Peer Review File

Manuscript Title: RAS-mutant leukemia stem cells drive clinical resistance to Venetoclax

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Caracamo et al find that ordered mutation acquisition is obligatory for leukemia initiation and provide a model of venetoclax resistance that incorporates genetics and differentiation states. This model reconciles conflicting views in the field and has high clinical relevance considering the widespread use of venetoclax. The importance of mutation order is well-supported by the work, and the model of venetoclax resistance is impactful. However, conclusions regarding the "cell of origin" are overstated. Furthermore, much of the work relies on few biological replicates. If these concerns can be addressed, the manuscript would be an important contribution to the field.

Abstract/introduction: The way the authors discuss the cell of origin is not optimal. While mouse GMPs can be transformed (Krivtsov 2006), it is likely that the first step leading to all cases of human myeloid leukemia is the acquisition of an oncogenic genetic mutation in an HSC. I don't think a RAS wildtype LSC with GMP characteristics acquiring RAS should be referred to as the LSC cell of origin (e.g., last sentence of the abstract). Please see https://pubmed.ncbi.nlm.nih.gov/16084092/ for the distinction between the cell of origin and the LSC.

Furthermore, the data does not establish the authors' bold claim that the cell of origin of RAS-mutated subclones is a GMP (e.g., the section title "RAS-mutant AML LSCs originate from GMPs" is an overstatement). Indeed, while it is concluded that "NRAS G12D can only transform GMPs that harbor previously acquired cooperating mutations," the results do not exclude that RAS mutations can transform primitive LSCs that harbor previously acquired mutations, especially if mutated RAS induces a GMP phenotype (i.e., CD45RA expression). In this light, it may be interesting to assess the RAS mutation frequency in sorted CMPs and GMPs (Figure 2c), since the alternative model would predict a higher RAS mutation frequency in GMPs. More importantly, the authors' claim could gain support if NRAS G12D expression only transforms sorted GMPs, and fails to transform sorted HSCs and CMPs. Alternatively, the conclusions could be rephrased to more closely reflect the results that leukemia-initiating capacity is exclusive to phenotypic GMPs. This would still be supportive of the model that RAS mutated LSCs with GMP characteristics and monocytic differentiation bias drive venetoclax resistance.

Page 3: "how [specific hierarchies] impact clinical outcomes remains obscure" – it is very well-established that stem cell signatures are associated with poor outcomes, at least in the era of chemotherapy.

Oncogene expression in human iPSC-derived HSCs followed by mouse injection is not a well-established leukemia model. The engraftment remains below 10%, and it appears the mice don't die (Figure S1i, Figure 1b). Additional support that the mice develop myeloid leukemias would strengthen the manuscript, such as phenotypic and morphological analysis. Even then, conclusions on page 4 ("generate leukemia," "induce leukemia") should be phrased within the limitations of the models.

Page 5-6: The bias of RAS mutated clones towards monocytic differentiation appears to be minor for both the PDX and the GoT analyses. Can the authors use a larger patient database with bulk sequencing and phenotyping to show that (high VAF) RAS mutations are associated with increased monocytic differentiation?

Many experiments in Figures 3 and 4 rely on two isogenic lines derived from a single patient and single-cell RNA-sequencing + GoT analysis of one other patient. Adding additional biological replicates would strengthen the manuscript.

Figure 4a-e: While the trial included 118 patients, only the survival curve shown in panel C adds up to 118. I also don't understand the rationale of subsetting this analysis to the monocytic cohort. Having only six RAS mutated patients calls into question the robustness of the conclusions.

Figure 4h-j: The data presentation in panel h is not ideal. For example, in h, y-axis ranges differ between panels for the same gene. It might be more clear to show a heatmap. More importantly, all panels are missing P-values, making it difficult to interpret the data.

Extended Data Figure 7a is vastly overclustered. Does the analysis hold up when reducing the resolution to analyze larger clusters?

Minor comments:
Page 3: "sizeable fraction" – can this be specified?

Page 4: Fig. 1h,i should be Extended Data Fig. 1h,i

Page 4: "R+SA or R+SA" should be "R+SA or SA+R"

Figure 2c-d: Information regarding the number of biological replicates is missing.

Figure 3, Panel B: The shades of blue are hard to distinguish. It would help to add some (broad) labels to the UMAP. Panel C: it may be more informative to have one UMAP colored by the difference in S phase between the PDX models, and another by the difference in local density of cells, thereby conveying increased monocytic differentiation in the RAS model. Also, please consistently show RAS WT left and RAS mutant right (e.g., panel D).

Page 6 or Methods: Please specify the efficiency of GoT (i.e., the proportion of cells in which a mutation was captured).

Referee #2 (Remarks to the Author):

The authors have used CRISPR/Cas9-mediated gene editing in human iPSCs and primary CD34+ HSPCs to explore the role of N/KRAS mutations in AML. They identify that transformation by mutant RAS is restricted to GMPs and that this only occurs when cells carry pre-existing oncogenic mutations, such as C-terminal truncated ASXL1 and SRSF2 P95L, but not either alone. Multiomic approaches were used to show that RAS transformed LSCs preferentially generate monocytic leukemias that are VEN resistant and that resistance is specific to RASmutant LSCs, rather than monocytic lineage. The authors propose a new paradigm whereby an oncogene can impact therapeutic outcome by targeting a specific cell subtype for transformation into an LSC with altered sensitivity to targeted therapies. In addition, the authors conclude that RAS mutations alone are not sufficient for leukemogenesis.

The authors generated iPSCs with DNMT3A, FLT3 and NPM1 and yet this did not form AML. Why was this the case? This combination is known to cause AML in mouse models and is a relevant combination in humans. Does this suggest some limitation of the diversity with which iPSC HSCs may be genetically transformed?

The group have previously engineered iPSCs with the variants described in this paper in their Cell Stem Cell paper in 2021 (DOI: 10.1016/j.stem.2021.01.011). In their current work, they have used this system to show that NRAS mutation increases cell proliferation and cell cycling without impact on differentiation. In contrast, SRSF2 and ASXL1 mutations impair differentiation without affecting proliferation. These results are consistent with our prior knowledge of how these variants function.

The authors identify that only the triple mutant (SAR) state resulted in transplantable AML but not other gene combinations. They also confirm the leukemogenic relevance of the SAR combination in primary human HSPCs via lentiviral transduction of SRSF2 P95L, ASXL1 del1900-1922 and NRAS G12D into cord blood CD34+ cells. These are impressive experiments and confirm their observations in iPSC derived HSPCs.

The authors identify that engraftment of transplanted HSPCs derived from iPSC lines only occurred for triple mutant cells, not other variations. It would be helpful to elucidate the biological reasons for this. For example, what are self-renewal programs re-wired by the presence of 3 variants not re-capitulated by the presence of just 2 and what does RAS mut add to the biology of the cell that allows this to happen? Are these alterations a result of synergistic epigenetic programs or just the additive impact of each gene variant individually? It would also be instructive, for example, to know the fate of the triple mutant cells after engraftment if the RAS mut was reverted back to a wild-type state to understand whether the properties of transformation are reversible or fixed.

Although the authors highlight interestingly that other iPSC lines with NRAS G12D combined with DNMT3A, FLT3 and NPM1 or RUNX1 and ASXL1 did not lead to robust engraftment, is this a consequence of the system used or do the authors propose that this is biological finding relevant to human AML in general?

What is the biological rationale for the RAS+ SA combination being unique and what specific pathways are invoked that are unique to SAR and not other genetic combinations?

The authors nicely show that SA+R cells generate a lethal myeloid leukemia, whereas the R+SA group showed no engraftment. Are the cellular programs modified by SAR and SA+R the same and how does this differ from R+SA to produce different biological outcomes? Could dissection of the single cell studies be pertinent here.?

The authors show that the HSPC populations in transduced cord blood cells result in skewed progenitor populations. R+SA and SAR cells showed reduction of GMPs with increase of CMPs, suggesting some degree of differentiation block with sorted engraftment showing that only the GMP cells gave rise to a lethal leukemia. The authors used bulk RNA-Seq and ATAC-Seq analyses in SA+R vs R+SA iPSC-HSPCs to show that NRAS G12D can only transform GMPs harboring SA variants. What signalling pathways explain this biological effect? Or are the outcomes mainly lineage dependent?

The authors suggest that RAS-mutated AML LSCs drive a monocytic phenotype. What is the mechanism for this? RAS mutation is not a lineage defining property of monocytic lineage cells. Do other kinase activating variants cause the same outcome? Is it perhaps the founding variants ASXL1 and SRSF2 which ultimately determine the likelihood of monocytic transformation, rather than RAS mut itself?

If RASm was transduced onto a different doublet variant background founded by eg IDH, NPM1 or a CBF rearrangement, does a monocytic leukemia result? Why did this not occur in the DNMT3A model shown in the extended data?

Combined presence of ASXL1 and SRSF2 variants are the commonest feature of CMML and so it would be useful to show that human AMML is frequently driven by co-presence of RAS mutation to strengthen the finding. The authors refer to some literature but a more detailed discussion of the frequency of this association would be useful.

The authors suggest that outcomes of monocytic and nonmonocytic AML were comparable for patients receiving VEN-DEC, suggesting that monocytic lineage alone did not determine VEN-based resistance. Although the authors show that N/KRAS mutant cases
within the monocytic AML cohort had significantly shorter DOR (3.4 months vs not reached in patients with WT N/KRAS, $p<0.001$), there was no difference in OS. Furthermore, the relative numbers of patients within these subgroups is small. A further validation using an independent dataset would strengthen this observation. Although 2 out of 6 (33%) patients with N/KRAS mutations achieved CR, compared to 60% (n=15/25) of those without N/KRAS mutations, a 6 patient observation is not sufficient

to be sure that response associations are not just a chance observation.

The authors show that monocytes from HSPCs with KRAS MT line were VEN-resistant with low expression of BCL2 and high expression of MCL1. Of interest, LSCs of the RAS MT clone had reduced BCL2 expression and higher MCL1 and BCL2L1 expression and lower expression of pro apoptotic BAX. This is an interesting observation and consistent with the author's recent studies suggesting that RAS mutation drives altered expression of pro-survival MCL1 (doi: 10.1038/s41392-021-00870-3). There is perhaps an opportunity to explore using the engineered cell lines to better understand whether the effect of KRAS variants on pro-survival expression occurs promiscuously in the other genetic settings, or only in SA cells. This would more sharply focus the effect of KRAS mut in relation to the explained effects on AML transformation vs venetoclax resistance, which are likely to engage different signalling pathways and be non-overlapping. Are the effects resulting from KRAS and NRAS the same or are there isoform specific differences in the impact of R/NRAS on survival signalling?

The authors suggest that there is strong rationale for combining VEN with MCL1 inhibitors and potentially BCL-xL inhibitors as frontline therapy in patients with detectable RAS mutations or all eligible patients. The potential limitations of using MCL1 and BCLX inhibitors for this purpose should also be highlighted.

Referee #3 (Remarks to the Author):

In this paper, the authors use gene editing in iPS as well as UCB CD34+ cells to induce different mutations especially focus on N-Ras and other pre-leukemic mutations to reveal that N-Ras induce leukemia transformation only if occurring in later stage as it target GMP-like cells. They also provide evidence that these Ras-mutant LSCs promote monocytic differentiation and are resistant to venetoclax treatment.
This paper is the continuation of the early paper by the same group (wang et al Cell Stem Cell 2021) who already demonstrate that the combination of N-Ras, ASXL.1 and SRSF.2 induced leukemia.

The paper provides some incremental informations related to the order of the mutations being important as well as the fact that N-Ras LSC induce Venetoclax resistance. There are nevertheless some major issues that can be raised from the present work.

1- They show that N-Ras alone is not enough to induce transformation but show in Figure 1 that in combination ASXL-1 and SRSF.2 iPS derived HSPC can engraft in NSG-S mice. They conclude that this is enough to prove transformation. It seems that the level of engraftment is quite low and thus is unclear whether the mice really died from leukemia. I supposed based on their original paper, the engraftment is mostly myeloid. But neither in this paper nor in the original one, they provided evidence that the cells out of these mice can produce leukemia after serial transplant. This should be done as a prove of clear leukemia development and invasion of the BM. They mentioned that other mutations like FLT3, DNMT3A and NPM or Runx1 or ASXL1 in conjunction with N-Ras are not able to induce transformation

but in extended Fig 2f, they show engraftment with AR at around 4.5% which is not dissimilar to what they show with NAS, especially as this time they injected the cell in NSSG instead of NSG-S. Nevertheless, when N-Ras is added (RAR), the level of engraftment is much lower. It will have been interested to investigate what happened there.

2- They next tested the effect of the mutation order and show indeed N-Ras need to be induce later using both iPS or CB CD34+ where they used lentivirus vector instead of Crisp-Cas.

In Fig1 d, they show that except the R-late group they did not retrieve any CD34+CD45+ cells. It is surprising that control CD34+ do not give rise to CD34+? In this experiment, it is also unclear whether after transduction, they sorted the triple transduced cells? To confirm the presence of the three mutations in the same cells out of the mice, they should provide at least genetic analysis of CFC. It is indeed unclear whether all cells are triple mutated. They should also show the level of engraftment in each arm as well as the proportion CD33/CD19 as it seems that even in the R-early, or R only, they have mostly only CD33 engraftment.

3- They also show in Fig 1h that the time of induction of N-ras is important. This might indicate two options that N-Ras induced transformation only when induced at later stage or that N-Ras is transforming a progenitor cell. Even through in Fig 2, they show that GMP and not CMP have LSC activity, it does not directly prove this point. As ex vivo expanded cells have been reported to changed phenotype like dowregulation of CD38+ ex vivo in liquid culture. Thus, they should sort GMP-CMP and HSC directly from UCB and induced again all three mutations at once compared to what they have done. Indeed, with the iPS I believe they have induced the three mutations at once and then induced there HSC differentiation.

4- They then switch the analysis to AML samples and their response to Venetoclax plus DEC. They show in a cohort of 117 patients treated with venetoclax and DEC that monocytic phenotype is not associate with a better DOR or survival but the presence of N-Ras mutation is associated with a shorter DOR but not overall survival. In these patients, it seems that a number have also TP53 which has already been associated with poor response. It might be of interest to eliminate the TP53 samples and run the analysis again.

5- Lastly, they used again iPS derived from AML samples and tested their response to venetoclax using enriched HSPC versus HSPC induced to differentiate into monocyte ex vivo. They show that all monocytic cells both WT and Nras mutant are resistant to venetoclax but that only the Ras mutated LSC are resistant. Here they provide a correlation between Ras mutation and resistance but not a direct prove as all iPS used have also potentially other confounding mutations.

Author Rebuttals to Initial Comments:

A detailed point-by-point response is provided below. The Referees' comments are in *Italics*. Our responses are in blue font. All changes in the manuscript text are also marked with blue font.

Referee #1:

Carcamo et al find that ordered mutation acquisition is obligatory for leukemia initiation and provide a model of venetoclax resistance that incorporates genetics and differentiation states. This model reconciles conflicting views in the field and has high clinical relevance considering the widespread use of venetoclax. The importance of mutation order is well-supported by the work, and the model of venetoclax resistance is impactful. However, conclusions regarding the "cell of origin" are overstated. Furthermore, much of the work relies on few biological replicates. If these concerns can be addressed, the manuscript would be an important contribution to the field.

RESPONSE: We are grateful to the Referee for their very supportive comments on the impact of our work. As we detail in our responses to the specific comments of the Referee below, we have now made extensive revisions to address both the "cell of origin" and biological replicates issues that the Referee identified.

Abstract/introduction: The way the authors discuss the cell of origin is not optimal. While mouse GMPs can be transformed (Krivtsov 2006), it is likely that the first step leading to all cases of human myeloid leukemia is the acquisition of an oncogenic genetic mutation in an HSC. I don't think a RAS wildtype LSC with GMP characteristics acquiring RAS should be referred to as the LSC cell of origin (e.g., last sentence of the abstract). Please see https://pubmed.ncbi.nlm.nih.gov/16084092/ for the distinction between the cell of origin and the LSC.

RESPONSE: We thank the Referee for raising this important point regarding the distinction between the "leukemia stem cell (LSC)" and the "leukemia cell-of-origin", i.e. the cell that can initiate and maintain leukemia in vivo (LSC) vs the cell in which the transforming event occurs (cell-of-origin). In response to this comment, we made modifications to the manuscript text to more accurately convey this point and performed additional experiments to clarify and advance our findings, specifically leukemogenesis in pre-sorted CB GMPs and CMPs, described in more detail in our response to this Referee's second point below.

More specifically: We removed the phrase "AML… may originate from hematopoietic stem cells (HSCs) or more committed progenitors" from our Introduction and replaced it with "originates from hematopoietic stem/progenitor cells (HSPCs)". To clearly state that the initiating mutation in most if not all cases of AML occurs in an HSC, we added this sentence: "The frequent multilineage presence of preleukemic mutations that drive clonal hematopoiesis suggests that in most human AML cases the initiating mutation is acquired by hematopoietic stem cells (HSCs) or long-lived multipotent progenitors (MPPs)[10,11]." In addition, we performed **additional experiments that establish that not only is the RAS-mutant LSC a phenotypic GMP, but also that the cell that acquires a RAS mutation to give rise to an LSC can be a GMP**. (The new experiments are outlined in detail in our response to this Referee's second point below and presented in new Figure panels 2g-i and Extended Data Fig. 5d-f). These results show that the cell in which a subsequent driver mutation is acquired and gives rise to a subclonal LSC can be a different and more mature cell type than the cell-of-origin of the ancestral AML major clone, i.e. the normal cell that acquired the initial oncogenic mutation (and which is likely an HSC/MPP in most AML cases).

