leads to decreases in the polarity of several proteins involved in cytoskeletal remodeling and asymmetric/symmetric division. In Borg4 or Septin7 cKO mice, there are minor alterations in progenitor populations suggesting minor defects in lineage development, although no differences in mature lineages were noted. However, upon transplantation in a competitive setting, Borg4 and especially Septin7 cKO HSCs appear defective in engraftment and lineage reconstitution. Septin7 cKO HSCs appeared dysfunctional in differentiation at several stages, and a massive increase in lymphoid progenitors was noted along with a relative decrease in myeloid output. Sept7 cKO HSCs also had deficiencies in cell division and viability in vitro. Overall, this study suggests a role for cell polarity in regulating HSC differentiation function, which is disrupted in aging, and a mechanism by which Cdc42-GTP increases with age and inhibits polarization via Borg4 and Septin7.

In general, I enjoyed this study and found their basic premise supported by their data. They used a variety of different experiments from gene expression, imaging, proximity ligation assays, and transplantation to build a case that the Cdc42-Borg4-Septin7 axis plays an important role in the age-related differentiation phenotype known for HSCs. Not all their data was clearly supportive, there were some inconsistencies in the data and how it was presented, and I didn't agree with some of their interpretations, but overall, I think this manuscript adds something novel to the literature and advances the field.

Major comments:

1) The decrease in donor chimerism in the Borg4 cKO in the blood (Figure 4H) appears different than the BM analysis (Fig 4 J, K, L). It's unclear how donor chimerism is being calculated in each case. For the blood, is it CD45.2 as a percentage of total CD45? For the BM, is it just within the CD45.2 cells? Both are labeled as "% of donor derived cells". The figure legends and methods sections don't explain this. This is important as the data appears different, with the blood data suggesting a defect in Borg4 cKO HSC engraftment, and the BM suggesting no defect.

Response: We used our standard approach to determine donor chimerism. The "% of donor derived cells" in Figure 4H reports the frequency of CD45.2 positive donor cells among cells from PB. After 20-21 weeks, we also determine chimerism in BM, and determine the frequency of CD45.2 positive donor cells like shown in Figure EV5 D. In Figures 4J to L, we determine the frequency of B-cells, T-cells and Myeloid cells among donor derived CD45.2 cells in bone marrow to test whether there is a difference in the differentiation potential of the transplanted HSCs. It is for example a possibility that there is both an overall difference in donor contribution (Figures 4H or I) and thus overall stemness, but no difference in differentiation (for example Figure 4J, K, L) for Borg4-/- HSCs, but in addition to the
difference in stemness also a difference in the differentiation profile for Septin 7-/- HSCs (for example Figure 4M,N,O). This type of presentation allows us to separate stemness from the differentiation potential, which is mechanistically very important. With respect to HSCs for example in the Septin 7-/- donor experiments, the relative contribution of the transplanted donor HSCs to HSC-derived donor LT-HSCs is increased. Due to the low number of overall donor chimerism though, the absolute number of donor derived HSCs remains very low (Figure EV6J).

3) The model implies that the loss of polarity contributes to the HSC aging phenotype, which is reduction in engraftment ability and a myeloid bias. So, if one were to examine cKO mice that lose an important polarity protein, like Borg4 or Sept7, then this would lead to a premature aging phenotype. And yet the transplant data implies a lymphoid bias in the Borg4 and Sept7 cKO mice, which have a defect in HSC polarity. This discrepancy isn't really explained in the discussion.

Response: The reviewer is correct that we observed what we call segmental phenotypes of aging: low engraftment, higher contribution to LT-HSCs upon transplantation, a reduced frequency of cell polar for polarity proteins. We do though also see elevated contribution of lymphoid differentiation, which has not been associated with aging, so indeed we do not see a premature aging phenotype, only a segmental or partial one. We added now line 392 to 393: “This premature aging phenotype though is only partial or segmental, as for example lymphoid differentiation is rather enhanced in these HSCs.”

Minor comments:

1) Fig. 1A/1B - Gene expression qPCR data presented here clearly does not use biological replicates (or that's the most consistent gene expression patterns I've ever seen). I don't know the journal's policy on presenting error bars for technical replicates, but I would request that the authors clearly state in the figure legend that the replicates are technical replicates or remove the error bars.