In order to convey with clarity the **distinction between the cell-of-origin** of the ancestral main AML clone (i.e. the normal cell that acquired the first driver mutation) **vs the target cell of the RAS-mutant subclone** (i.e. the cell that acquired the RAS mutation), we made the following additional modifications to the manuscript text:

- We rephrased the two instances in the abstract where the word "cell-of-origin" or "cell type of origin" appeared. Specifically, we changed the last sentence from "by imposing a specific LSC cell-of-origin restriction" to "by imposing a specific LSC target cell restriction" and the phrase "advanced leukemic clones have a different cell type of origin from earlier clones" to "advanced leukemic clones originate from a different cell type than ancestral clones".

- We added the following statement in the first paragraph of Discussion (page 11): "While our studies do not exclude that RAS mutations may also originate in a more primitive HSC/MPP that can give rise to GMP LSCs, they firmly establish that GMPs can be the target cell from which RAS-MT LSCs originate. We thus demonstrate that the subclonal RAS-mutated AML LSC can emerge from a different and more mature cell type than the cell-of-origin of the major AML clone, which in most cases originates from a primitive HSC/MPP (Extended Data Fig. 9f and Extended Data Fig. 10a)."

- We modified panel a of Extended Data Fig. 10 (previous Extended Data Fig. 8) and rephrased the corresponding legend as follows: "RAS mutations acquired by a GMP harboring previously acquired driver mutations can give rise to an LSC. The latter generates leukemic cells with mature monocytic immunophenotype, whereas the major AML clone without RAS mutations gives rise to leukemic cells with more immature features. Thus, the LSC of the RAS-MT subclone originates from a different and more mature type of cell in the hematopoietic hierarchy (a GMP) than the LSC of the major ancestral RAS-WT clone, which most commonly originates from an HSC/MPP/CMP."

- We added the following statement to the Discussion (page 12) referencing the Wang and Dick 2005 Review article that the Referee cites: "It has long been debated whether the phenotype of leukemic blasts is determined by the degree of differentiation of the LSC cell-of-origin or, alternatively, by the transforming event and its effects on the developmental program of the LSCs[59]. Our results propose a new paradigm, whereby the oncogenic event (i.e. RAS mutation) selects for a specific differentiation state of a progenitor cell (i.e. a committed myelomonocytic progenitor) that is the target cell of transformation, with the resulting blast phenotype (i.e. monocytic differentiation) being the result of the interaction between both the target cell type and the mutational event. Furthermore, importantly, we show that this interaction between the genetic and developmental AML hierarchy determines not only the phenotype, but also critical properties of the disease with far-reaching implications for its treatment."

We thank the Referee for highlighting this important matter that prompted us to add critical conceptual clarity to our work and refine our proposed model of transformation by RAS. The additional experiments in support of a GMP being the cell from which RAS-mutant subclones emerge are described in our response to this Referee's second point below.

Furthermore, the data does not establish the authors' bold claim that the cell of origin of RAS-mutated subclones is a GMP (e.g., the section title "RAS-mutant AML LSCs originate from

GMPs" is an overstatement). Indeed, while it is concluded that "NRAS G12D can only transform GMPs that harbor previously acquired cooperating mutations," the results do not exclude that RAS mutations can transform primitive LSCs that harbor previously acquired mutations, especially if mutated RAS induces a GMP phenotype (i.e., CD45RA expression). In this light, it may be interesting to assess the RAS mutation frequency in sorted CMPs and GMPs (Figure 2c), since the alternative model would predict a higher RAS mutation frequency in GMPs. More importantly, the authors' claim could gain support if NRAS G12D expression only transforms sorted GMPs, and fails to transform sorted HSCs and CMPs.
Alternatively, the conclusions could be rephrased to more closely reflect the results that leukemia-initiating capacity is exclusive to phenotypic GMPs. This would still be supportive of the model that RAS mutated LSCs with GMP characteristics and monocytic differentiation bias drive venetoclax resistance.

RESPONSE: We thank the Referee for these insightful comments that prompted us to perform additional experiments as suggested:

- First, we assessed RAS mutation frequency in CMPs and GMPs. These results are shown in new Extended Data Fig. 5c and show that the **frequency of NRAS G12D positive cells is comparable or lower in GMPs, compared to CMPs**.

- Second, we performed two **additional experiments that show that *NRAS G12D* only transforms sorted GMPs and fails to transform sorted CMPs** (also transduced with SA). These data are shown in new panels in Fig. 2g-i and Extended Data Fig. 5d-f and are described in the Results (page 6) as follows:

"To test if *RAS* mutation acquired by a GMP can cause leukemia, we next sorted phenotypic CMPs and GMPs prior to *NRAS G12D* induction (but after SA transduction, SA+R) or prior to simultaneous transduction with all 3 transgenes (SAR) (Fig. 2g-I and Extended Data Fig. 5d-f). In both cases, only SA+R and SAR GMPs, but not CMPs, could initiate leukemia (Fig. 2g-i). These results indicate that not only are RAS-MT GMPs LSCs, i.e. cells able to initiate and maintain leukemia in vivo, but, additionally, that GMPs derived from ancestral AML clones with previously acquired cooperating driver mutations are the target cell of transformation by RAS mutations."

Of note, we were not able to perform transplantation experiments with sorted HSC/MPPs and therefore it is still possible that these can be transformed by SA+R and give rise to LMPP/GMPs with LSC activity. To clearly state this, we added the following to the manuscript text:

- Our conclusion in Results (page 6) was changed from "NRAS G12D can only transform GMPs that harbor previously acquired cooperating mutations" to "GMPs derived from ancestral AML clones with previously acquired cooperating driver mutations are the target cell of transformation by RAS mutations."

- Last paragraph of Introduction (page 3) – "because transformation by mutant RAS is restricted to GMPs with pre-existing oncogenic mutations" was changed to "because mutant RAS transforms GMPs with pre-existing oncogenic mutations".

- First paragraph of Discussion (page 11) – we added the sentence: "While our studies do not exclude that RAS mutations may also originate in a more primitive HSC/MPP that can give rise to GMP LSCs, they firmly establish that GMPs can be the target cell from which RAS-MT LSCs originate."

We hope that the Reviewer would agree that, in view of these additional results and text modifications, the section title “RAS-mutant AML LSCs originate from GMPs” is now justified.

We are sincerely grateful to the Referee for these suggestions, which we believe truly deepened and strengthened our understanding and presentation of our proposed model for the role of RAS mutations in human AML.

Page 3: “how [specific hierarchies] impact clinical outcomes remains obscure” – it is very well-established that stem cell signatures are associated with poor outcomes, at least in the era of chemotherapy.

RESPONSE: We appreciate the Referee’s comment and have rephrased this sentence in the Introduction to acknowledge that stem cell signatures have been associated with poor outcomes to chemotherapy and allogeneic stem cell transplantation and that it is rather the mechanisms by which specific hierarchies are generated and impact clinical outcomes that remain obscure (page 3):

*“*Associations between specific hierarchical organizations and AML genetics as well as drug responses and disease relapse have been described[16] and stem cell signatures have been associated with poor outcomes after chemotherapy and allogeneic HSC transplantation[17]. However, how these hierarchies are determined and the mechanisms by which they impact clinical outcomes remain obscure.”

Oncogene expression in human iPSC-derived HSCs followed by mouse injection is not a well-established leukemia model. The engraftment remains below 10%, and it appears the mice don’t die (Figure S1i, Figure 1b). Additional support that the mice develop myeloid leukemias would strengthen the manuscript, such as phenotypic and morphological analysis. Even then, conclusions on page 4 (“generate leukemia,” “induce leukemia”) should be phrased within the limitations of the models.

RESPONSE: We appreciate the Referee’s comment and have accordingly included morphological analysis (Extended Data. Fig. 1j) showing human leukemic blasts in the bone marrow of mice transplanted with iPSC-derived SAR cells and rephrased the descriptions and conclusions on page 4 with these limitations of the iPSC models in mind. Specifically, we have replaced all instances of statements, such as “generate leukemia” and “induce leukemia” referring to the gene edited iPSC-derived cells, with more appropriate descriptions, such as “give rise to transplantable myeloid cells”, “induce leukemic features”, “generate engraftable cells”, “promote leukemogenesis”.

We agree with the Reviewer that transplantation of genetically engineered iPSC-derived HSPCs is a less well-established leukemia model, compared to that of patient-derived AML-iPSCs that we previously reported (Kotini et al. CSC 2017; Wesely et al, Cell Reports 2020; Kotini et al. BCD 2023) and present in Fig. 3a, and of genetically engineered primary HSPCs, like the ones we present in this manuscript. We have however found them useful in the initial experiments presented in Extended Data Fig. 1 to define the minimal mutation requirements for leukemic potential, taking advantage of two characteristics that distinguish these experiments from those using genetically engineered primary HSPCs: (a) the ability to assess clonal gene edited populations without contaminating unedited WT cells; (b) the fact that baseline engraftment of

normal iPSC-HSPCs is zero and thus any level of detectable engraftment can serve as readout of leukemic potential. This is in contrast to the transplantation of genetically engineered primary (CB-derived) HSPCs that is evaluated against the backdrop of normal engraftment of unmodified HSPCs that are invariably also present at varying proportions in the transplanted cell pool. This complicates evaluation of leukemic vs normal human hematopoiesis in the xenografts, especially in the absence of clinical signs and symptoms of illness. (Transplantation of genetically engineered primary HSPCs has of course a number of other important advantages, such as high-level engraftment and more reliable assessment of immunophenotypic HSPC types, among others, as we demonstrate here as well.)

Page 5-6: The bias of RAS mutated clones towards monocytic differentiation appears to be minor for both the PDX and the GoT analyses. Can the authors use a larger patient database with bulk sequencing and phenotyping to show that (high VAF) RAS mutations are associated with increased monocytic differentiation?

RESPONSE: We acknowledge that the previous presentation of these results on the bias of RAS-mutant clones towards monocytic differentiation was suboptimal and did not adequately convey the magnitude of the differences in hierarchical organization between the RAS mutant and WT clones. We now show **additional analyses of the GoT data** to better highlight these differences, specifically a new panel in Fig. 3i showing that *NRAS* MT cells have significantly higher expression of a monocytic priming gene module score from *Velten et al. NCB 2017*.

Additionally, as the Referee suggested, we now **analyzed a larger patient database** with mutational and phenotypic (bulk RNA-Seq, as well as FAB classification) information **available (**Alliance cohort), consisting of 599 AML patients, of which 94 have *NRAS* mutations, 16 have *KRAS* mutations and 47 have *PTPN11* mutations (3 patients have co-occurring *NRAS* and *KRAS* mutations and 6 have co-occurring *NRAS* and *PTPN11* mutations). These data, presented in new Fig. 3j,k, provide **strong evidence for the association between RAS mutations and monocytic differentiation** (specifically, with fraction of CD14+ monocytes and M4/M5 FAB types). (Cell type fractions were determined using cell type-specific gene expression profiles derived from a single-cell RNA-seq AML dataset.) These findings are described in the Results section (page 7) as follows:

"In a cohort of 599 AML patients with bulk RNA-Seq, genotyping and FAB classification information available[36], patients with *NRAS* mutations or any RAS pathway mutation (in *NRAS*, *KRAS* or *PTPN11* genes) had significantly higher fraction of CD14+ monocytic blasts and higher frequency of AML with myelomonocytic (FAB M4) or monoblastic/monocytic (FAB M5) morphology, compared to those without RAS mutations (Fig. 3j,k). These results corroborate at the AML patient population level the association between RAS mutations and monocytic differentiation uncovered in our intra-patient investigations (AML-iPSC-xenografts and GoT, Fig. 3a-i)."

Finally, we also provide new scRNA-Seq datasets of CB HSPCs and analyses (data described in more detail in our responses to Referee #2's comments below), showing that RAS-MT GMPs are biased towards the monocytic lineage at the expense of the granulocytic lineage (Fig. 5c,f, Extended Data Fig. 6f and Extended Data Fig. 9c,f).

Many experiments in Figures 3 and 4 rely on two isogenic lines derived from a single patient and single-cell RNA-sequencing + GoT analysis of one other patient. Adding additional biological replicates would strengthen the manuscript.

RESPONSE: We now performed additional experiments in **3 additional isogenic pairs of RAS-mutant vs RAS-WT LSCs from AML patient-derived iPSC lines** (in addition to AML-4.24 already presented earlier) of various AML genetic groups, specifically: MLLr (AML.9.9); Core Binding Factor (AML-37.16); and splicing factor-mutated (AML-47.1). These data are included in new Fig. 5h,i and show that **RAS mutations confer VEN resistance** (Fig. 5h), **increase MCL1 and BCL-xL and decrease BCL2** (Fig. 5i) and that a **RAS multi inhibitor reverses these changes** (Fig. 5h,i).

Figure 4a-e: While the trial included 118 patients, only the survival curve shown in panel C adds up to 118. I also don't understand the rationale of subsetting this analysis to the monocytic cohort. Having only six RAS mutated patients calls into question the robustness of the conclusions.

RESPONSE: We agree with this criticism and have now revised and updated these analyses based on the Referee's suggestions. Specifically, we repeated the analyses comparing outcomes in AML patients with vs without RAS mutations **without subsetting to monocytic disease** and have now included **additional patients with significantly longer follow up**. The updated dataset with data cut off of May 1, 2023 includes more patients since the analysis reported for the monocytic analysis in the original submission and with significantly longer median follow up.

Our analyses presented in the original version of the manuscript included 118 patients with newly diagnosed AML enrolled at the time of that earlier data cut off. The overall survival (OS) analysis presented in panel 4c includes all those 118 patients. Panel 4b shows duration of response (DOR) in the subset of patients from panel 4c who achieved CR/CRi response and excluded patients who either achieved MLFS response or were refractory, following ELN2017 recommendations (Dohner et al. Blood 2017) and initial phase 1b trial of VEN with HMA (NCT02203773) (DiNardo et al. Lancet Oncol 2018).

We now present an updated analysis in Fig. 4d,e, which includes a larger cohort and an updated data set with significantly longer follow up. In addition, in response to Referee #3 comment #4, we also repeated these analyses after excluding *TP53*-mutated cases, as these are known to have poor prognosis. These revised analyses are presented in new Fig. 4d,e (*TP53*-WT only patients), Extended Data Fig. 7a,b (all patients, including *TP53*-mutated) and Supplementary Tables 4,5. They include 31 (26 *TP53*-WT) patients with *N/KRAS* mutations, of which 18 (17 *TP53*-WT) could also be assessed for DOR, based on the criteria mentioned above, and show **significantly worse DOR** (regardless of exclusion of *TP53*-mutated cases) and **significantly shorter OS** in *TP53*-WT patients with *N/KRAS* mutations, compared to those without *N/KRAS* mutations. Specifically, a RAS mutation significantly increased the risk of relapse with HR 5.32, 95% CI 1.81, 15.68, p<.001 and significantly increased the risk of death with HR 2.42, 95% CI 1.28, 4.60, p<.001. (Of note, the cohort used for the comparison of outcomes between monocytic and non-monocytic disease was not updated – hence panels Fig. 4b,c remain unchanged – because no information on monocytic differentiation status is available for the new patients enrolled since data cut off of the prior analysis. This is because monocytic differentiation is not routinely reported, but needs to be manually adjudicated for each patient by a pathologist after detailed review of flow cytometric markers.)
In addition, we would also like to point out that these data on patient outcomes (monocytic vs non-monocytic, as well as RAS-mutated vs RAS-WT) are very much in agreement with results of the larger VIALE-A study, reported at the most recent EHA 2023 meeting

(https://journals.lww.com/hemasphere/fulltext/2023/08003/p521__findings_from_an_analysis_of_patients_with.422.aspx) and included in a manuscript in preparation (Konopleva et al.).

These updates **firmly establish that RAS mutations, but not monocytic differentiation, have a strong negative impact on AML patient outcomes on VEN combination therapy**.

Figure 4h-j: The data presentation in panel h is not ideal. For example, in h, y-axis ranges differ between panels for the same gene. It might be more clear to show a heatmap. More importantly, all panels are missing P-values, making it difficult to interpret the data.

RESPONSE: We have revised these panels with range of values in the y axis more comparable across genes (with the exception of MCL1 in monocytes, which is very high and out of range in comparison to the others) and added p values. We modified the wording in the Results section accordingly. We have opted to show violin plots, instead of a heatmap, in panels showing single-cell data, i.e. 4h and 4i, as the former also contain information on the distribution of the expression across all cells. We changed the presentation of the bulk RNA-Seq iPSC-HSPC expression data in Fig. 4j to barplots so that we can add p values for all comparisons.
We would like to caution that single-cell RNA-Seq data comparing small cell populations (as in our subclustered LSCs and GoT analyses) can be underpowered and not reach statistical significance, due to “dropout” (whereby genes expressed at mid- and low levels are randomly not detected, resulting in false-zero values). Because of this limitation, we complement these studies with models and other orthogonal complementary measurements of levels of BCL2 family genes and proteins throughout the manuscript. Importantly, as mentioned in our response to an earlier comment of this Referee, we have now performed additional experiments with more and genetically diverse AML-iPSC-derived LSCs in vitro, with or without mutant RAS, also using an active state-selective RAS multi inhibitor (RASi), and show at the protein level (by Western blots) that both *NRAS* G12D and *KRAS* G12D mutations decrease BCL2 and increase MCL1 and BCL-xL (new Fig. 5h,i) and that these effects are reversed by the RASi.

Extended Data Figure 7a is vastly overclustered. Does the analysis hold up when reducing the resolution to analyze larger clusters?

RESPONSE: We have included this analysis in reduced resolution in a new panel in Extended Data Fig. 8a. The trends overall hold and BAX now reaches statistical significance. The differences in BCL2 expression are diminished. We believe that, given that BCL2 expression is restricted to primitive LSCs, as others have also shown (*Waclawiczek et al*. PMID 36892565), subclustering to identify the more primitive LSC cluster is useful and justified for these analyses. Importantly, now we include additional data, described in our response above, that more firmly establish that RAS mutations decrease BCL2 and increase MCL1 and BCL-xL in AML LSCs of diverse genetic groups (new Fig. 5h,i).

Minor comments:

Page 3: “sizeable fraction” – can this be specified?

RESPONSE: We added the following to specify this: “20-30% of patients are refractory to VEN-based combination regimens and more than 40% of those responding ultimately relapse[19,21-23].”

Page 4: Fig. 1h,i should be Extended Data Fig. 1h,i

RESPONSE: We thank the Referee for pointing out this mistake, which has been corrected.

Page 4: "R+SA or R+SA" should be "R+SA or SA+R"

RESPONSE: We thank the Referee for pointing out this mistake, which has been corrected.

Figure 2c-d: Information regarding the number of biological replicates is missing.

RESPONSE: The experiment presented in the previous Fig. 2c,d has now been moved to Extended Data Fig. 5a,b and replaced with new extended experiments with more replicates in pre-sorted CMPs and GMPs (Fig. 2g-i).

Figure 3, Panel B: The shades of blue are hard to distinguish. It would help to add some (broad) labels to the UMAP. Panel C: it may be more informative to have one UMAP colored by the difference in S phase between the PDX models, and another by the difference in local density of cells, thereby conveying increased monocytic differentiation in the RAS model. Also, please consistently show RAS WT left and RAS mutant right (e.g., panel D).

RESPONSE: We thank the Referee for this suggestion, which we agree improves the visualization of these data. We have added broad labels directly on the UMAP in panel Fig. 3b and replaced panels 3c with updated ones based on the Referee's suggestions. Specifically, we present two density UMAP plots, one colored by sample (AML-4.24 in green and AML-4.10 in red gradient) and one by phase of the cell cycle.

We have changed all panels to consistently show RAS WT on the left and RAS MT on the right throughout all figures of the manuscript.

Page 6 or Methods: Please specify the efficiency of GoT (i.e., the proportion of cells in which a mutation was captured).

RESPONSE: The NRAS genotyping efficiency in the GoT experiment was 8.3%, with 576 cells genotyped as mutant and 423 as WT. This information is now included in the Methods ("*The genotyping efficiency was 8.3% with 576 cells genotyped as NRAS MT and 423 as NRAS WT.*") and in the legend of the corresponding figure (Fig 3f: "*423 cells could be genotyped as NRAS WT and 576 as NRAS MT.*").

Referee #2:

The authors have used CRISPR/Cas9-mediated gene editing in human iPSCs and primary CD34+ HSPCs to explore the role of N/KRAS mutations in AML. They identify that transformation by mutant RAS is restricted to GMPs and that this only occurs when cells carry pre-existing oncogenic mutations, such as C-terminal truncated ASXL1 and SRSF2 P95L, but not either alone. Multiomic approaches were used to show that RAS transformed LSCs

preferentially generate monocytic leukemias that are VEN resistant and that resistance is specific to RASmutant LSCs, rather than monocytic lineage. The authors propose a new paradigm whereby an oncogene can impact therapeutic outcome by targeting a specific cell subtype for transformation into an LSC with altered sensitivity to targeted therapies. In addition, the authors conclude that RAS mutations alone are not sufficient for leukemogenesis.

The authors generated iPSCs with DNMT3A, FLT3 and NPM1 and yet this did not form AML. Why was this the case? This combination is known to cause AML in mouse models and is a relevant combination in humans. Does this suggest some limitation of the diversity with which iPSC HSCs may be genetically transformed?

RESPONSE: We thank the Reviewer for raising this important point that prompted us to re-evaluate these data, presented in Extended Data Fig. 2c and e. The Reviewer is correct that the DNMT3A, FLT3 and NPM1 mutation combination is known to cause AML in mice and humans. One potential caveat with the DNMT3A^{R882H}-FLT3^{ITD}-NRAS^{G12D}-NPM1c (DFRN) cells is that the NPM1c mutation – which is known to be required for AML – was introduced with a lentiviral vector (as was depicted in the schematic in the previous Extended Data Fig. 2c). We resorted to this solution because of technical problems with gene editing of the NPM1 locus that did not allow us, after multiple attempts, to successfully introduce the NPM1c mutation in the endogenous locus through CRISPR gene editing, as we did for all other mutations presented in theses series of experiments in the iPSCs. Furthermore, because lentiviral vectors profoundly silence upon differentiation from iPSCs to HSPCs, transduction with the NPM1c lentiviral vector was performed in the HSPC stage. These two experimental modifications set apart this group from the others in these series and both the lentiviral ectopic expression, as well as the delayed expression after differentiation, may well confound the results. We think that this technical issue may confound interpretation of the lack of engraftment ability of this specific group. Therefore, while it is possible that this is an indication of some limitation of the iPSC-HSPC modeling system, this cannot be concluded from these data, given the technical caveats. We therefore decided, out of caution, to remove this specific line from this dataset. This removal does not impact in any way the remaining data in this figure panel or their interpretation nor the broader conclusions drawn from this Figure. The DNMT3A^{R882H}-FLT3^{ITD}-NRAS^{G12D} (DFR) group remains (since the above technical issues don't apply there). That the DFR line has no engraftment potential is not surprising from what we know from mutational combinations in human AML.

The group have previously engineered iPSCs with the variants described in this paper in their Cell Stem Cell paper in 2021 (DOI: 10.1016/j.stem.2021.01.011). In their current work, they have used this system to show that NRAS mutation increases cell proliferation and cell cycling without impact on differentiation. In contrast, SRSF2 and ASXL1 mutations impair differentiation without affecting proliferation. These results are consistent with our prior knowledge of how these variants function.

The authors identify that only the triple mutant (SAR) state resulted in transplantable AML but not other gene combinations. They also confirm the leukemogenic relevance of the SAR combination in primary human HSPCs via lentiviral transduction of SRSF2 P95L, ASXL1 del1900-1922 and NRAS G12D into cord blood CD34+ cells. These are impressive experiments and confirm their observations in iPSC derived HSPCs.

The authors identify that engraftment of transplanted HSPCs derived from iPSC lines only occurred for triple mutant cells, not other variations. It would be helpful to elucidate the biological reasons for this. For example, what are self-renewal programs re-wired by the

presence of 3 variants not re-capitulated by the presence of just 2 and what does RAS mut add to the biology of the cell that allows this to happen? Are these alterations a result of synergistic epigenetic programs or just the additive impact of each gene variant individually? It would also be instructive, for example, to know the fate of the triple mutant cells after engraftment if the RAS mut was reverted back to a wild-type state to understand whether the properties of transformation are reversible or fixed.

RESPONSE:
We have described the transcriptional and chromatin accessibility changes in SAR, compared to SA, iPSC-HSPCs previously in *Wang et al. Cell Stem Cell, 2021*. Briefly, these included upregulation combined with increased accessibility of genes related to ribosome biogenesis, Rho GTPase signaling, interleukin signaling, innate immune signaling and RUNX1 targets and downregulation/decreased accessibility of genes related to adhesion, MHC class II antigens and histone methylation.

In the present manuscript, we have now significantly expanded our genomics analyses to include:

1. Bulk RNA-Seq and ATAC-Seq of SA+R iPSC-HSPCs (compared to SA+Ctrl and R+SA)
2. scRNA-Seq of CB-derived HSPCs with various S,A,R permutations
3. Bulk and sc RNA-Seq of CB-derived SA+R sorted CMPs and GMPs

These data are described in new Figure panels: Fig. 2 panels a-o, Fig. 5 panels a-g, Extended Data Fig. 4 panels e,f, Extended Data Fig. 6 panels a-c and f, and Extended Data Fig. 9 panels a-d.

We also present additional functional and signaling analyses presented in Fig. 2 panels j, Fig. 5 panels h,i, and Extended Data Fig. 9 panel e.

In summary these additional data and analyses show that:

- RAS as an early mutation (alone or with SA) causes **a block in formation of GMPs**, identified both immunophenotypically and transcriptionally (Fig. 2a-f and Extended Data Fig. 4e,f).

- RAS mutations **can transform pre-sorted GMPs but not CMPs** (Fig. 2g-l and Extended Data Fig. 5c-e).

- RAS mutations **activate ERK at comparable or lower levels** in CB sorted GMPs, than in CMPs or HSC/MPPs transduced with SA+R (Fig. 2j), but **upregulate non-overlapping genes** in GMPs vs in CMPs (Fig. 2m and Extended Data Fig. 6a). The genes upregulated by mutant RAS selectively in GMPs are more accessible in GMPs compared to CMPs, suggestive of a cell lineage effect whereby this set of genes are **"primed" at the chromatin level in GMPs** (Fig. 2n,o).

- RAS mutation as late mutation in GMPs results in upregulation of pathways, such as inflammatory pathways, NFkB, IFN alpha and gamma, mTORC1 signaling, hypoxia, glycolysis (Fig. 2k,l and Extended Data Fig. 9c,d). Because these changes, identified by bulk RNA-Seq and bulk ATAC-Seq in genetically engineered iPSC-derived HSPCs (Fig. 2k) and scRNA-Seq in genetically engineered CB-derived HSPCs (Fig. 2l), likely reflect

the sum of differences caused by RAS mutation vs no RAS mutation, as well as by GMP vs CMP state, or, in other words, the composite effect of RAS mutational status and cell type – which are intertwined in these analyses since RAS-Early blocks GMP formation –, in an attempt to better separate these signals, we generated a third scRNA-Seq dataset from sorted SA+R CB-derived CMPs and GMPs and used the reads of the reporter genes linked to the S,A,R transgenes (mCherry, GFP and ΔLNGFR) as a means to call mutant cells vs cells WT for all mutations (Fig. 5c,f and Extended Data Fig. 9b). These analyses show that RAS mutation, specifically in GMPs, downregulates a number of ribosomal protein genes at the transcriptome level and upregulates pathways such as MTORC1 signaling and others, suggestive of changes in protein synthesis and metabolism (Extended Data Fig. 9c,d).

- The same genomics analyses, together with functional experiments (VEN treatment) and Western blot detection of BCL2 family proteins, show that the **GMP state per se does not confer VEN resistance** and GMPs express comparable levels of BCL2 family genes, as CMPs (Fig. 5d,e). In contrast, RAS mutation upregulates MCL1 in GMPs (Fig. 5g). In addition, LSCs from additional AML-iPSC lines of various AML genotypes with ectopic expression of either NRAS G12D or KRAS G12D become VEN resistant, with increased MCL1 and BCL-xL and concomitant decrease of BCL2 (Fig. 5i). In addition, treatment with an **active state-selective RAS multi inhibitor** (RASi) reverses these changes, and restores both sensitivity to VEN and expression of MCL1 and BCL-xL to levels comparable in those of RAS-WT cells (Fig. 5h,i).

- The genomics analyses outlined above also provide support that **RAS mutations cause a monocytic bias in GMPs**. Specifically, RAS-mutant, compared to WT, GMPs upregulate monocytic lineage genes and reciprocally downregulate granulocytic genes (such as MPO, AZU1, ELANE) (Extended Data Fig. 6f, Extended Data Fig. 9c). This is not the case with SA alone (Extended Data Fig. 6f), neither do we find an association of the S or A mutations with monocytic disease, as we do for RAS pathway mutations, by examining a large AML patient dataset (Fig. 3j,k and m,n).

These additional data and analyses significantly extend our studies of the biological effects of RAS mutations and their mechanistic underpinnings with regards to leukemic transformation, monocytic bias and VEN resistance, as summarized above and further described and elaborated in our responses to this Referee's subsequent comments.

Although the authors highlight interestingly that other iPSC lines with NRAS G12D combined with DNMT3A, FLT3 and NPM1 or RUNX1 and ASXL1 did not lead to robust engraftment, is this a consequence of the system used or do the authors propose that this is biological finding relevant to human AML in general?

RESPONSE: We thank the Reviewer for raising this important issue that prompted us to re-evaluate these data. As stated in our response to this Reviewer's first comment, because of technical differences in the engineering of the DNMT3A^{R882H}-FLT3^{ITD}-NRAS^{G12D}-NPM1c (DFRN) cells that might confound interpretation (as they may account for the negative engraftment result), we decided to remove this group from this dataset.

Regarding the two other mutational combinations, DNMT3A^{R882H}-FLT3^{ITD}-NRAS^{G12D} (DFR) and RUNX1-ASXL1-NRAS^{G12D} (RAR), these are not common co-mutations in human AML datasets,

in contrast to the SAR. (We selected to engineer the RAR combination before comprehensive analyses of population genetics data on co-mutations in RUNX1-FPD patients progressing to AML – such as: Homan et al. Blood Adv 2023, PMID: 37406166 – became available. These human data no longer support the RAR combination as very relevant to human AML.)

These data thus support that our findings with the different edited iPSC lines are biologically relevant to human AML. However, this is still a small set of combinations and certainly these data by no means exclude that the iPSC-HSPC system may have limitations in AML modeling, but we feel that at this stage, these limited data do not support such a conclusion either.

What is the biological rationale for the RAS+ SA combination being unique and what specific pathways are invoked that are unique to SAR and not other genetic combinations?

The authors nicely show that SA+R cells generate a lethal myeloid leukemia, whereas the R+SA group showed no engraftment. Are the cellular programs modified by SAR and SA+R the same and how does this differ from R+SA to produce different biological outcomes? Could dissection of the single cell studies be pertinent here.?

RESPONSE: We thank the Reviewer for this very important question. We now performed extensive additional bulk and single-cell RNA-Seq and ATAC-Seq analyses in both genetically engineered iPSC-HSPCs and CB CD34+ cells and the results are summarized in our response to this Referee's earlier comment.

The authors show that the HSPC populations in transduced cord blood cells result in skewed progenitor populations. R+SA and SAR cells showed reduction of GMPs with increase of CMPs, suggesting some degree of differentiation block with sorted engraftment showing that only the GMP cells gave rise to a lethal leukemia. The authors used bulk RNA-Seq and ATAC-Seq analyses in SA+R vs R+SA iPSC-HSPCs to show that NRAS G12D can only transform GMPs harboring SA variants. What signalling pathways explain this biological effect? Or are the outcomes mainly lineage dependent?

RESPONSE: We thank the Referee for these insightful comments, which prompted us to expand our signaling, genomics and functional analyses, as outlined in our earlier response. In brief, extensive additional data we provide argue that it is **rather the different chromatin milieu of GMPs, than differences in signaling activation**, that determine the ability of RAS mutations to transform GMPs but not CMPs. This is based on the following data: ERK/MAPK pathway activation by SA+R is comparable or lower in GMPs vs CMPs (new Fig. 2j), but the transcriptional changes impacted by RAS mutation in GMPs (R-Late) are distinct and not overlapping with those in CMPs (R-Early) (Fig. 2m and Extended Data Fig. 6a) and the genes upregulated by mutant RAS selectively in GMPs, are more accessible in GMPs compared to CMPs at the chromatin level (new Fig. 2n,o). These results suggest that a **permissive cellular and chromatin milieu in GMPs may underlie their transformation by RAS**.

The relevant Results section (page 6) describing these new data is pasted below:

"To understand the signaling and genomic underpinnings of the selective transformation of GMPs but not CMPs by mutant RAS, we first asked whether RAS mutations differentially activate the MAPK pathway in different stem/progenitor cell stages along the hematopoietic hierarchy. Mutant RAS activated ERK at comparable or lower levels in CB sorted GMPs, than in

CMPs or HSC/MPPs transduced with SA+R and expressing comparable levels of all transgenes (Fig. 2j, Extended Data Fig. 5g). To next interrogate the transcriptional and genomic consequences of RAS mutation, we defined “RAS-late genes” as the genes upregulated in iPSC-HSPCs selectively in SA+R and not in any other condition (Extended Data Fig. 6a, Supplementary Table 1). In addition, we defined “RAS-late peaks” as the peaks more accessible selectively in SA+R cells (Extended Data Fig. 6b, Supplementary Table 2). The latter were enriched for motifs of transcription factors (TFs) classically associated with MAPK signaling, such as AP1, CREB and ETS family TFs (Extended Data Fig. 6c). “RAS-late genes” were enriched for pathways primarily related to inflammatory responses (Fig. 2k). Similar pathways were enriched in genes differentially expressed between SA+R and Ctrl CB cells in the GMP cluster identified by scRNA-Seq (Fig 2l).