Response: The data is indeed derived from biological repeats (n=3 biological repeats, aka three separately sorted populations of HSCs), and not simply technical repeats. As we need to harvest bone marrow from multiple animals to obtain enough HSCs from one sort for qPCR, even one biological repeat is already an average on cells from multiple animals, which might explain an overall lower variation in our data. In addition, the deviation in the graphs shows SEM values and the data is plotted on a log-scale, which might also contribute to the perception of simply technical repeats in Figure 1A/1B. The overall trend in the expression profile of borgs and septins in HSCs as described in Figure 1A/1B is similar to publicly available gene expression datasets (Figure EV2 and EV3).

2) On page 8 (of the pdf file), the authors state: “This strongly implies a strong feedback loop in which changes in the septin 7 localization and thus likely a disturbed septin network will in return influence the localization of Cdc42 as well as borg4.” The changes in polarity in Figs 3C and F to me don't justify use of the word “strong”, let alone twice in one sentence. I recommend toning this down.

Response: We agree with the reviewer that the change in the frequency of cell polar for polarity proteins is not all or nothing, but turns from an average of 60-70% polarity to an average of about 40% polarity in the absence of Borg4/Septin 7. While the data still implies a feedback loop on polarity, the magnitude of the influence remains indeed open for discussion. We thus removed the word “strong” from the sentence (now line 242-250).

3) Fig. 4A/4D - The bone marrow contains few T cells. The blood would be a better place to assess differences in lineage output, in my opinion.
Response: We added novel data presenting lineage output among peripheral blood cells (Figure EV6 C&D). We did not observe a difference in lineage output in PB of Borg4 or Septin7-/- animals, which is consistent with our analysis of cells in BM.

4) Fig. 4B/4E. Either graph LSK as a percentage of total BM cells or LT-HSCs as a percentage of total BM cells. Fig 4B/E only shows LT-HSCs as a percentage of LSK, but total LSK could be decreasing in the KO and this wouldn't show that. Same with CLP. They are plotted as a percentage of the gate above them. It would be helpful to plot them as a percentage of total BM cells.

Response: We provide information on now the absolute number of stem and progenitor cells in bone marrow (Figure EV6), which mirrors our data obtained with relative numbers (percentages of cells in the parent population. Total LSK cells are thus not affected by deletion of borg4 or septin 7.

5) Fig. 4C/4F - CLP are also Flk2+. If Flk2 is in the stain, it would be good to gate CLP as Flk2+ IL7Ra+ (ckit low Sca1 low Lin-). In fact, if the lineage stain, Flk2 and IL7Ra stains are all good, you don't even need to gate ckit low and Sca1 low.

Response: We did not use Flk2 for the analysis of common lymphoid progenitors. We gated for ckit low Sca1 low Lin-. In Fig EV5 B we now report our gating strategy, CLPs: gated as IL7R+ population among Lin- Sca-1 (kit c-Kit low) cells, common myeloid progenitors (CMPs, gated as Lin- c-kit CD34 CD16/32), megakaryocyte-erythrocyte progenitors (MEPs gated as Lin- c-kit CD34 CD16/32) and granulocyte-macrophage progenitors (GMPs gated as Lin- c-kit CD34 CD16/32)

6) Fig. 4H - Hard to see which are squares and which are circles with the error bars there. Perhaps enlarge the symbols or make one open symbol to make it easier to distinguish.

Response: We now provide Figure 4H with easier to distinguish symbols.

7) Fig. 4J/4M - CD3 and B220 are switched between 4J and 4M.

Response: We switched CD3 and B220 in 4J and 4M for consistent labeling.

8) Fig. 4B/4E and 4K/4N - one graph uses LMPPs, the other graph uses MPPs. In the results for 4E, it refers to LMPP but the bar graph is labelled MPP.

Response: In Figure 4E, MPP was replaced with LMPP for consistent labeling.

9) Fig. 4N - Why does the graph make it look like LT-HSC chimerism is increased in the Sept7 cKO when the blood data indicates a massive reduction in donor chimerism in the blood? It appears that this is also explained by the way the LT-HSC data is reported, as a percentage of total LSK cells, and not as a percentage of total BM cells.