We then asked if RAS mutation activates shared or distinct genes as an early or late mutation. Among genes classically associated with RAS signaling activation, distinct sets of genes were upregulated by RAS as early vs late mutation, with almost no overlap with each other (Fig. 2m). 65 genes were activated selectively by late but not by early RAS (Fig. 2m, cluster 4, Supplementary Table 3). Interestingly, these 65 genes were more accessible in the SA+Ctrl and SA+R groups – which, as shown earlier, represent GMPs (Fig 2f, Extended Data Fig. 4f) – and less accessible in the R+Ctrl and R+SA groups that resemble CMPs (Fig. 2n,o). These results taken together support a scenario whereby RAS leads to comparable ERK signaling activation in GMPs as in more primitive HSPCs, but with different transcriptional output, resulting in upregulation of a distinct set of genes that are more accessible (or “primed”) at the chromatin level in the GMP state.”

The authors suggest that RAS-mutated AML LSCs drive a monocytic phenotype. What is the mechanism for this? RAS mutation is not a lineage defining property of monocytic lineage cells. Do other kinase activating variants cause the same outcome? Is it perhaps the founding variants ASXL1 and SRSF2 which ultimately determine the likelihood of monocytic transformation, rather than RAS mut itself?

RESPONSE: We thank the Referee for raising these important questions. We now provide extensive additional data to address those points.

- *What is the mechanism for this (RAS mutation driving monocytic phenotype)?*

We provide new data showing that RAS-mutant GMPs have upregulated monocytic lineage genes and downregulated granulocytic genes (Extended Data Fig. 6f and Extended Data Fig. 9c). This is consistent with RAS mutation driving monocytic differentiation of GMPs at the expense of granulocytic differentiation. While the mechanistic underpinnings of this need further investigations, we show upregulation of inflammatory pathways, NFkB and TNFa signaling, and others that could account for this skewing (Fig. 2k,l, Extended Data Fig. 9d).

- *Do other kinase activating variants cause the same outcome?*

Our expanded analyses in a large patient dataset (Alliance cohort) with genotyping, morphology and bulk gene expression data (shown in new Fig. 3j,k), show that all RAS pathway mutations (*NRAS*, *KRAS* and *PTPN11*) are associated with monocytic AML.

On the other hand, current evidence strongly suggests that FLT3-ITD mutations do not cause monocytic differentiation of leukemic blasts. On the contrary, FLT3-ITD is associated with more primitive AML phenotype. We have shown this in our recent *Kotini et al. BCD, 2023* paper by comparing xenografts from a pair of isogenic AML-iPSC lines with and without FLT3-ITD using scRNA-Seq (https://aacrjournals.org/view-large/figure/15259321/318fig7.jpeg).
Association between FLT3-ITD and primitive AML phenotype is also reported in *van Galen et al. Cell, 2019* (shown in Fig. 5E: https://www.cell.com/cms/attachment/2fd99f16-4f8f-4b97-ae4c-2e09539572fc/gr5.jpg) and in *Zeng et al. Nat Med, 2022* (in Fig. 2e: https://www.nature.com/articles/s41591-022-01819-x/figures/2).
We thank the Referee for the opportunity to comment on this. We have added the following comment in our Discussion section (page 12): "Consistent with our proposition that VEN resistance and monocytic differentiation are independent processes, FLT3-ITD mutations are not associated with monocytic differentiation, and, on the contrary, appear to give rise to more primitive leukemic blasts[16,32,57]."

In contrast, interestingly, as shown in the same Fig. 5E of the *van Galen et al. Cell* paper, FLT3-TKD may be associated with monocytic state, but the data are currently limited.

- *Is it perhaps the founding variants ASXL1 and SRSF2?*

This is an excellent question. New data that we present in the revised manuscript argue against a role for *ASXL1 or SRSF2* mutations in driving monocytic differentiation.

First, by examining the same patient cohort as above (Alliance cohort), we do not find any association between *ASXL1* mutations, *SRSF2* mutations or their combination and monocytic features. Specifically, these data, presented in new panels Fig. 3m,n, show that the founding mutations show no association with either percentage of CD14+ monocytic blasts or AML with myelomonocytic (FAB M4) or monoblastic/monocytic (FAB M5) morphology. (In contrast, we show in Fig. 3j,k that RAS pathway mutations are associated with both a higher fraction of CD14+ monocytic blasts and higher frequency of FAB M4 and M5 AML).

Second, we find that, in contrast to RAS mutation, SA mutations alone (without R) do not result in upregulation of monocytic lineage genes in CB GMPs (new Extended Data Fig. 6f), again arguing against an effect of the S or A mutation in instructing monocytic fate.

These are all great points and we again thank the Referee for these suggestions.

If RASm was transduced onto a different doublet variant background founded by eg IDH, NPM1 or a CBF rearrangement, does a monocytic leukemia result? Why did this not occur in the DNMT3A model shown in the extended data?

RESPONSE: The GoT experiment was performed with cells from a patient with DNMT3A and IDH1 mutations and shows increased monocytic differentiation in the RAS-mutated subclone (Fig. 3h,i). We also now include analysis of a large patient cohort with bulk RNA-Seq and phenotyping, as mentioned above, showing strong association between RAS pathway mutations and monocytic disease, presented in new Fig. 3j,k.

These data show that RAS mutations cause monocytic leukemia in a wide range of AML genotypes.

Combined presence of ASXL1 and SRSF2 variants are the commonest feature of CMML and so it would be useful to show that human CMML is frequently driven by co-presence of RAS mutation to strengthen the finding. The authors refer to some literature but a more detailed discussion of the frequency of this association would be useful.

RESPONSE: We have now included a new panel in Fig. 3l, showing that, indeed, as the Referee suggests, *RAS* mutations frequently co-exist with mutations of *ASXL1* and *SRSF2* in human CMML. For this we analyzed all 399 patients with CMML (classified based on WHO 2016 guidelines) from the IWG cohort of 3328 MDS patients from Bernard et al. 2022 (NEJM Evidence).

The authors suggest that outcomes of monocytic and nonmonocytic AML were comparable for patients receiving VEN-DEC, suggesting that monocytic lineage alone did not determine VEN-based resistance. Although the authors show that N/KRAS mutant cases within the monocytic AML cohort had significantly shorter DOR (3.4 months vs not reached in patients with WT N/KRAS, p<0.001), there was no difference in OS. Furthermore, the relative numbers of patients within these subgroups is small. A further validation using an independent dataset would strengthen this observation. Although 2 out of 6 (33%) patients with N/KRAS mutations achieved CR, compared to 60% (n=15/25) of those without N/KRAS mutations, a 6 patient observation is not sufficient to be sure that response associations are not just a chance observation.

RESPONSE: We agree with this criticism and have now revised and updated these analyses to include **additional patients with longer follow up** from the DEC10-VEN trial of MD Anderson Cancer Center. In addition, in response to Referee #1's comment, we repeated the analyses comparing outcomes in AML patients with vs without RAS mutations without subsetting to monocytic disease and, in response to Referee #3's comment, we also repeated these analyses after excluding *TP53*-mutated cases, as these are known to have poor prognosis.

These revised analyses are presented in new Fig. 4d,e (*TP53*-WT only patients), Extended Data Fig. 7a,b (all patients, including *TP53*-mutated) and Supplementary Tables 4,5. They include 31 (26 *TP53*-WT) patients with *N/KRAS* mutations, of which 18 (17 *TP53*-WT) could also be assessed for duration of response (DOR), and show **significantly worse DOR** (regardless of exclusion of *TP53*-mutated cases) and **significantly shorter overall survival** (OS) in *TP53*-WT patients with *N/KRAS* mutations, compared to those without *N/KRAS* mutations. Specifically, a RAS mutation significantly increased the risk of relapse with HR 5.32, 95% CI 1.81, 15.68, p<.001 and significantly increased the risk of death with HR 2.42, 95% CI 1.28, 4.60, p<.001. (Of note, the cohort used for the comparison of outcomes between monocytic and non-monocytic disease was not updated – hence panels Fig. 4b,c remain unchanged – because no information on monocytic differentiation status is available for the new patients enrolled since data cut off of the prior analysis. This is because monocytic differentiation is not routinely reported, but needs to be manually adjudicated for each patient by a pathologist after detailed review of flow cytometric markers.)

In addition, we would also like to point out that these data on patient outcomes (monocytic vs non-monocytic, as well as RAS-mutated vs RAS-WT) are very much in **agreement with results of the larger VIALE-A study,** reported at the most recent EHA 2023 meeting (https://journals.lww.com/hemasphere/fulltext/2023/08003/p521__findings_from_an_analysis_of_patients_with.422.aspx) and included in a manuscript in preparation (Konopleva et al.).

These updates firmly establish that RAS mutations, but not monocytic differentiation, have a strong negative impact on AML patient outcomes on VEN combination therapy.

The authors show that monocytes from HSPCs with KRAS MT line were VEN-resistant with low expression of BCL2 and high expression of MCL1. Of interest, LSCs of the RAS MT clone had reduced BCL2 expression and higher MCL1 and BCL2L1 expression and lower expression of pro apoptotic BAX. This is an interesting observation and consistent with the author's recent studies suggesting that RAS mutation drives altered expression of pro-survival MCL1 (doi: 10.1038/s41392-021-00870-3). There is perhaps an opportunity to explore using the engineered cell lines to better understand whether the effect of KRAS variants on pro-survival expression occurs promiscuously in the other genetic settings, or only in SA cells.
This would more sharply focus the effect of KRAS mut in relation to the explained effects on AML transformation vs venetoclax resistance, which are likely to engage different signalling pathways and be non-overlapping.
Are the effects resulting from KRAS and NRAS the same or are there isoform specific differences in the impact of R/NRAS on survival signalling?

RESPONSE: We thank the Referee for raising these important points, which we are now addressing with additional data, as follows:

- *There is perhaps an opportunity to explore using the engineered cell lines to better understand whether the effect of KRAS variants on pro-survival expression occurs promiscuously in the other genetic settings, or only in SA cells.*

The data on pro-apoptotic and pro-survival gene expression shown in Fig. 4h (iPSC-derived xenografts) and Fig. 4i (GoT) are from a patient with t(1;7;14) with del7q (AML-4, Fig. 4h) and from a patient with DNMT3A and IDH1 (Fig. 4i), respectively. (This information is shown in schematics in Figs 3a and 3e, respectively.) The data on pro-apoptotic and pro-survival gene expression in the SA+R cells are shown in Fig. 4j.

In addition, we now present data on **additional iPSC lines derived from patients of various AML genetic groups**, including MLLr (AML-9.9), Core Binding Factor (AML-37.16), and splicing factor-mutated (AML-47.1). In paired isogenic iPSC-LSCs with *N/KRAS* G12D or without RAS mutations, we show that RAS mutation confers VEN resistance, with downregulation of BCL2 and upregulation of MCL1 and BCL-xL (new Fig. 5h,I and Extended Data Fig. 9e). In addition, treatment with an **active state-selective RAS multi inhibitor** (RASi) reverses these changes, and restores both sensitivity to VEN and expression of MCL1 and BCL-xL to levels comparable in those of RAS-WT cells (Fig. 5h,i).

The relevant Results section (page 10) describing these new data is pasted below:

"Finally, to further confirm that *N/KRAS* mutations confer VEN resistance and test whether the effect of both *NRAS* and *KRAS* mutations is similar and generalizable across diverse AML genetic types, we ectopically expressed *NRAS* G12D or *KRAS* G12D in 4 patient-derived AML-iPSC lines of different genetic groups: splicing factor (SF)-mutated (AML-47.1); core binding factor (CBF, AML-37.1); del7 (AML-4.24, see also Fig. 3a) and MLL-rearranged (MLLr, AML-9.9)[32]. Mutant NRAS and KRAS significantly decreased VEN sensitivity of LSCs derived from all lines (Fig. 5h and Extended Data Fig. 9e). This was reversed by treatment with an active state-selective RAS multi inhibitor (RASi)[42,43] (Fig. 5h). Additionally, and consistent with this, both

NRAS G12D and KRAS G12D expression led to increase in MCL1 and BCL-xL and decrease in BCL2 in all LSCs of all genetic groups (Fig. 5i). The RASi reversed the increase of MCL1 and BCL-xL in all cases, with more variable effects on BCL2 levels across different lines (Fig. 5i)."

These results confirm and expand our data presented in the original manuscript in a range of AML genotypes (6 different AML genotypes in total).

- *This would more sharply focus the effect of KRAS mut in relation to the explained effects on AML transformation vs venetoclax resistance, which are likely to engage different signalling pathways and be non-overlapping.*

This is an excellent point that the Reviewer raises. Prompted by this comment, we now present extensive additional analyses with bulk multiomics and sc RNA-Seq to dissect the effect of RAS mutations in transformation and VEN resistance, described in our earlier responses, and shown in new Fig. 5a-g, Fig. 2a-f and j-o, Extended Data Fig. 4e,f, Extended Data Fig. 6a-c and f, and Extended Data Fig. 9a-d.

With regards to VEN resistance, our findings can be summarized as follows:
SA+R CMPs and GMPs show comparable sensitivity to VEN and comparable expression of BCL2 family genes at the transcriptional level (Fig. 5d,e). These results argue against the GMP cell state per se conferring VEN resistance.

Rather, our results are consistent with RAS mutation conferring VEN resistance to GMPs (as well as to other HSPC types, Fig. 5g-i), at least in part, by altering their BCL2 expression profiles, specifically by increasing MCL1, BCL-xL and decreasing BCL2 (Fig. 5g,i).

Indeed, our new results support the Referee's hypothesis that the effects of RAS mutations on AML transformation vs VEN resistance seem to be separate processes that engage different mechanisms, as transformation seems to require the specific permissive chromatin and cellular milieu of a GMP, while VEN resistance appears to be independent of cell type, since RAS mutations seem to confer VEN resistance to all types of HSPCs. We have added the following in our Discussion to make this point:

"Our data strongly point to monocytic differentiation and VEN resistance being two independent effects with a common cause, RAS mutations. This can explain the observed associations between these two independent processes in some studies but not others. In addition, we show here that leukemic transformation by RAS mutations is dependent on the cellular milieu and chromatin landscape, whereas VEN resistance is conferred more broadly in all HSPC types by RAS mutations, again pointing to different mechanistic underpinnings of these processes."

- *Are the effects resulting from KRAS and NRAS the same or are there isoform specific differences in the impact of K/NRAS on survival signalling?*

We present new results showing that both *KRAS* and *NRAS* mutations mediate similar effects. Specifically: First, we found all RAS pathway mutations (*KRAS*, *NRAS* and *PTPN11*) to be associated with monocytic AML in a large patient cohort (new Fig. j,k) (technically the association of *KRAS* mutations does not reach statistical significance, likely because there are much fewer patients with *KRAS* mutations compared to *NRAS* and *PTPN11*, but the trend is there). Second, we show that both *KRAS* G12D and *NRAS* G12D mutations mediate VEN resistance and alter BCL2 family protein levels (increase MCL1 and BCL-xL and decrease

BCL2) in a variety of LSCs from AML patient-derived iPSC lines, as described above (new Fig. 5h,i).

The authors suggest that there is strong rationale for combining VEN with MCL1 inhibitors and potentially BCL-xL inhibitors as frontline therapy in patients with detectable RAS mutations or all eligible patients. The potential limitations of using MCL1 and BCLX inhibitors for this purpose should also be highlighted.

RESPONSE: We agree with the Referee and added the statement (page 11): "However the development of MCL1 and BCL-xL inhibitors has been hampered by on-target dose-limiting toxicities."

Referee #3:

In this paper, the authors use gene editing in iPS as well as UCB CD34+ cells to induce different mutations especially focus on N-Ras and other pre-leukemic mutations to reveal that N-Ras induce leukemia transformation only if occurring in later stage as it target GMP-like cells. They also provide evidence that these Ras-mutant LSCs promote monocytic differentiation and are resistant to venetoclax treatment.
This paper is the continuation of the early paper by the same group (wang et al Cell Stem Cell 2021) who already demonstrate that the combination of N-Ras, ASXL.1 and SRSF.2 induced leukemia.

The paper provides some incremental informations related to the order of the mutations being important as well as the fact that N-Ras LSC induce Venetoclax resistance. There are nevertheless some major issues that can be raised from the present work.

1- They show that N-Ras alone is not enough to induce transformation but show in Figure 1 that in combination ASXL-1 and SRSF.2 iPS derived HSPC can engraft in NSG-S mice. They conclude that this is enough to prove transformation. It seems that the level of engraftment is quite low and thus is unclear whether the mice really died from leukemia. I supposed based on their original paper, the engraftment is mostly myeloid. But neither in this paper nor in the original one, they provided evidence that the cells out of these mice can produce leukemia after serial transplant. This should be done as a prove of clear leukemia development and invasion of the BM.

RESPONSE: The Reviewer is correct that the SAR iPSC-derived HSPCs are not serially transplantable nor do they cause lethal disease. However, they are able to give rise to engraftable human myeloid blasts in every single animal (engraftable at levels up to ~10% in the BM), which is remarkable, given that normal unmodified hPSC-derived HSPCs – unlike primary human HSPCs – are not engraftable at baseline. We and others have thus considered **any level of engraftment of hPSC-derived HSPCs as a surrogate phenotype of leukemic potential** (Kotini et al. Cell Stem Cell 2017; Chao et al. Cell Stem Cell 2017; Wesely et al. Cell Reports 2020; Wang et al. Cell Stem Cell 2021; Kotini et al. Blood Cancer Discov 2023).