Response: We used our standard approach to determine donor chimerism. The “% of donor derived cells” in Figures 4H&I reports the frequency of CD45.2 positive donor cells among all blood cells. In Figure 4N, we determine the frequency of LT-HSCs derived CD45.2 cells among donor LSK cells to test whether there is a difference in the differentiation potential of the transplanted HSCs. It is for example a possibility that there is both an overall difference in donor contribution (Figures 4H&I) and thus overall stemness, but no difference in differentiation (for example Figure 4N). This type of presentation allows us to separate stemness from the differentiation potential, which is mechanistically very important. With respect to HSCs for example in the septin 7-/- donor experiments, the relative contribution of the transplanted donor HSCs to HSC-derived donor LT-HSCs is increased. Due to the low
number of overall donor chimerism though, the absolute number of donor derived HSCs remains low (Figure EV6J).

Fig. 5A - Does this include only live cells or does it include the dying cells as well? It probably should only include live cells as that would focus on whether the division kinetics are slower. If it includes dead cells then the difference could easily be explained by the difference in dead cells as shown in 5B.

Response: Figure 5A: We only sorted live LT-HSCs and also controlled presence of cells and their morphology in the first hours after a sort. After that, we checked individual wells every 8h hours for the number of cells division (1st division consider as 2 cells in the well, 2nd division considered as at least 3 cells) as well as for dying/dead cells.

Referee #3:
The manuscript "A Cdc42-Borg4-Septin 7 axis regulates HSCs polarity and function" by Kandi et al investigates the role Borg4 and Sept7 in hematopoietic function. Septins are emerging as novel regulators of cytoskeletal remodeling with potential input in many cellular processes. However, they remain fairly uncharacterized and their role in physiological processes as well as their regulation and mechanisms of action are fairly unknown. Here the authors address this issue by investigating the role of septins in hematopoietic function. In addition, they explore the potential of Borg proteins (the only known upstream regulators of septins) and the regulation and function of septins in this system.

1) Using a vav-1 driven cre recombinase model, the authors specifically delete Borg4 and Sept7 in hematopoietic stem cells (HSCs), showing a reduced engraftment potential upon transplantation and skewed differentiation programs. Characterisation of HSC function and differentiation is very exhaustive, although very hard to follow.

Response: We thank the reviewer for the encouraging overall comments on our work on the exhaustive analysis of the function of septins and borgs in primary HSCs, which were performed primarily with IF analyses and in competitive transplantation experiments. Competitive transplant experiments are regarded as the gold standard for the determination of the function of HSCs.

The authors show that deletion of Borg4 or Sept7 affects the proportions of different hematopoietic lineages at steady state, with more profound phenotypes after deletion of Sept7. Interestingly, phenotypes were more evident after stress (ie. BM transplantation), suggesting a particular role of this axis in this context. Although these findings are interesting, their relevance in health/disease is obscure. At this point, all the results are purely observational, without clear mechanistic explanation.

Response: We agree with the reviewer that our data does not reveal whether the lack of borg4 or septin 7 contributes to disease. Mechanistically, our data though demonstrate that borg4 and septin 7, due to being downstream effectors of Cdc42 activity, at least in part contribute to segmental HSCs aging initiated by elevated Cdc42 activity. Currently, we do not have additional insights into how for example septin 7 influences aging of HSCs in detail.

2) It is not clear why sept7/borg4 function is only evident during transplantation, only affects certain levels of differentiation potential and not others, presents some characteristics of aged HSCs but not others, etc. Thus, most of the conclusions are purely speculative.

3) Furthermore, the mechanistic insights are very limited and it is not clear if (or how) borg4 and septins modulate HSC function. The rationale for investigating specifically Borg4 and Sept7 in HSC illustrated in the first part of the manuscript is also very arbitrary.
Response: The general consensus in the field is that borgs and septins act cell-type specific. The determination of the function of borgs and septins need thus to be done in the cell-type under investigation, in our case primary hematopoietic stem cells (HSCs), and transplantation experiments are the gold standard to measure the various functions HSCs have. Septin 7 for example is thought to have a particular role in the septin family due to the fact that it is the only member of its subgroup that cannot be replaced by any other septin in a hexameric or octameric septin assembly (Kremer et al, 2005; Tooley et al, 2009; Kim et al, 2011). We therefore respectfully disagree that our conclusions are purely speculative. The rationale on focusing on borg4 and septin 7 is the fact that these were the only ones among the borg-septin pairs tested that showed colocalization in HSCs. Our data with respect to the polarity and function of HSCs in the absence of either borg4 or septin 7 or the data on a cdc42-borg4-septin7 axis and within HSCs are valid and solid, including our conclusions. What is missing, but which in our opinion is also beyond the current scope of the manuscript, is indeed information on how for example the absence of septin 7 affects differentiation. The novelty we report is that it affects differentiation, polarity and reconstitution. For example, lack of septin 6 does not affect these parameters to a great detail (Senger et al, 2017).

4) The authors focus in gene expression analysis to define their primary candidates, but expression is not directly associated with function, and they did not confirm their qPCR results by alternative ways (thus, confirming that low levels of expression are not just a result of faulty primer design). Although the intrincacies of investigating Borg proteins are acknowledged (lack of tools or specific antibodies), more robust results are required to convincingly determine the relevance of these factors (i.e. Borg4 and not others) in the process.

5) This may include a loss-of-function genetic screen. For example, it is not clear why the specific requirement for Sept7 and not other septins, if they may be equally required for septin network assembly. If Sept2 and Sept6 do not relocalize after Cdc42 modulation, does it imply that this is a septin-network-independent function of Sept7? Or are other septins involved?

Response: We agree with the reviewer that faulty primer pairs can result in an underestimation of expression. We thus included novel information from other, publicly available databases which to a large extent confirm, with a completely distinct approach, our expression pattern reported in the manuscript. We report data on knock-out animals to convincingly determine the relevance of borg 4 and septin 7. Our data does not exclude that also other septins and borgs might play important roles in the biology of HSCs. The purpose of this manuscript is not to study the function of all known septins and borgs within HSCs.

Septin 7 has a particular role in the septin family due to the fact that it is the only member of its subgroup that cannot be replaced by any other septin in a hexameric or octameric septin assembly. We performed additional experiment to determine septin 2 and septin 6 distribution and active Cdc42 in young and aged LT-HSCs, as well as in Borg4 KO LT-HSCs (Figure EV1 L to P; Figure EV4 G). The data implies that there might be functions of septins that are indeed independent of the contribution to the published basic septin network, as they components of the basic network do not strongly co-express or colocalize.

6) Finally, their working model (Figure 6) is based in perturbations that are particularly noisy and that might have multiple undesired off-target effects (i.e. Cdc42 inhibition, young vs matured HSC). The validity of this model needs to be supported by additional, more robust perturbations including the modulation of the binding capacity of Borg4 towards Cdc42 and septins, using already described mutants.

Response: Our model is based on co-IF staining, proximity ligation assays, polarity stainings and results from competitive transplantation of knock-out HSCs and analyses of the function of HSCs in standardize transplantation assays and very specific inhibition of the activity of Cdc42. These perturbations are not regarded as being particularly noisy, nor as having
multiple undesired off-target effects, including inhibition of Cdc42 activity by CASIN (see published data). We do therefore respectfully disagree with the reviewer that additional genetic data will be necessary to support our model, especially as genetic manipulation of HSCs ex vivo and subsequent analyses are prone to noisy data and undesired effects due for example random integration of the construct into the genome. We are convinced that co-staining and the results of the proximity ligation assay confirm, especially in the presences of Cdc42 activity inhibition, support our model.

7) Given that Borg4 requires Cdc42 function for their correct positioning, it is not clear how interaction with Septin 7 is increased in low Cdc42-GTP conditions. For these assays, it is particularly important to analyse Cdc42 activity spatially.