However, we acknowledge that this is a limitation of the iPSC system and have thus complemented and expanded these experiments using primary CB-derived HSPCs in all subsequent transplants.

We also appreciate the Reviewer's concern about overstating the results of the iPSC-HSPC engraftment experiments and we have accordingly changed the wording of our descriptions of these findings to eliminate references to "leukemia" (such as "generate leukemia" and "induce leukemia") and replaced these with: "give rise to transplantable myeloid cells", "induce leukemic features", "generate engraftable cells", "promote leukemogenesis".

They mentioned that other mutations like FLT3, DNMT3A and NPM or Runx1 or ASXL1 in conjunction with N-Ras are not able to induce transformation but in extended Fig 2f, they show engraftment with AR at around 4.5% which is not dissimilar to what they show with NAS, especially as this time they injected the cell in NSSG instead of NSG-S. Nevertheless, when N-Ras is added (RAR), the level of engraftment is much lower. It will have been interested to investigate what happened there.

RESPONSE: We thank the Referee for the opportunity to clarify these data. Extended Fig. 2f of the original manuscript shows engraftment with AR at 1.5%, not 4.5%. Of note, this one flow cytometry dot plot shows the highest engrafted mouse, while the range of engraftment in 6 mice was 0%-1.5%, shown in the cumulative data in previous Extended Data Fig. 1i, which is now Extended Data Fig. 1h, (with 3 out of 6 mice showing no detectable engraftment, one mouse at 0.6%, one mouse at 0.8% and the one shown in previous Extended Data Fig. 2f at 1.5%). This level of engraftment is very dissimilar to SAR, and this difference is statistically highly significant, as shown in Extended Data Fig. 1h. In the case of the SAR group, a total of 27 mice were transplanted with cells derived from several independent differentiations of different SAR clones, with a range of engraftment of 1.2% to 6.3%. All the groups shown in Extended Data Fig. 1h, including the SAR and AR (and the mouse that was shown in previous Extended Data Fig. 2f), were injected into NSG mice. The engraftment level of SAR is even higher in NSGS mice, at 3-9%, as we have shown in Fig 1i of the Wang et al. Cell Stem Cell 2021 manuscript. (For the present study we did not transplant SAR into NSGS mice.)

The comparison of AR (6 mice with engraftment levels as stated above) vs RAR (5 mice total with 4 mice without detectable engraftment and one mouse at 0.1%, as shown in Extended Fig. 2e) is not statistically significant. Additionally, the RAR line was generated from a different parental iPSC line (from a RUNX1-FPD patient with a germline RUNX1 mutation) and is thus not isogenic to the AR and all other lines in this set of experiments, which weakens any conclusions that can be drawn from directly comparing engraftment between AR and RAR. Rather, the conclusion we can draw from this set of experiments, as stated in the manuscript, is that none of the other engineered lines come close to the engraftment levels achieved with the SAR combination, where every single mouse engrafts at levels >1%.

We appreciate that this panel, previously included as Extended Data Fig. 2f, was confusing because the flow plots that were previously selected to be shown were not the most representative of the full range of engraftment levels of each group and, furthermore, its previous placement in Extended Data Fig. 2, together with the data from the non-isogenic RAR line, added to the confusion. We therefore now replaced the flow cytometry plots with more representative ones of the groups R, AR, SR and SAR and moved this panel to a more appropriate place in Extended Data Fig. 1, as panel i.

We again thank the Reviewer for bringing this to our attention.

2- They next tested the effect of the mutation order and show indeed N-Ras need to be induce later using both iPS or CB CD34+ where they used lentivirus vector instead of Crisp-Cas.
In Fig1 d, they show that except the R-late group they did not retrieve any CD34+CD45+ cells. It is surprising that control CD34+ do not give rise to CD34+?

RESPONSE: We thank the Reviewer for allowing us to clarify this point. Transplantation of normal human CD34+ HSPCs gives rise to mostly CD34- cells, as the majority of the cells present in the mice several weeks post-transplantation are no longer HSPCs, but rather more mature CD34- cells.
The fraction of CD34+ cells in both NSG and NSGS mice transplanted with CB CD34+ cells is no more than 5-10% (see, for example, Figure 3d of Kaufmann et al. Nat Immunol 2021 (https://www.nature.com/articles/s41590-021-00925-1/figures/3); Figure 1H of Wunderlich et al. PLOS One 2018, (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0209034#pone-0209034-g001) and review by Goyama et al. Blood 2015, PMID 25762176).

Therefore in the case of transplantation of genetically engineered CB-derived HSPCs, we consider detection of primitive CD34+ cells in the mice at a high fraction as a feature of leukemic engraftment (specifically, the R-Late group shown in Fig. 1d).

In this experiment, it is also unclear whether after transduction, they sorted the triple transduced cells? To confirm the presence of the three mutations in the same cells out of the mice, they should provide at least genetic analysis of CFC. It is indeed unclear whether all cells are triple mutated.

RESPONSE: Here the Reviewer raises a very important point, which is whether the CB HSPCs transplanted into the mice had all 3 mutations present in the same cells, since these mutations were transferred via 3 separate lentiviral vectors, and whether the human cells that engrafted in the mice were triple-mutant. To facilitate detection of transduction with each vector and easily estimate and track the fraction of cells transduced with all 3 mutant transgenes, we used vector designs that allow co-expression of each mutant gene with a distinct fluorescent protein, as shown in the schematic of Fig. 1c. Specifically, $ASXL1^{del}$ and $NRAS^{G12D}$ were co-expressed with GFP and LNGFR, respectively, through a P2A peptide, and $SRSF2^{P95L}$ with mCherry via a bidirectional hPGK promoter.

Extended Data Fig. 3a-c show flow cytometry data assessing transduction efficiency with each vector. Specifically, in Extended Data Fig. 3c upper panels we show that the pre-transplant cells were composed of 27% Cherry/LNGFR double positive, (i.e. $NRAS^{G12D}$/$SRSF2^{P95L}$ double transduced), and 96% of these were also GFP+, i.e. triple transduced with NRAS/SRSF2/ASXL1. This means that 26% of total pre-transplant cells had all 3 mutations. Importantly, as shown in Extended Data Fig. 3c middle and lower panels, more than 80-90% of human cells out of the mice from both the R-Early and R-Late groups had all 3 mutations.

To clarify and emphasize this important point, we have added a sentence in the Results section (page 4: “The fraction of triple-transduced cells was ~26% before transplantation and increased to over 80% in the cells retrieved post-transplant from all mice (Extended Data Fig. 3a-c).”) and expanded the respective legends of Extended Data Fig. 3a-c panels to better explain these data, as follows: “Evaluation of transduction efficiency in lentivirally transduced CB CD34+ cells shown in Fig. 1c. Panels a,b show transduction efficiency with each individual lentiviral vector (estimated on the basis of the fraction of cells expressing the respective linked co-expressed

fluorescent protein) in cells of the 4 groups shown in the schematic of Fig. 1c (R-Early, R-Late, R-Only and Cherry) before and after transplantation. (Note that the pre-transplant cells are the same for both R-Early and R-Late groups.) Panel c shows the fraction of triple-transduced cells, estimated at approximately 27 x 0.96 = 26% pre-transplant; 82 x 1 = 82% for the R-Early; and 91 x 0.97 = 88% for the R-Late cells."

They should also show the level of engraftment in each arm as well as the proportion CD33/CD19 as it seems that even in the R-early, or R only, they have mostly only CD33 engraftment.

RESPONSE: The level of engraftment in each arm is shown in Extended Data Fig. 3e. The CD33+ fraction is shown in Fig. 1e. Indeed, as the Reviewer notes, the cells engrafted in these mice are mostly CD33+ in the R-Early and R-Only groups as well, unlike what would be expected with normal CB CD34+ cells (like the Cherry control group), in which case approximately 20-30% of total human cells are expected to be CD33+ (as per the references mentioned in our response to this Reviewer's second point above). Because the R-Early mice also developed a lethal disease with CD33+ but CD34- human cell engraftment, we interpret these data as indicative of a lethal myeloproliferative disease (as opposed to a myeloid leukemia with CD34+ blasts in the R-Late group) and describe it in the manuscript text as follows (page 4): "Induction of *NRAS G12D* immediately after transplantation ("R-Early" cohort) gave rise to a lethal myeloproliferative disease caused predominantly by CD34- mature myeloid cells". RAS alone (R-Only) also appears to drive myeloproliferation, as the Reviewer notes, although these mice did not develop a lethal disease within the timeframe of the experiment.

3- They also show in Fig 1h that the time of induction of N-ras is important. This might indicate two options that N-Ras induced transformation only when induced at later stage or that N-Ras is transforming a progenitor cell. Even through in Fig 2, they show that GMP and not CMP have LSC activity, it does not directly prove this point. As ex vivo expanded cells have been reported to changed phenotype like dowregulation of CD38+ ex vivo in liquid culture. Thus, they should sort GMP-CMP and HSC directly from UCB and induced again all three mutations at once compared to what they have done. Indeed, with the iPS I believe they have induced the three mutations at once and then induced there HSC differentiation.

RESPONSE: We agree with the Referee's concern about reliability of phenotypic markers, primarily CD38, in ex vivo cultured cells. To address this, we performed extensive additional experiments and analyses:

- Per the Referee's suggestion, we performed **two additional sorting/transduction and transplantation experiments**. As we show in new Fig. 2g-h and new Extended Data Fig. 5d-f, we sorted CMPs and GMPs either (1) before induction of the RAS mutation and after induction of the SA mutations (Fig. 2g); or (2) before induction of all three SAR mutations simultaneously (Fig. 2i). These experiments confirm that **only pre-sorted GMPs, but not CMPs, are the target cell of transformation by RAS mutations**. The relevant Results section (page 6) describing these new data is pasted below:

"FACS-sorting and transplantation of GMPs and CMPs from the SA+R group expressing comparable levels of all 3 transgenes, revealed that only the GMPs had leukemia initiating or leukemia stem cell (LSC) activity (Extended Data Fig. 5a-c). To test if *RAS* mutation acquired by a GMP can cause leukemia, we next sorted phenotypic CMPs and GMPs prior to *NRAS G12D*

induction (but after SA transduction, SA+R) or prior to simultaneous transduction with all 3 transgenes (SAR) (Fig. 2g-l and Extended Data Fig. 5d-f). In both cases, only SA+R and SAR GMPs, but not CMPs, could initiate leukemia (Fig. 2g-i). These results indicate that not only are RAS-MT GMPs LSCs, i.e. cells able to initiate and maintain leukemia in vivo, but, additionally, that GMPs derived from ancestral AML clones with previously acquired cooperating driver mutations are the target cell of transformation by RAS mutations."

- We expanded our genomics analyses with **bulk RNA-Seq and ATAC-Seq of iPSC-HSPCs and scRNA-Seq of CB-derived HSPCs that corroborate at the transcriptional and chromatin level that SA+R cells resemble GMPs** not only immunophenotypically, but also in their transcriptome and chromatin accessibility landscape. These analyses, presented in new panels in Fig. 2d-f and Extended Data Fig. 4e,f, should greatly aid in alleviating concerns over reliability of immunophenotypic markers. The relevant Results section (pages 5-6) describing these new data is pasted below:

"We confirmed these observed changes of immunophenotypically defined populations with transcriptional analyses using single-cell RNA-Sequencing (scRNA-Seq) of the CB cells engineered with the different transgene iterations (Fig. 2a). Clusters were manually assigned to cell types based on expression of known cell-type-specific marker genes found amongst the most differentially expressed genes in each cluster (Fig. 2b and Supplementary Fig. 1). These analyses confirmed decrease of GMPs in all R-Early groups (R, R+SA and SAR), with concomitant increase in CMPs and erythroid progenitors (Fig. 2c). In contrast, the GMP population was preserved in the SA and SA+R groups (Fig. 2c).

These results, which corroborate the immunophenotypic analyses (Fig. 2a), indicate that acquisition of *NRAS G12D* mutation before or at the same time as the other two mutations causes arrest at the CMP stage with loss of the GMP population and failure to induce leukemia.

To further explore the cellular state of the R-Late LSCs, we performed bulk RNA-Seq and ATAC-Seq analyses in SA+R vs R+SA iPSC-HSPCs (Fig. 2d). Principal component analysis revealed that cells with initial *NRAS G12D* mutation were very distinct from those with initial *SRSF2* and *ASXL1* mutations, in both their transcriptome and chromatin landscape, regardless of subsequent mutational acquisition (Fig. 2d). Acquisition of *SRSF2* and *ASXL1* mutations following *NRAS G12D*, resulted in only modest change of cell state, consistent with a maturation arrest by early *NRAS G12D* in our functional experiments (Fig. 2d and Extended Data Fig. 4a,b, left panels: "R+SA vs R+Ctrl"). In contrast, acquisition of *NRAS G12D* as a late event, after *SRSF2* and *ASXL1* mutations, resulted in profound changes (Fig. 2d and Extended Data Fig. 4a,b, right panels: "SA+R vs SA+Ctrl"). Gene set enrichment analysis (GSEA) showed enrichment of gene expression signatures of primary human AML in SA+R cells, corroborating our functional experiments (Extended Data Fig. 4c). We then interrogated the similarity of SA+R cells with cell subsets along the developmental hierarchy of primary human AML using gene signatures characterized by Zeng et al. (LSPC-Quiescent, LSPC-Primed, LSPC-Cycle, GMP-like, ProMono-like, Mono-like and cDC-like)[16]. The GMP-like gene signature was highly enriched in SA+R cells and, conversely, depleted in R-Early cells (R+Ctrl vs SA+Ctrl) (Fig. 2e and Extended Data Fig. 4d). Similar positive and negative enrichment in these comparisons was found for the LSPC-Cycle signature (Fig. 2e). Additionally, we asked what cells along the normal hematopoietic hierarchy are R-Early and R-Late iPSC-HSPCs more similar to, using hematopoietic cell type-specific accessible chromatin regions and, more specifically, distal cis-regulatory elements[30]. R-Late cells were more similar to GMPs and monocytes in their accessible chromatin landscape, whereas R-Early cells showed higher similarity to primitive

cells – HSC/MPP and CMP –, as well as progenitors of the megakaryocytic and erythroid lineage (Fig. 2f and Extended Data Fig. 4e,f)."

We believe that these additional data support that GMPs, and not CMPs, have LSC activity. They further support that the target cell in which RAS mutations are acquired in AML is a GMP.

4- They then switch the analysis to AML samples and their response to Venetoclax plus DEC. They show in a cohort of 117 patients treated with venetoclax and DEC that monocytic phenotype is not associate with a better DOR or survival but the presence of N-Ras mutation is associated with a shorter DOR but not overall survival. In these patients, it seems that a number have also TP53 which has already been associated with poor response. It might be of interest to eliminate the TP53 samples and run the analysis again.

RESPONSE: We re-run these analyses after **excluding *TP53*-mutated cases** as per the Referee's suggestion. Indeed excluding *TP53*-mutated cases helped better uncover the effects of RAS mutations in patient outcomes and we thank the Referee for this suggestion. We also now revised and updated our analyses comparing outcomes in AML patients with vs without RAS mutations to include **additional patients with longer follow up** and to **remove subsetting to monocytic disease**, in response to comments by Referees #1 and 2.

These revised analyses are presented in new Fig. 4d,e (*TP53*-WT only patients), Extended Data Fig. 7a,b (all patients, including *TP53*-mutated) and Supplementary Tables 4,5. They include 31 (26 *TP53*-WT) patients with *N/KRAS* mutations, of which 18 (17 *TP53*-WT) could also be assessed for duration of response (DOR), and show **significantly worse DOR** (regardless of exclusion of *TP53*-mutated cases) and **significantly shorter overall survival** (OS) in *TP53*-WT patients with *N/KRAS* mutations, compared to those without *N/KRAS* mutations. Specifically, a RAS mutation significantly increased the risk of relapse with HR 5.32, 95% CI 1.81, 15.68, p<.001 and significantly increased the risk of death with HR 2.42, 95% CI 1.28, 4.60, p<.001. (Of note, the cohort used for the comparison of outcomes between monocytic and non-monocytic disease was not updated – hence panels Fig. 4b,c remain unchanged – because no information on monocytic differentiation status is available for the new patients enrolled since data cut off of the prior analysis. This is because monocytic differentiation is not routinely reported, but needs to be manually adjudicated for each patient by a pathologist after detailed review of flow cytometric markers.)

In addition, we would also like to point out that these data on patient outcomes (monocytic vs non-monocytic, as well as RAS-mutated vs RAS-WT) are very much **in agreement with results of the larger VIALE-A study**, reported at the most recent EHA 2023 meeting (https://journals.lww.com/hemasphere/fulltext/2023/08003/p521__findings_from_an_analysis_of_patients_with.422.aspx) and included in a manuscript in preparation (Konopleva et al.).

These revised analyses more firmly establish that RAS mutations, but not monocytic differentiation, have a strong negative impact on AML patient outcomes on VEN combination therapy. We again thank the Referee for this suggestion.

5- Lastly, they used again iPS derived from AML samples and tested their response to venetoclax using enriched HSPC versus HSPC induced to differentiate into monocyte ex vivo. They show that all monocytic cells both WT and Nras mutant are resistant to venetoclax but that only the Ras mutated LSC are resistant. Here they provide a correlation between Ras mutation

and resistance but not a direct prove as all iPS used have also potentially other confounding mutations.