Response: The question on how the interaction of borg4 with septin 7 is affected by Cdc42 activity in HSCs is indeed interesting. It is though not evident to us how the spatial determination of Cdc42 activity will address this question unequivocally. We also feel that these in depth mechanistic studies on how the complex is ultimately formed within HSCs are beyond the current scope of the current manuscript. Finally, until very recently, other than in cell lines, it was not really possible to reliably determine the spatial distribution of Cdc42 activity, especially not in primary HSCs. We though very recently confirmed specificity of an antibody against Cdc42-GTP (Althoff et al., 2020; Amoah et al., 2021; Nalapareddy et al., 2021) and provide novel data on the localization of Cdc42-GTP, borg4 and septin 7 in in Figure EV1N-P. As anticipated, there is co-localization among these proteins in HSCs, while only the PLA assay (Figure 2) will allow to determine whether they indeed interact.

8) It is possible that polarity arises from concentrated activation of Cdc42 in certain areas (leading to the recruitment of Borg and septin in those regions), whereas high global Cdc42 activity leads to the disruption of Cdc42-GTP gradients and the inability to generate concentrated modules of Cdc42-GTP/Borg4/Sept7. There may be a competition between different Borgs for binding to Sept7, that is affected by Cdc42 modulation... Unfortunately, the authors did not exhaustively investigated alternative explanations.

Response: We fully agree with the reviewer that these are all very interesting question which we would also like to address in the future. They are very detailed questions on the biochemistry of the complex, especially in HSCs, and we do not per se exclude alternative models, but see the current model as the best fit on our data, in combination with published knowledge. As septin-borg-Cdc42 complexes are likely to act very cell-type specific, surrogate cell line experiments as suggested by the reviewer are likely not informative and will anyhow require verification of the biochemistry in HSCs, which is still very difficult to achieve due to the low number of HSCs that can be obtained from a single mouse, and HSC cell lines do not exist. We thus regard such experiments, while again they address very interesting novel questions, beyond the current scope of the manuscript.

9) Borg and septins are relatively novel and thus fairly uncharacterized factors related to Cdc42 function and cytoskeletal regulation. However, their pathobiological role and regulation is still not well understood. Overall, this manuscript provides evidence of a possible new biological role for Borgs and septins in hematopoietic cells. However, it provides very limited advances in the function and regulation of Borgs and septins, or the underlying molecular mechanisms affecting HSCs.

Response: We strongly agree with the reviewer that borg and septins are fairly uncharacterized factors related to Cdc42 activity and polarity regulation. The novelty within our work is that we demonstrate the existence of an Cdc42-Borg4-Septin7 axis, and can demonstrate a role for lack of Borg4 as well as Septin 7 for HSCs function and regulation of polarity, and by this means at least a segmental role for aging of primary HSCs and animals. We thus provide a significant contribution to their biological and pathobiological role in vivo.
Other comments:

1) Use alternative sources/material to confirm expression data (e.g. Human Cell Atlas).
Response: We have included alternative sources to confirm the expression data in normal mouse hematopoietic stem cells. The data is now included as Figure EV2 in the manuscript.

2) Expression does not determine relevance. Perform genetic screen - is there any other Borg modulation affecting Sept7 phenotype?
Response: We agree with the reviewer that expression per se does not determine relevance. We thus provide a large number of additional data (co-localization, response to attenuation of Cdc42 activity, and function of knock-out HSCs for either septin 7 or borg4 to demonstrate relevance of borg4 and septin 7 for HSCs. Our data does not exclude an influence of other borgs on septin 7. The current manuscript though establishes a Cdc42-borg4-septin7 axis in HSCs which affects polarity and HSCs function.

3) Explain why polar for Sept7 and not the rest, if septin filaments are oligo heteromers. Is this a septin-network-independent function of Sept7? Or are other septins involved? Which?
Response: Septin 7 has a particular role in the septin family due to the fact that it is the only member of its subgroup that cannot be replaced by any other septin in a hexameric or octameric septin assembly. We performed additional experiment to determine septin 2 and septin 6 distribution and active Cdc42 in young and aged LT-HSCs, as well as in Borg4 KO LT-HSCs (Figure EV1 L to P). The data implies that there might be functions of septins that are indeed independent of the contribution to the published basic septin network, as they components of the basic network do not strongly co-express or colocalize. We are currently investigating the overall nature of the septin network in HSCs, which will likely contribute to a better understanding of the role of the network for HSCs function.

4) If Borg are Cdc42 effectors, why reduction of Cdc42 activity is associated with redistribution? What is determining the localization of borg/sept in Cdc42-GTP low cells leading to polarity?
Response: These are indeed very interesting questions. Our data supports a feed-back mechanism, in which the localization of the Borg4 effector can also affect localization of Cdc42 itself. That indeed poses the question what determines the localization of the complex in Cdc42 activity low cells (if not Cdc42 GTP activity), which we currently do indeed not know.

5) Expression of candidate factors after CASIN treatment is not relevant unless you show mechanism controlling their expression.
Response: We show that the expression of borg and septin themselves can be altered by the changes in the activity of Cdc42. That information is important and novel and will contribute to our understanding of the regulation of the complex relationship of these genes and proteins (see above), even in the absence of knowledge on how Cdc42 activity regulates expression of borgs and septins.

6) Experiments in Fig 2 are over interpreted - if there is no Cdc42 activity, how can borg reach septins? It implies that septin regulation is independent of Cdc42. Analysis of effect of Cdc42-binding and septin-binding mutants of Borg4 is required. Interaction between Cdc42 and Borg4, and Borg4 and Sept7 needs to be shown.
Response: The reviewer is not correct in his/her statement that there is no Cdc42 activity in experiments listed in figure 2. We only shift between normal and elevated levels of Cdc42 activity.

7) Analysis of polarization are mainly phenotypic and therefore prone to observational bias. In order to add rigour, additional readouts are required, as well as a quantitative ways to illustrate significant differences between the experimental points. Independent readouts of
polarity (not dependent on the main effector under study i.e. Cdc42) are required. This is particularly important since candidate perturbations (Borg4/Sept7) seem to affect Cdc42 localization, landing on a chicken and egg scenario.

Response: We agree with the reviewer that rigor is required to read-out polarization. We established long-term, reliable read-out criteria that span multiple years and researchers to determine polarity to precisely avoid observational bias, which allows us to provide reliable, quantitative analyses (Althoff et al., 2020; Amoah et al., 2021; Florian et al., 2012; Florian et al., 2018; Florian et al, 2013; Grigoryan et al, 2018; Grigoryan et al, 2021; Guidi et al, 2017; Kared et al, 2020; Kumar et al, 2021; Nalapareddy et al., 2021; Sacma et al., 2019; Senger et al., 2017; Tiwari et al, 2021). Cdc42 is in itself a polarity protein, and is thus suitable to serve as a polarity protein. We though added new data on the distribution of the histone 4 acetylated on lysine 16 (H4K16ac), which serves as a nuclear polarity marker also controlled by Cdc42 activity (Grigoryan et al., 2018), also in HSCs deleted for septin 7 or borg 4. The frequency of cells polar for H4K16ac was significantly decreased in septin 7∆/∆ HSCs (Figure EV4 E&H) whereas it was not affected upon deletion of borg4 (Figure EV4 F&I). The distribution of another cytosolic polarity marker (Crumbs3) was also not affected by the absence of borg4 (Figure EV4 K&L), which imply distinct roles of borg 4 and septin 7 for the regulation of the distribution of proteins beyond Cdc42, or that the absence of borg 4 can be compensated by another borg protein, while the absence of septin 7 cannot be compensated.

8) The link between polarity and function is not clear.
Response: We previously demonstrate that there is a tight link between polarity and the mode of HSCs division (Florian et al, 2018). Polar HSCs prefer asymmetric divisions, while apolar cells prefer symmetric divisions.

9) PLA analysis is particularly hard to interpret, as it involves what appears clusters of cells. Why there is PLA mainly occurring in the perinuclear region? Why is it not polarized?
Response: Hematopoietic stem cells are round and nonadherent cells with a only a small volume of cytoplasm, which is a very distinct appearance compared to cells from cell lines or fibroblasts that stick to a dish. So almost all cytoplasmic proteins might thus be regarded to be peri-nuclear in HSCs. We did indeed not determine the frequency of polar cells in these assays, as the number of cells necessary to reliably determine the frequency of cells polar for the PLA signal would have been much higher compared to the number of cells that were analyzed in the PLA assay to determine proximity. We can thus not provide data on whether the signal is polarized or not polarized.