RESPONSE: To investigate the effect of *N/KRAS* mutation **without confounding effects of other mutations** we use **isogenic models and conditions**. In the original manuscript we used (A) an isogenic pair of iPSC lines (derived from the same patient and from the same founder clone with a driver t(1;7;14) translocation, see schematic in Fig. 4f) and (B) GoT to measure expression of pro- and anti-apoptotic BCL2 family genes again in isogenic conditions, i.e. in NRAS-WT and NRAS-MT cells within the same patient – all harboring the same earlier driver mutations and genetic lesions, namely *DNMT3A*^{R882H}, *IDH1*^{R132C}, trisomy 8 and +1q (shown in the respective schematics in Fig. 3a and e). The VEN treatment experiment that the Referee mentions also used this isogenic pair of iPSCs with and without a KRAS mutation (see schematic in Fig. 4f).

In addition, we have now **extended these analyses to additional isogenic pairs of RAS-MT vs RAS-WT LSCs from AML patient-derived iPSC lines** (in addition to AML-4.24 already presented earlier) **of various AML genetic groups**, specifically: MLLr (AML.9.9); Core Binding Factor (AML-37.16); and splicing factor-mutated (AML-47.1). These data are included in new Fig. 5h,i and show that RAS mutations confer VEN resistance (Fig. 5h) and increase of MCL1 and BCL-xL with concomitant decrease of BCL2 (Fig. 5i). In addition, we show that treatment with an **active state-selective RAS multi inhibitor** (RASi) reverses these changes, and restores both sensitivity to VEN and expression of MCL1 and BCL-xL to levels comparable in those of RAS-WT cells (Fig. 5h,i). The relevant Results section (page 10) describing these new data is pasted below:

“Finally, to further confirm that *N/KRAS* mutations confer VEN resistance and test whether the effect of both *NRAS* and *KRAS* mutations is similar and generalizable across diverse AML genetic types, we ectopically expressed *NRAS* G12D or *KRAS* G12D in 4 patient-derived AML-iPSC lines of different genetic groups: splicing factor (SF)-mutated (AML-47.1); core binding factor (CBF, AML-37.1); del7 (AML-4.24, see also Fig. 3a) and MLL-rearranged (MLLr, AML-9.9)[32]. Mutant NRAS and KRAS significantly decreased VEN sensitivity of LSCs derived from all lines (Fig. 5h and Extended Data Fig. 9e). This was reversed by treatment with an active state-selective RAS multi inhibitor (RASi)[42,43] (Fig. 5h). Additionally, and consistent with this, both NRAS G12D and KRAS G12D expression led to increase in MCL1 and BCL-xL and decrease in BCL2 in all LSCs of all genetic groups (Fig. 5i). The RASi reversed the increase of MCL1 and BCL-xL in all cases, with more variable effects on BCL2 levels across different lines (Fig. 5i).”

We believe that these additional data convincingly and definitively show that RAS mutations confer VEN resistance to LSCs regardless of other mutations.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

Sango et al have substantially improved the manuscript by increasing replicates, adding experiments, and clarifying the text. It's interesting and supportive that sorted CMPs do not transform upon RAS transduction but GMPs do. The clear distinction between the ancestral cell-of-origin and the subclonal RAS-mutated LSC is appreciated. My remaining minor comments and suggestions are as follows:

Sometimes, the authors include PTPN11 as an NRAS/KRAS pathway gene; sometimes, they do not. It would be helpful to be consistent, especially going forward if the presented insights are going to be used for treatment decisions.

In several places, the authors should add "n=…" to the legends.

Some of the genes in Supplementary Table 3 are interesting. I suggest highlighting a few on the right of Figure 2m (ETS1, ID2, IL1B…author's discretion).

Page 10: "Furthermore, this effect of RAS mutations…" – please clarify the effect being referred to. Also, check the referral to Fig. 5e at the end of the sentence.

In my opinion, given the limitations of the model systems, the word "must" should be removed on Page 3 in the following context: "N/KRAS mutations MUST occur late in AML pathogenesis."

The authors do not discuss why some patients develop Venetoclax resistance in the absence of RAS mutations. This point might be of sufficient interest to include.

Referee #2 (Remarks to the Author):

I thank the authors for their comprehensive and detailed responses to my review and the high quality of the added data, which I agree has led to a substantially more impressive manuscript.

In Figure 2a: this looks like transduction by SA, yet there are 3 colors of virus shown. Is the red NRAS G12D virus present in error?

Regarding the comment:
Strikingly, the LSCs of the RAS-MT clone had reduced BCL2 expression compared to the LSCs of the RASWT clone, as well as potentially higher MCL1 and BCL2L1 expression and lower expression of

pro-apoptotic BAX (Fig. 4h, upper panels).

As neither of the MCL1 or BCL2L1 expression changes in LSCs are significant, should this statement be amended?

Fig 4h: What is the expression alteration in MCL1 that is significantly different in monocytic cells? Is it higher MCL1 expression related to WT or KRAS mut cells? By eye it looks higher in the WT population.

Referee #3 (Remarks to the Author):

In this revised paper the authors provided an extensive revision of their original paper.
Nevertheless, there are still some concerns related to their interpretations of some of the data.

Major comments:
- In Fig extended Fig 1, they show that only triple N-Ras g12D/ SRSF2 and ASXL1 mutant iPS induced HSPCs can engraft in immunodeficient mice. Nevertheless, as these cells are not able to repopulate secondary transplant, it is unclear what are the " leukaemia" features that are associated with the engraftment.
- When looking at the order of the mutations, they also show that induction of N-Ras mutation only induce engraftment when added after SRSF2 and ASXL1. Nevertheless, later on they also show that the target of N-Ras effect is on GMP. The authors should first induced iPS differentiation, then introduced the expression of N-RAS and at the same point add or not SRSF2 and AXSL1.
- Using CD34+ UCB, they show that the induction of R-early induced a lethal myeloproliferation, constitute of CD34neg cells. What do they conclude that these are not leukemia? Leukemia cells from patients and LSC in some patients could be CD34neg. The question is whether these cells could be transplanted back to a secondary recipient.
- They then induce Nras before or after transduction of SA. In the late induction, all three lentivirus were transduced at the same time in CD34+ and only the induction of N-Ras was delayed via inducible vector used. In the other scenario, N-Ras was induced before. The transduction of SA after N-Ras, in this case questioned, whether the SA was not introduced to the HSC and thus none of the triple transduced cells were transplanted? The transduction efficient of SA on early induce N-Ras will need to be shown. Again, the question is whether by using constitutive vector for N-Ras but using inducible vectors for SA, the authors could indeed verify that their conclusion are correct. Also, it is surprising that in early R-SA, they show no human cells engraftment. As they injected UCB, it is quite surprising to see no engraftment of R-SA. Also, what characteristic of the engraftment of SA-R making them conclude that it is a leukemia.
- By the transduction of CMP versus GMP, they show that GMP is the target to induce what they called "Leukemia". Leukemia should be better defined. Are there again transplantable in secondary recipient?
- It seems that the transduction of GMP with SAR or the transduction of SA and then R induced the same results. This question whether the order of the mutation is indeed essential as the authors claimed. Indeed it seems that all three mutations could be induced at once as long as the target are GMP cells.

- They also investigate in a cohort of AML patients treated with DEC-10-VEN, whether the presence of monocytic versus non-monocytic features, correlated with response. They show that it does not per se, but the presence of Ras does correlate with non -responder. They then used iPS lines derived from the same patient and differentiate these iPS into CD34+ or monocytes as well as another iPS control or Ras mutant lines. In both cases, monocytes derived from normal or AML mutant clones are resistant to VEN contradicting that monocytes are not implicated in the resistance observed clinically. They also show that monocytic derived iPS have lower bcl2 and high MCL1 and thus are intrinsically resistant to VEN.
- They then investigate what they called LSC of Ras-WT versus MT. How they defined LSC is unclear. Even if some CD34+ iPS derived are able to engraft, what is the frequency of the LSC into the CD34+ fraction?
- Lastly, they also wanted to generalise that N/K-Ras confer resistance, they then overexpressed N-Ras on 4 patients derived AML iPS lines with different mutations background. Again, they differentiate these cells and mentioned that LSCs with N-Ras are more resistant. Unclear how LSC were defined. Also they showed before that N-Ras is not able to transform iPS with specific type of mutations like DNMT3A , FLT3 or Runx1 and ASXL1). How do they know that in that case N-Ras is able to transform these iPS lines? Are the ectopic expression of N-Ras in these lines changing their transforming capacity, their phenotype? Are overexpression of N-ras in CD34+ from UCB also provided a more resistant phenotype to normal HSC?

Author Rebuttals to First Revision:

The Referee's comments are in *Italics*.
Our responses are in **blue font**.
Our proposed changes to a revised manuscript are in **red font**.

Referee #3

In this revised paper the authors provided an extensive revision of their original paper. Nevertheless, there are still some concerns related to their interpretations of some of the data.

We thank the Referee for acknowledging the extensive revision of our original paper, which included 52 new figure panels (31 in main Figures and 21 in Extended Data Figures), 2 new Supplementary Figures and 5 Supplementary Tables.

We believe that the additional revisions we outline below, including **additional data and clarifications**, can resolve the Referee's remaining concerns regarding interpretations of some of our data.

In summary, the Referee raises 3 main issues, which we believe we can address as follows:

1. **Definition/criteria of AML in xenograft assays.** We understand that the multitude of human models we employ throughout the manuscript requires more clear explanation of the criteria we use to call an experimental outcome leukemia or not. We will clarify, as outlined in detail below in our point-by-point response, that we use uniform criteria across all models, based on accepted practices in the field of study of human hematopoiesis in xenografts. We can also explain how considerations relating to the different sources and preparations of the transplanted cells (iPSC-derived vs CB-derived HSPCs and time in culture prior to transplantation) impact the outcome of the experiments. We believe that by **clarifying these criteria and features of our models** and by **providing more detailed documentation of the leukemic features** in the xenograft mice (mainly blast % by immunophenotype and/or morphology/histology), we can address the Referee's concerns regarding the interpretation of our data. We also consulted with other leaders in the field of study of human hematopoiesis and leukemia in xenograft models and we and they do not consider serial transplantation to be a requirement to define AML (but rather an assay to quantify LSCs). Nevertheless, these are feasible experiments and we can provide data on secondary transplants in a very reasonable timeframe, if the editorial team finds them necessary.

2. **Interpretation of mutational order experiments**. In our point-by-point response we justify in detail our rationale for focusing on interrogating the acquisition of RAS mutation either before or after the other mutations, rather than at the same time (considering relevance to human AML development) and clarify how the design of our experiments supports the interpretation of our data. We believe that by clarifying the above, by providing data confirming the transplantation of triple-transduced transplanted cells in all experiments, and by clarifying that additional scenarios are not excluded by our study, we can reach a consensus on interpretation of these data.

3. **Attribution of LSC properties to CD34+ cells derived from patient-derived AML-iPSC lines.** The AML-iPSC lines used in the experiments of the second part of the manuscript (Figs 3-5), have been extensively characterized before in previous publications by our group and characterized for LSC activity. We will clarify this and provide additional data from an in vivo VEN treatment experiment.

Major comments:
#1- In Fig extended Fig 1, they show that only triple N-Ras g12D/ SRSF2 and ASXL1 mutant iPS induced HSPCs can engraft in immunodeficient mice. Nevertheless, as these cells are not able to repopulate secondary transplant, it is unclear what are the " leukaemia" features that are associated with the engraftment.

RESPONSE: In various points the Referee raises the issue of what leukemic features we evaluate and how we define leukemia in our xenograft assays in the different models. We appreciate that the multitude of novel human models we employ in this manuscript warrants more detailed explanations. In addition, criteria of human AML in xenograft mouse models are not the same as criteria of mouse AML in mouse models.

Below we will first define the AML criteria we use throughout the study and then describe how they apply to each specific model.

Based on broadly accepted practices in the field of study of human hematopoiesis with xenograft assays (for example PMIDs 21112565, 26834243, 21251617, 28123069, 27377587, 19626050, 25762176, 28159741, 30659850), we adopt and **apply across all experiments throughout our study the following definition of AML in a xenograft: engraftment of myeloid-restricted immature human cells harboring AML driver mutations**. This is the minimum set of criteria that apply to all our models across all experiments.

Considering that: (a) transplantation of normal unmodified CB CD34+ cells into NSG and NSGS mice gives <5-10% CD34+ and 20-30% CD33+ cells (CD34+ cells upon engraftment give rise to more differentiated CD34- cells and the majority of normal human cells in xenograft models are B cells) (PMIDs 33958784, 30540841, 25762176) and (b) as per the **clinical guidelines for the diagnosis of human AML** (cutoff of 20% blasts to diagnose AML), we define "myeloid-restricted" as predominantly myeloid (50% or more - of note, in almost all our experiments we obtain exclusively, nearly 100%, myeloid cells) and we define "immature" as >20% blasts – defined by immunophenotype or morphology.

Although not universal criteria, additional considerations that are highly suggestive of AML in a xenograft are:

- **Engraftment initiated by an HSPC population that should not normally show durable engraftment.** As further discussed below, this applies to all our experiments, with the exception of the CB experiment shown in Fig. 1c. In all other experiments of our study we obtain engraftment by normally non-engraftable cells, namely: iPSC-derived HSPCs (Extended Data Fig. 1 and Fig. 1a,b); sorted committed progenitors (CMPs and GMPs) (Fig. 2g,i and Extended Data Fig. 5); unfractionated CB HSPCs after prolonged culture (11 days) (Figs. 1h-l and 2a and Extended Data Fig. 3a-e).
- **Lethal disease.** This also applies to all our experiments with the exception of the experiments using gene edited iPSCs (Extended Data Fig. 1 and Fig. 1a,b).

The Referee mentions secondary transplantation, however this assay is not required to define AML, but rather to quantify the frequency of leukemia stem cells (LSCs). We therefore did not provide secondary transplant data for the experiments spanning Figs 1-2, aimed at reading out leukemic potential of the various cells engineered with various mutational combinations in different order, for which we used the criteria we outline above and we describe on a case-by-case basis below. We believe this detailed description of our criteria can add clarity and resolve any perceived ambiguity regarding our definition of human AML in xenografts. While there are many examples in the literature of studies defining AML LSC phenotypes that have not

performed secondary transplants (for example PMIDs 21112565, 26834243, 28123069), of note, our experiments showing VEN resistance and monocytic bias of RAS-mutant LSCs (Figs 3-5, specifically panels 3a-d, 4f-h and 5h,i) utilized CD34+ HSPCs derived from various patient-derived AML-iPSC models previously demonstrated in various publications from our group to have LSC activity with serial transplantation and limiting dilution assays (more on this below).

Because our study uses a multitude of different models of human AML with different characteristics, below we explain the characteristics of each model in detail and how the criteria for AML that we described above apply to them. **We also include the same information in a more concise way in Table 1 below.**

- Model #1: Genetically engineered iPSCs (used in Extended Data Figs. 1 and 2 and main Fig. 1a,b).
Leukemic features:
"engraftment of myeloid-restricted immature human cells": 1.2%–6.3% engraftment 13-15 weeks post-transplantation (Extended Data Fig. 1h) of CD33+ (Extended Data Fig. 1i) cells with immature morphology (Extended Data Fig. 1j)
"harboring driver AML mutations": the transplanted cells were derived from clonal triple mutant SAR iPSC lines
"initiated by an HSPC population that should not normally show durable engraftment": HSPCs derived from pluripotent stem cells (including embryonic stem cells, ESCs, and induced pluripotent stem cells, iPSCs) do not have the ability to durably engraft, unlike primary human CB CD34+ cells. Transplantation of any normal genetically unmodified iPSC-derived HSPC population never produces detectable engraftment of any level. (There is extensive literature on the topic, as generating engraftable iPSC-HSPCs is one of the holy grails of stem cell research and regenerative medicine, for example see PMIDs 35484246, 27723718, 37611730, 36939073. This lack of engraftment potential is believed to reflect the inability to generate true HSCs from pluripotent stem cells, due to technical limitations of the differentiation protocols and/or developmental immaturity of the cells derived from pluripotent stem cell sources in general.)

We and the Majeti lab previously demonstrated in two back-to-back papers in Cell Stem Cell (Kotini et al. CSC 2017 PMID: 28215825 and Chao et al. CSC 2017 PMID: 28089908) that HSPCs differentiated from iPSCs which were derived by reprogramming leukemic cells from patients with AML (which we refer to in this and previous manuscripts as "AML-iPSCs") show engraftment of myeloid-restricted immature human cells. Subsequently, in Kotini et al. Blood Cancer Discov. 2023, we demonstrated engraftment of myeloid-restricted immature human cells in a larger collection of AML-iPSC lines from different patients and genetic backgrounds (some of which we use in the last figure of this manuscript to show VEN resistance mediated by RAS mutations – more on this later). Based on these observations, we (and others) subsequently used transplantation assays in xenografts as a readout of leukemic potential of our genetically engineered "synthetic leukemia" models, including the SAR model in our Wang et al. Cell Stem Cell 2021 paper and the present study.