10) Show data not shown.
Response: The only data not shown is the data on the septins which did not show a signal in our RT-PCR analysis for the specific gene. A low level of expression of these septins and borgs has been further confirmed by the addition of expression data from publicly available databases (Figure EV2,3). As it is not quite common to show simple non-signal CT values of 40 and over, and the data cannot be simply added to the bar diagram (value of 0 is also not informative), we would like to list this primary data also in the current version as not shown.

Reference point-to-point response to the reviewers
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Dear Dr. Geiger,

Thank you for the submission of your revised manuscript to EMBO reports. Since my colleague Achim Breiling is currently not in the office, I have temporarily taken over the handling of your manuscript. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and request only minor changes to clarify the limitations of the current study in the text/discussion.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Please enter all information on funding in the relevant fields in our online submission system.

- Please reduce the number of keywords to 5.

- Please add a Data availability section at the end of Materials and Methods. If you have no data that requires deposition in an external database, please simply state this in this section. You could add a link to Dryad, if you deposit primary data there.

- Please note that our editorial policies require that all data described in the manuscript are included. In this respect we note that you refer to "data not shown" twice on page 5. Please include the relevant data in the manuscript.

- Please add callouts to the following figure panels in the text where appropriate: Fig 2C, Fig. EV2 panels, and Fig. EV3 A-F panels.

- Please add a callout to Fig EV6.

- Please note that only up to 5 EV figures can be typeset. You currently have 6. Please either combine figures to reduce the number of EV figures to 5 or alternatively, move some of the figures to an Appendix. The Appendix is a single pdf including figures and their legends as well as a title page with a table of content (incl. page numbers). The nomenclature is Appendix Fig. Sx.

- Please add the Expanded view figure legends into the main Article file after the main figure legends.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. I noticed that information on the number of samples is currently missing from many EV figure legends. In case the quantification was based on n<3, please do not use mean/median, error bars and statistical analysis but show the data as scatter blots instead. If the data are based on technical replicates, please do not perform statistical analysis.

- I noticed that you used Bloodspot to generate data/graphs. Please add a citation to the original paper, in case you have not already done so.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of
the findings and their significance, and B) 2-3 bullet points highlighting key results. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

Referee #1:

The authors convincingly replied to the initial concerns raised. While I still think that fetal liver data is relevant to this manuscript, I appreciate how polarity and the role of Septin7 in fetal liver HSC may require more indepth followup and warrants independent investigation. I have no further questions towards this revised manuscript and support it’s publication.

Referee #2:

My concerns have been address satisfactorily. I thank the authors for such well-thought and detailed responses.

Referee #3:

The authors have done their best to address my comments in this revised manuscript. I acknowledge the technical limitations of their system and tools, that have prevented them to provide additional mechanistic data regarding the core axis, which they claim is beyond the scope of the current manuscript. I strongly suggest that these limitations and shortcomings are discussed (at least briefly) in the manuscript and not just in the Letter to reviewers.
In my opinion, this manuscript is of particular interest to researchers specialised in HSC biology with limited interest to a broader public.
Point-to-Point response to the remaining on comment of one reviewer and the editor

EMBOR-2021-52931-V2: A Cdc42-Borg4-Septin7 axis regulates HSC polarity and function by Kandi et al.

Reviewer 3:

The authors have done their best to address my comments in this revised manuscript. I acknowledge the technical limitations of their system and tools, that have prevented them to provide additional mechanistic data regarding the core axis, which they claim is beyond the scope of the current manuscript. I strongly suggest that these limitations and shortcomings are discussed (at least briefly) in the manuscript and not just in the Letter to reviewers.

Response: We added additional sentences to the Discussion part to further discuss these limitations.
Line 342: “It is likely that borgs and septins act cell-type specific”.
Line 349: “The data presented in this manuscript is focused on HSCs. The role of specific borgs and septins might be distinct in other cell types”.

Comments from the Editor

1) Please enter all information on funding in the relevant fields in our online submission system.
Response: We have entered funding information now also in the online submission system.

2) Please reduce the number of keywords to 5.
Response: We reduced the number of keywords to 5.

3) Please add a Data availability section at the end of Materials and Methods. If you have no data that requires deposition in an external database, please simply state this in this section. You could add a link to Dryad, if you deposit primary data there.
Response: We added a Data Availability section at the end of Materials and Methods.

4) Please note that our editorial policies require that all data described in the manuscript are included. In this respect we note that you refer to "data not shown" twice on page 5. Please include the relevant data in the manuscript.
Response: We provide now these data (real-time RT PCR data) as an additional Excel sheet.

5) Please add callouts to the following figure panels in the text where appropriate:
   - Fig 2C, Fig. EV2 panels, and Fig. EV3 A-F panels.
   Please add a callout to Fig EV6.
Response: We included these figures callouts to the manuscript.

6) Please note that only up to 5 EV figures can be typeset. You currently have 6. Please either combine figures to reduce the number of EV figures to 5 or alternatively, move some of the figures to an Appendix. The Appendix is a single pdf including figures and their legends as well as a title page with a table of content (incl. page numbers).
The nomenclature is Appendix Fig. Sx.
Response: We decided to use the Appendix format and move EV6 to the Appendix. We adjusted the call-outs in the manuscript.

7) Please add the Expanded view figure legends into the main Article file after the main figure legends.
Response: done

8) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. I noticed that information on the number of samples is currently missing from many EV figure legends. In case the quantification was based on n<3, please do not use mean/median, error bars and statistical analysis but show the data as scatter blots instead. If the data are based on technical replicates, please do not perform statistical analysis.
Response: We addressed the comments of the data editor. We added the number of samples to all figure legends.

9) I noticed that you used Bloodspot to generate data/graphs. Please add a citation to the original paper, in case you have not already done so.
Response: We included the reference to the website in the manuscript (Legend of Figure EV2 and EV3).

10) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, and B) 2-3 bullet points highlighting key results. Please send us this information along with the revised manuscript.
Short summary and bullet points are attached.
Dear Dr. Geiger,

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication’s Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: “No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case.”

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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our Production Office; you should return your corrections within 2 days of receiving the proofs.

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Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.
**A- Figures**

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 authorship guidelines on Data Presentation.

**2. Captions**

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

- A specific description of the experimental system investigated (e.g., 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.
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- The specifications of the experimental system investigated (e.g., cell line, species name).
- A statement of how many times the experiment shown was independently replicated in the laboratory.
- Definitions of statistical methods and measures:
  - Common terms, such as t-test (please specify whether paired or unpaired), simple 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.

**B- Statistics and general methods**

1a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

1b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

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.

4a. Were any steps taken to minimize the effects of subjective bias during group allocation or (e.g., blinding of the investigator)? If yes please describe.

4b. For animal studies, include a statement about blinding even if no blinding was done.

5. For every figure, are statistical tests justified as appropriate?

6. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

7. Is there an estimate of variation within each group of data?
| Reagents | Provide a statement only if it could. |
|----------|-------------------------------------|
| G- Dual use research of concern | 
| F- Data Accessibility | 
| E- Human Subjects | 
| D- Animal Models | 
| C- Reagents | 

**G- Dual use research of concern**

1. Could your study fall under dual use research restrictions? Please check biosecurity documents in a public repository or included in supplementary information.

**F- Data Accessibility**

26. 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-Sequencing: Gene Expression Omnibus GSE13462, Proteomics data: PRIDE PXD00028 etc.) Please refer to our author guidelines for 'Data Deposition'.

**C- Reagents**

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1Degreewhite (see link list at top right).

2. Identify the source of cell lines and report if they were recently authenticated (e.g., by S1P profiling) and tested for mycoplasma contamination.

**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.

9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

10. We recommend consulting the ARRIVE guidelines (see link list at top right) (Rutledge B, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.

**E- Human Subjects**

11. Identify the committee(s) approving the study protocol.

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.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.

17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.

**F- Data Accessibility**

20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access repositories such as Dryad (see link list at top right) or figshare (see link list at top right).

**C- Reagents**

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3. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

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**G- Dual use research of concern**

1. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

2. Identify the source of cell lines and report if they were recently authenticated (e.g., by S1P profiling) and tested for mycoplasma contamination.

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