- Model #2: CB HSPCs with minimal in vitro culture (shown in main Fig. 1c-g and Extended Data Fig. 3a-e).
Leukemic features:
"engraftment of myeloid-restricted immature human cells": ~10-50% engraftment (Extended Data Fig. 3e) of myeloid-restricted, i.e. >50% CD33+ (Fig. 1e) immature, i.e. >20% CD34+ (Fig. 1d) cells

"harboring driver AML mutations": documented triple-transduced cells (Extended Data Fig. 3a,c)
"lethal disease": Extended Data Fig. 3d

- Model #3: CB HSPCs with extended in vitro culture to query induction of RAS mutation before or after the SA mutations (shown in main Fig. 1c-g and Extended Data Fig. 3a-e).
Leukemic features:
"engraftment of myeloid-restricted immature human cells": ~20-50% engraftment (Fig. 1j) of myeloid-restricted, i.e. >50% CD33+ immature, i.e. >20% CD34+ cells and/or immature blasts by morphology (we can add representative panels to document these – we did not include more panels documenting CD33 and CD34 expression to avoid repetition and due to space restrictions before, but we can include them now)
"harboring driver AML mutations": transduction efficiency (Extended Data Fig. 3g)
"lethal disease": Fig. 1i
"initiated by an HSPC population that should not normally show durable engraftment": CB CD34+ cells cannot normally be maintained in in vitro culture without loss of engraftment potential. (There is extensive literature on this topic, as expansion of long-term HSCs in culture in vitro is another at present unattainable holy grail of stem cell research and regenerative medicine.) In this set of experiments (presented in Figs 1h-l and 2a-c), the CB CD34+ cells are cultured for a period of 11 days prior to transplantation: 8 days shown in the schematic of the respective figures plus 3 days of prestimulation with cytokines prior to day 1 (corresponding to the first transduction in the schematics), necessary to induce cycling and enable efficient lentiviral transduction. After this culture period in the culture conditions we use (which are standard culture conditions for human HSPCs), **CB cells are no longer engraftable**, as these culture conditions are unable to preserve HSCs/MPPs with engraftment potential.

- Model #4: Sorted CMPs and GMPs from CB CD34+ cells (shown in main Fig. 2g,i and Extended Data Fig. 5).
Leukemic features:
"engraftment of myeloid-restricted immature human cells": engraftment of myeloid-restricted, i.e. >50% CD33+ immature, i.e. >20% CD34+ cells and/or immature blasts by morphology (we can add more panels to document these)
"harboring driver AML mutations": transduction efficiency (Extended Data Fig. 5c-g)
"lethal disease": Fig. 2g,i
"initiated by an HSPC population that should not normally show durable engraftment": Sorted GMPs and CMPs are committed progenitors without engraftment potential unless they are transformed.

- Model #5: CD34+ HSPCs derived from AML-iPSCs, i.e. iPSC lines generated through reprogramming of AML patient cells (shown in Figs. 3a-d, 4f-h and 5h-i).
Leukemic features:
"engraftment of myeloid-restricted immature human cells": these iPSC lines have been extensively characterized by us in previous publications, showing durable (13-15 weeks or lethal disease) engraftment of exclusively CD33+ blasts (by CD34 expression and/or morphological assessment): PMIDs 28215825, 32492433, 37067914
See for example:
Fig. 4 of PMID 28215825:
https://www.cell.com/cell-stem-cell/fulltext/S1934-5909(17)30031-0?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS1934590917300310%3Fshowall%3Dtrue
Fig. 2 of PMID 32492433:

https://www.cell.com/cell-reports/fulltext/S2211-1247(20)30641-0?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS2211124720306410%3Fshowall%3Dtrue
Figs 4-7 of PMID 37067914:
https://aacrjournals.org/view-large/figure/15259311/318fig4.jpeg
"harboring driver AML mutations": these cells are derived from clonal iPSC lines with documented AML mutations from AML patients, PMIDs 28215825, 32492433, 37067914
For example, see:
https://aacrjournals.org/view-large/figure/15259297/318fig2.jpeg
"lethal disease": PMIDs 28215825, 32492433, 37067914
"initiated by an HSPC population that should not normally show durable engraftment": As mentioned above, normal iPSC-HSPCs are not engraftable cells.

	Model	Figure in manuscript	Leukemic features			
			Engraftment of myeloid-restricted immature human cells	Harboring driver AML mutations	Initiated by an HSPC population that should not normally show durable engraftment	Lethal disease
#1	**Genetically engineered iPSCs**	Extended Data Figs 1 and 2 and Fig. 1a,b	1.2%–6.3% engraftment 13-15 weeks post-transplantation (Extended Data Fig. 1h) of CD33+ (Extended Data Fig. 1i) cells with immature morphology (Extended Data Fig. 1j)	The transplanted cells were derived from clonal triple mutant SAR iPSC lines	Transplantation of any normal genetically unmodified iPSC-derived HSPC population never produces detectable engraftment of any level	No
#2	**CB HSPCs with minimal in vitro culture**	Fig. 1c-g and Extended Data Fig. 3a-e	~10-50% engraftment (Extended Data Fig. 3e) of myeloid-restricted, i.e. >50% CD33+ (Fig. 1e) immature, i.e. >20% CD34+ (Fig. 1d) cells	Triple-transduced cells (Extended Data Fig. 3a,c)	No	Extended Data Fig. 3d
#3	**CB HSPCs with extended in vitro culture**	Fig. 1c-g and Extended Data Fig. 3a-e	~20-50% engraftment (Fig. 1j) of myeloid-restricted, i.e. >50% CD33+ immature, i.e. >20% CD34+ cells and/or immature blasts by morphology (we will add more panels)	Transduction efficiency (Extended Data Fig. 3g)	CB HSPCs are no logner engraftable after 11 days of in vitro culture	Fig. 1i
#4	**Sorted CMPs and GMPs from CB CD34+ cells**	Fig. 2g,i and Extended Data Fig. 5	~20-50% engraftment of myeloid-restricted, i.e. >50% CD33+ immature, i.e. >20% CD34+ cells and/or immature blasts by morphology (we will add more panels)	Transduction efficiency (Extended Data Fig. 5c-g)	Sorted GMPs and CMPs are committed progenitors without engraftment potential	Fig. 2g,i
#5	**CD34+ HSPCs derived from AML-iPSCs**	Figs 3a-d, 4f-h and 5h-i	Extensively documented in previous publications durable (13-15 weeks or lethal disease) engraftment of exclusively CD33+ blasts (by CD34 expression and/or morphological assessment): PMIDs 28215825, 32492433, 37067914	Derived from clonal iPSC lines with documented AML mutations from AML patients, PMIDs 28215825, 32492433, 37067914	Normal iPSC-HSPCs are not engraftable cells	PMIDs 28215825, 32492433, 37067914

Table 1. Leukemic features of each model used in this study.

In summary, we propose to address this comment and related subsequent comments by:

1. Clearly articulating the definition and criteria for documenting AML in xenograft models in the manuscript in the Methods section and/or at first appearance in the Results.

2. Introducing additional textual revisions in the Results to better guide the reader to understand the interpretation of data and conclusions.

3. Adding more figure panels to document engraftment of "myeloid-restricted immature human cells" by flow cytometry and morphology (Wright-Giemsa-stained cytospin preparations or H&E histology of mouse bone marrow) for all models.

4. We believe that the above clarifications and additional documentation of leukemic features will establish that we apply stringent, well-defined and uniform across experiments criteria to define AML in our xenograft assays and will alleviate the Referee's concerns. While serial transplantation is not a requirement to define AML, if it is perceived by the editorial team that it will strengthen the study, we can provide secondary transplantation data for 1 or 2 representative experiments (specific experiments indicated below in our responses to the Referee's subsequent comments).

#2- When looking at the order of the mutations, they also show that induction of N-Ras mutation only induce engraftment when added after SRSF2 and ASXL1. Nevertheless, later on they also show that the target of N-Ras effect is on GMP. The authors should first induced iPS differentiation, then introduced the expression of N-RAS and at the same point add or not SRSF2 and AXSL1.

RESPONSE: Here and in subsequent comments, the Referee raises concerns about our conclusions regarding the order of mutations. Below we explain our rationale for our experimental design and the interpretation of our results regarding mutational order.

- The starting point of our study is the well-documented observation that RAS mutations occur as late events in AML. By late events we mean that in the natural history of the disease development in patients RAS mutations are acquired at later stages of the disease, as documented in population genetics studies in large cohorts of AML patients: RAS mutations are acquired late in de novo AML or upon transformation of a prodromic disease, such as myelodysplastic syndrome, MDS, or myeloproliferative neoplasm, MPN, to AML – or during relapse of AML (note that RAS mutations are not always required for AML transformation, they can also appear for the first time at relapse). The evidence that RAS mutations are late events in human AML is extensive and emerges from 4 lines of evidence: (1) Differences in the frequency of mutations in AML compared to prodromic disease states, namely MDS and clonal hematopoiesis (CH), PMIDs 27992414, 30670442, 32430504. Mutations with similar frequencies across CH, MDS and AML (including de novo AML and sAML) occur early in the disease course, while mutations that are more common in AML than MDS arise later upon disease progression. The latter include RAS mutations. (2) Bulk DNA sequencing of targeted gene panels of the most recurrently mutated genes. Late mutations have significantly smaller variant allele frequencies (VAF) than earlier mutations within each sample. RAS mutations are almost always low-VAF subclonal (i.e. VAF<0.5) mutations. (3) Mutational analysis of paired longitudinally sampled cells from MDS patients who progressed to sAML show that RAS mutations are acquired upon progression, PMIDs 27992414, 22417201, 32430504, 23443460, 28429724, 25550361, 28090092. (4) More recent single-cell DNA sequencing studies have also shown that RAS mutations are typically subclonal, PMIDs 33116311, 33087716.

That RAS mutations are late events in human AML is thus a very well-documented indisputable fact.

- To understand why RAS is typically a late mutation (which is in contrast to the case of most solid tumors, as classically epithelial cancers are driven by RAS mutations as early truncal events) we sought to develop a human model that allows us to manipulate the mutational order and read out the outcome as induction of AML. We started with the iPSC SAR model from our previous study (Wang et al. Cell Stem Cell 2021) to obtain information on how to set up this model. From these initial experiments (Extended Data Fig. 1) we learned that the SAR combination of mutations is best and that both the S and A mutations are needed together with RAS ®.

- To then query mutational order we tested mainly two experimental configurations: RAS after the other mutations (SA+R) or before (R+SA). Acquisition of all driver mutations at the same time is not a plausible scenario in the development of human AML, as the chance of simultaneous acquisition of 3 driver mutations by the same cell at once is infinitesimally small, practically impossible. Our experiments were thus designed to test RAS before or after the SA mutations rather than at the same time. The R+SA mutational order did not generate AML in either iPSC-HSPCs (Fig. 1a,b) or CB HSPCs (Fig. 1h-l) (and therefore showed no detectable engraftment as iPSC-HSPCs and CB-HSPCs after 11 days of culture are non-engraftable unless transformed). We interpret this result to suggest that this experimental model captures at least one scenario where RAS mutation transforms HSPCs when acquired after the other mutations but not before and can account for the observed late occurrence of RAS mutations in human patients.

(As a side note, here, with regards to the experiment shown in Fig. 1c that the Referee mentions in their next comment, the "R-Early" group of this experiment is not testing RAS before the other mutations, like the experiments presented before and after this one. Rather in this case, RAS is induced with a short delay after SA – delay of ~24 h due to Dox induction, so as we discuss more below, this is not a very informative experiment regarding the order.)

- As the Referee stated in their original comments ("*They also show in Fig 1h that the time of induction of N-ras is important. This might indicate two options that N-Ras induced transformation only when induced at later stage or that N-Ras is transforming a progenitor cell.")*, one potential, in fact likely, explanation for why RAS is a late event could be that RAS mutation transforms progenitors that arise from an ancestral clone with previous mutations. The next key finding was that R+SA (as well as R alone and SAR, basically all groups with RAS as first mutation) have increased CMPs and MEPs and markedly decreased GMPs (Fig. 2a-f). This led us to test and find that it is the GMPs (depleted when RAS is the first mutation, but preserved in SA+R, when RAS comes after SA) that transplant AML. To then definitively test that RAS can transform GMPs with SA mutations, we performed the experiment suggested by the Referee in their original comments, namely inducing the mutations in sorted GMPs (Fig. 2g,i), which provides strong evidence that RAS mutation can transform GMPs harboring previous mutations.

- In view of the above, we consider this **finding that RAS mutation transforms GMPs with previously acquired mutations as a plausible explanation for why RAS mutation transforms only when acquired after and not before SA** in our models. In a later point the Referee states "*It seems that the transduction of GMP with SAR or the transduction of SA and then R induced the same results. This question whether the order of the mutation is indeed essential as the authors claimed. Indeed it seems that all three mutations could be induced at once as long as the target are GMP cells."* The finding that in experimental conditions when GMPs are the target cell of transformation there is no longer a requirement for RAS to be

induced after the other mutations is in fact in agreement with (and not antithetical to) our proposition that the targeting of mutant GMPs by RAS mutation can be the explanation for why RAS mutation transforms when acquired after other mutations and not before, as it targets a GMP that is the progeny of a more ancestral HSC or long-lived MPP with SA mutations. While the experiment with simultaneous induction of SAR in sorted GMPs proves that RAS mutations can transform GMPs with additional mutations, the simultaneous acquisition of all 3 mutations by a GMP is not relevant to human AML. This is not a scenario that can occur in human AML development, because GMPs are not self-renewing cells and the chance of simultaneous acquisition of 3 driver mutations by the same cell at once is infinitesimally small. Rather the relevant scenario in human AML is that the initiating mutation(s) are acquired by an HSC or a long-lived MPP which gives rise to mutant GMPs that can then acquire a RAS mutation. As we discuss in our manuscript, it is well-documented that initiating mutations are acquired by HSCs/MPPs.

- Finally, as we stated in the revised manuscript, we cannot exclude the scenario that RAS mutation can also transform an HSC/MPP that has previously acquired SA mutations – the scenario that the Referee is possibly referring to as the first of the two options in their previous statement: "*This might indicate two options that N-Ras induced transformation only when induced at later stage or that N-Ras is transforming a progenitor cell.*". In the previously revised manuscript we included a statement to clearly state that this is still a possible and not mutually exclusive scenario: First paragraph of Discussion (page 11): "While our studies do not exclude that RAS mutations may also originate in a more primitive HSC/MPP that can give rise to GMP LSCs…" So, while our data support the scenario whereby RAS transforms GMPs arising from HSCs/MPPs from a clone with ancestral mutations, it is also possible and non-mutually exclusive that RAS can be acquired in HSCs/MPPs that had previously acquired ancestral mutations. Importantly, as Referee #1 also noted in their original critique, this additional scenario does not affect any downstream studies and conclusions regarding monocytic differentiation and VEN resistance of RAS-mutant LSCs.

We should also point out that this potential additional scenario cannot be thoroughly and conclusively tested due to experimental constraints. CB CD34+ cells (without any culture) contain HSCs and progenitors (including GMPs) and these HSCs differentiate to give rise to more progenitors over time in culture. However culture is necessary for lentiviral transduction, as well as for temporal control of induction of the driver mutations. It is therefore hard to conceive and execute an experiment that could effectively and exclusively restrict SA+R transduction in HSCs, excluding the possibility that a progenitor cell is targeted, even with repeated sorting, and generating cells suitable for transplantation in a mouse. We sincerely hope that the Referee can appreciate the formidable technical challenges of the experiments in this manuscript, involving triple lentiviral transduction, FACS-sorting and transplantation and the amount of optimization and attention to detail they required to be successful. (Referee #2 noted that these are "impressive experiments".)

Thus, while we acknowledge that the targeting of a GMP by RAS may not be the only possible scenario, it is one that is supported by our data (sorted GMPs with SAR or SA+R transplant leukemia) and plausible based on patient data and based on previous literature showing that progenitors can be the target cells of transformation into LSCs by other oncogenes.

With regards to the specific experiment that the Referee is proposing here ("*The authors should first induced iPS differentiation, then introduced the expression of N-RAS and at the same point add or not SRSF2 and AXSL1.*"): We do not think that this experiment will be informative and cannot identify strong rationale for it.

If the Referee's rationale is to test the simultaneous introduction of all 3 mutations (SAR), we do not think that simultaneous SAR acquisition is relevant to human AML development, as we discuss above. Additionally, iPSCs do not generate bona fide HSCs, further complicating interpretation. If simultaneous SAR does not yield AML, it supports that RAS needs to be after SA; if alternatively it does generate AML in the experiment, this is not relevant for human AML (mutations are not acquired at once over the course of human AML). If the editorial team finds this experiment necessary or useful, we can do it, but we propose to do it using CB cells instead of iPSC-HSPCs so that we can also track the immunophenotypic HSPC populations.

In summary, for the reasons outlined above, we do not think that the suggested experiment will generate strong evidence regarding the importance of mutational order regardless of its outcome. However, if the editorial team finds it necessary, we can do it.

#3- Using CD34+ UCB, they show that the induction of R-early induced a lethal myeloproliferation, constitute of CD34neg cells. What do they conclude that these are not leukemia? Leukemia cells from patients and LSC in some patients could be CD34neg. The question is whether these cells could be transplanted back to a secondary recipient.

RESPONSE: As discussed earlier, we define AML in a xenograft as **engraftment of myeloid-restricted immature human cells harboring AML driver mutations** with >20% blasts as the cutoff – defined by immunophenotype or morphology.
We conclude that the CD34+ CB R-Early cells are not leukemia by comparison with the R-Late group of the same experiment. While both R-Early and R-Late cells give engraftment of myeloid-restricted human cells (Extended Data Fig. 3e and Fig. 1e) harboring all 3 driver mutations (Extended Data Fig. 3a,c), the R-Early mice have <20% immature (CD34+) cells, whereas the R-Late have >20% CD34+ cells (Fig. 1d). Therefore we consider R-Late cells as AML, **using the threshold of 20% blasts to parallel clinical guidelines for the diagnosis of human AML**. See also our response to the Referee's first point (Model #2: CB HSPCs with minimal in vitro culture).
While the Referee is correct that some AMLs are CD34neg, the fact that the R-Late group of the same experiment with the same mutational combination (S,A,R) gives a CD34pos leukemia makes this an unlikely scenario. Secondary transplantation is not a criterion of leukemia. A leukemia can be non-serially transplantable, and, conversely, a myeloproliferative neoplasm can be serially transplantable, for example see PMIDs 28159736 and 35259128.

Of note also, in the "R-Early" group of this experiment RAS mutation is induced at the same time as the SA mutations (in fact with a slight delay of ~24h, required for Dox to induce mutant RAS expression) and is thus distinct than the R+SA groups of the other experiments shown in the same Figure (Fig. 1), which show that R+SA does not generate leukemia (Fig. 1a,b in iPSC-HSPCs and Fig. 1h-l in CB HSCPs). For this reason, this experiment (which was the first experiment we performed with CB cells for this study) is not particularly informative with regards to the effects of mutational order and can be omitted from the manuscript.

In summary, we can address this comment by explaining the use of the cutoff of 20% blasts per clinical guidelines for the diagnosis of human AML. Alternatively, as this experiment is not particularly informative with regards to the effects of mutational order, we can also remove it (Fig. 1 panels c-g) without any impact on the conclusions or flow of the narrative of the manuscript.

#4- They then induce Nras before or after transduction of SA. In the late induction, all three lentivirus were transduced at the same time in CD34+ and only the induction of N-Ras was delayed via inducible vector used. In the other scenario, N-Ras was induced before. The transduction of SA after N-Ras, in this case questioned, whether the SA was not introduced to the HSC and thus none of the triple transduced cells were transplanted? The transduction efficient of SA on early induce N-Ras will need to be shown. Again, the question is whether by using constitutive vector for N-Ras but using inducible vectors for SA, the authors could indeed verify that their conclusion are correct. Also, it is surprising that in early R-SA, they show no human cells engraftment. As they injected UCB, it is quite surprising to see no engraftment of R-SA. Also, what characteristic of the engraftment of SA-R making them conclude that it is a leukemia.

RESPONSE:

"*The transduction of SA after N-Ras, in this case questioned, whether the SA was not introduced to the HSC and thus none of the triple transduced cells were transplanted? The transduction efficient of SA on early induce N-Ras will need to be shown. Again, the question is whether by using constitutive vector for N-Ras but using inducible vectors for SA, the authors could indeed verify that their conclusion are correct*."

The Referee here is concerned about our lentiviral strategy to induce SA after R using a second transduction instead of Dox-inducible SA vectors. Specifically, the Referee is concerned about the transduction efficiency of this approach and about whether the transplanted population contains indeed triple-transduced cells, including HSCs. We agree it is important to clearly demonstrate that we have carefully excluded these alternative explanations. Since we designed our lentiviral vectors with such concerns in our minds, we paired each transgene (S, A, R) with a different reporter gene (mCherry, GFP and ΔLNGFR, respectively) to facilitate easy evaluation of transduction efficiency by flow cytometry. We have used these to quantify and monitor the transduction efficiency and fraction of triple-transduced cells in every group of every experiment throughout the study. For reasons of readability and space, we included only representative panels from representative experiments in Extended Data Figs 3 and 5 before.

Here in Figure 1 below we show the transduction efficiency of the R+SA group per the Referee's request, showing **10-15% triple-transduced cells** – total, as well as within the HSC/MPP (CD34+/CD38-/CD45RA-) population.

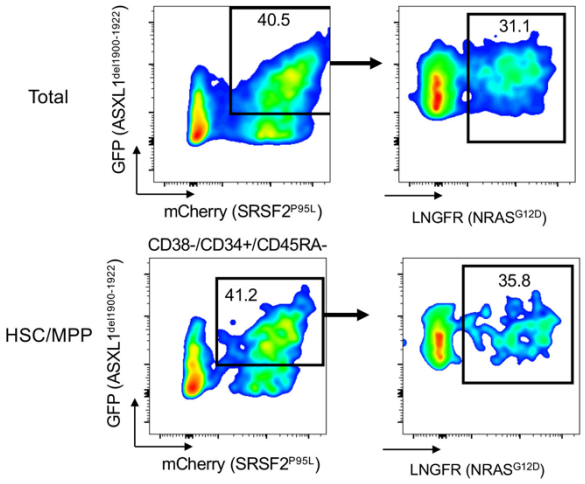


Fig. 1. Transduction efficiency of R+SA CB cells.

We believe that these data – which can be added to Extended Data Fig. 3 – should alleviate the Referee's concern.

"*Also, it is surprising that in early R-SA, they show no human cells engraftment. As they injected UCB, it is quite surprising to see no engraftment of R-SA*"
As we discussed in our response to the Referee's first point, in this experiment the CB cells of all groups were injected after 11 days of in vitro culture in standard HSPC expansion media. This results in loss of engraftment potential of normal cells and thus only transformed cells are able to engraft. The lack of engraftment of the R+SA cells thus indicates that the R+SA mutational order does not generate transformed AML initiating cells.

"*Also, what characteristic of the engraftment of SA-R making them conclude that it is a leukemia*."
Here again we use the same criteria stated earlier, namely **engraftment of myeloid-restricted immature human cells harboring AML driver mutations**. For clarity and consistency, we can add more panels in Extended Data showing the fraction of immature cells by flow cytometry (CD34) as in Figure 2 below, and/or by morphology and histology.

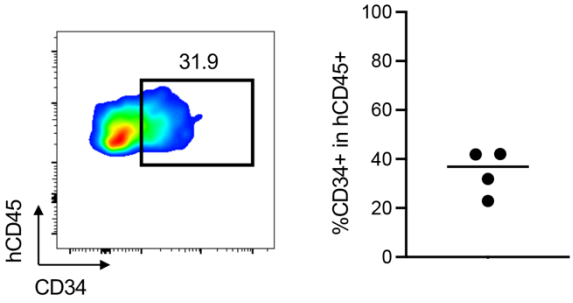


Fig. 2. Fraction of immature human cells (CD34+) in mice transplanted with SA+R CB cells.

In summary, we can address this comment as outlined in our response to the Referee's first point, i.e. by textual revisions to explain how we define AML, by adding panels in Extended Data Figures 3 and 5 showing the presence of triple-transduced R+SA cells in the transplanted population and the detailed leukemic features of the SA+R CB AML (myeloid-restricted and immature, by flow and/or morphology). Although secondary transplantation is not a criterion of AML, we can provide secondary transplantation data.

#5- By the transduction of CMP versus GMP, they show that GMP is the target to induce what they called "Leukemia". Leukemia should be better defined. Are there again transplantable in secondary recipient?

As in our response to the Referee's previous comments, **the definition of AML in a xenograft that we apply across all experiments throughout our study is: engraftment of myeloid-restricted immature human cells harboring AML driver mutations**. How these criteria apply to this specific experiment was outlined in our response to the Referee's first comment "Model #4: Sorted CMPs and GMPs from CB CD34+ cells".

As in our response to the Referee's point above in the case of unsorted SA+R cells, here again for the GMP-sorted SA+R cells, we can add more panels in Extended Data to clearly document

the “myeloid-restricted” and “immature” components of the AML definition, like those shown in Figure 3 below.

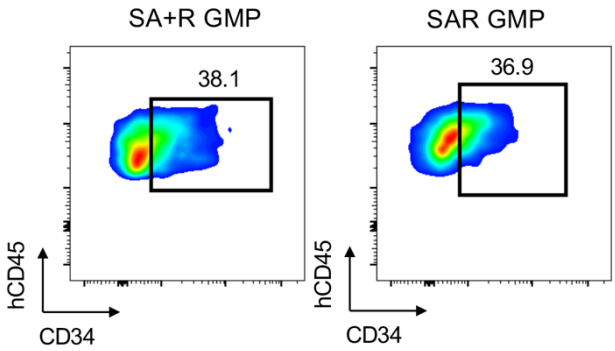


Fig. 3. Fraction of immature human cells (CD34+) in mice transplanted with sorted GMPs transduced with SA+R and SAR.

In summary, we can address this comment as outlined in our response to the Referee’s previous point and in our response to the Referee’s first point, i.e. by textual revisions to explain how we define AML, by adding panels in Extended Data Fig. 5 with more details on the leukemic features of the SA+R GMP-initiated AML (myeloid-restricted and immature, by flow and/or morphology). Although secondary transplantation is not a criterion of AML, we can provide secondary transplantation data for this experiment as well.

#6- It seems that the transduction of GMP with SAR or the transduction of SA and then R induced the same results. This question whether the order of the mutation is indeed essential as the authors claimed. Indeed it seems that all three mutations could be induced at once as long as the target are GMP cells.

RESPONSE:
We addressed this point earlier in our response to the Referee’s second point. Briefly:
We believe that the finding that in experimental conditions when GMPs are the target cell of transformation there is no longer a requirement for RAS to be induced after the other mutations is in agreement with and not antithetical to our proposition that the targeting of mutant GMPs by RAS mutation can be the explanation for why RAS mutation transforms when acquired after other mutations and not before, as it targets a GMP that is the progeny of a more ancestral HSC or long-lived MPP with SA mutations.

Furthermore, while the experiment with simultaneous induction of SAR in sorted GMPs proves that RAS mutations can transform GMPs with additional mutations, the simultaneous acquisition of all 3 mutations by a GMP is not relevant to human AML. This is not a scenario that can occur in human AML development, because GMPs are not self-renewing cells and the chance of simultaneous acquisition of 3 driver mutations by the same cell at once is infinitesimally small. Rather the relevant scenario in human AML is that the initiating mutation(s) are acquired by an HSC or a long-lived MPP which gives rise to mutant GMPs that can then acquire a RAS mutation. As we discuss in our manuscript, it is well-documented that initiating mutations are acquired by HSCs/MPPs.

We would also point out here that, based on this and previous comments by the Referee in this and their previous critique, there appears quite possibly to be a disconnect between what we

mean when we refer to the "order of mutations" – which is the order by which driver mutations are acquired over the natural history of AML development during the life of a patient – and the meaning that the Referee attributes to it, which appears to be an experimental succession of events separated only by time as in the sequential or simultaneous acquisition of mutations by the same cell(s). It is only under this light that we can understand the Referee's concern that our finding that GMPs can be transformed by simultaneous SAR transduction *"question whether the order of the mutation is indeed essential".* We believe this might be also the reason why the Referee seems to attribute weight on experimentally contrasting the successive vs simultaneous transduction (SA+R vs SAR) conditions – however, as we discussed above, this is not a relevant comparison, as there is no plausible scenario in which mutations are simultaneously acquired in leukemogenesis.

This point was addressed in our response to the Referee's point #2 and we can address with textual revisions/clarifications.

#7- They also investigate in a cohort of AML patients treated with DEC-10-VEN, whether the presence of monocytic versus non-monocytic features, correlated with response. They show that it does not per se, but the presence of Ras does correlate with non -responder. They then used iPS lines derived from the same patient and differentiate these iPS into CD34+ or monocytes as well as another iPS control or Ras mutant lines. In both cases, monocytes derived from normal or AML mutant clones are resistant to VEN contradicting that monocytes are not implicated in the resistance observed clinically. They also show that monocytic derived iPS have lower bcl2 and high MCL1 and thus are intrinsically resistant to VEN.

RESPONSE:
As we explain in detail below, **monocytes are resistant to VEN but do not drive clinical resistance**. It is exactly this apparent contradiction that our study sheds light into. This is our study's main point and major contribution to the field of AML: the **reconciliation of these seemingly contradictory findings** from the previous literature **into one coherent model of VEN resistance**, supported by the **key finding** of this study that **RAS pathway mutations drive both VEN resistance of LSCs and their monocytic differentiation**. Critically, it is the metabolic rewiring of the LSCs by mutant RAS that drives clinical relapse/resistance and not the monocytic differentiation, which is a confounder with a common cause (the RAS mutation). **It is the resistance of the RAS-mutant LSCs and not the resistance of the monocytes that determines the therapeutic response.**

Our model, schematically summarized in Extended Data Fig. 10, is powerful as, for the first time, it unifies and explains all prior observations. Not only do we solve a puzzle by offering a biologically meaningful and intellectually satisfying explanation that synthesizes all previous evidence, our findings furthermore have **far-reaching, real-world and immediate implications for clinical practice**: they indicate that patients with AML (and likely other myeloid malignancies) with RAS mutations should not be treated with VEN, at least not without concurrent chemotherapy, as VEN administration in these patients can likely accelerate disease progression. Thus timely publication of these findings can have a measurable **impact on many patients' lives**.

Below we briefly review the medical need and the seemingly contradictory observations reported in the literature until now and how our findings allow us to synthesize everything in one coherent model:

- VEN is a relatively new agent (BCL2 inhibitor) that in 2020 gained US FDA approval as combination therapy with hypomethylating agents (azacytidine, decitabine) or low-dose chemotherapy for patients with AML who are age 75 years or older, or who are ineligible for intensive induction chemotherapy. VEN has already widespread use, and its use is rapidly expanding, as it is an oral compound with limited toxicities that is well tolerated. Numerous ongoing clinical studies are aimed at expanding its use to additional indications, such as myelodysplastic syndrome (MDS), younger patients and even pediatric AML (which, of note, is enriched for RAS pathway mutations, compared to adult AML) and ALL.

- A significant fraction (~30% and as high as 50%) of patients who receive VEN/HMA combination therapy show resistance or relapse. Patients with VEN resistance do not benefit from VEN administration and instead lose precious time during which they could try other potentially beneficial treatments. There is therefore an urgent need to identify ways to predict who will respond and who is not likely to benefit and should be directed to other treatments earlier.

- Earlier efforts to uncover determinants of VEN resistance/relapse in AML patients reported an association between VEN resistance/relapse and monocytic differentiation of blasts (ref 24 in our manuscript). This observation, together with findings showing independently that monocytes are resistant to VEN ex vivo (refs 24-26 in our manuscript), led to the idea that monocytic differentiation of AML blasts per se causes resistance. However, this proposition was not compatible with extensive evidence that LSCs, and not more differentiated blasts, drive patient outcomes in AML and, specifically, that LSCs are the primary targets of VEN whose elimination determines the therapeutic outcome (ref 28 in our manuscript). In addition, other studies were not able to confirm the association between monocytic disease and adverse patient outcomes to VEN combination therapies (for example ref 28 in our manuscript). Independently, RAS mutations were found to be associated with poor VEN outcomes (ref 26 in our manuscript).

- Against this backdrop, our study provides a compelling model to synthesize and explain all these observations. The key to solving the puzzle of how monocytic blasts can be associated with adverse patient outcomes to VEN therapy without being themselves the targets of VEN, is our observation that RAS mutations generate LSCs that are VEN resistant (unlike RAS-WT LSCs that are sensitive to VEN) and that produce blasts with monocytic differentiation – the latter without having an impact in clinical outcomes. In other words, VEN resistant AML and monocytic AML are both parallel effects of RAS mutations with a common cause – the RAS mutation – but not causally related to each other.

- Thus, going back to the Referee's comment, all monocytes (normal, leukemic, RAS-MT or RAS-WT) are resistant to VEN in vitro, ex vivo or in vivo, because monocytes do not express BCL2, but instead express high levels of MCL1, and are thus resistant to BCL2 inhibition by VEN. However, critically, **monocytes are irrelevant to clinical responses, as they are differentiated non-self-renewing leukemic cells and are indeed not implicated in clinical resistance to VEN**, which is instead determined by the resistance of the RAS-MT LSCs. Again this is a finding of **high significance that will impact clinical practice and change clinical guidelines**.

- Finally, this study will put for the first time RAS mutations in the spotlight in human AML. The RAS/MAPK pathway is not considered an attractive target for AML, but our findings and the widespread use of VEN are already spurring the interest of industry developers of RAS and MEK inhibitors and can build rationale to initiate clinical trials of these inhibitors for VEN-resistant AML.

In summary, we believe that this point is already addressed in our manuscript, but we can carefully review the text and identify opportunities to get this message through more clearly and forcefully.

#8- They then investigate what they called LSC of Ras-WT versus MT. How they defined LSC is unclear. Even if some CD34+ iPS derived are able to engraft, what is the frequency of the LSC into the CD34+ fraction?

RESPONSE: The Referee here is referring to the data presented in Fig. 4f,g. These CD34+ are derived from iPSC lines AML-4.24 and AML-4.10 that have been extensively characterized in previous publications for LSC activity and frequency, specifically in Kotini et al. Cell Stem Cell 2017, PMID 28215825; Wesely et al. Cell Reports 2020, PMID 32492433 and Kotini et al. Blood Cancer Discov 2023, PMID 37067914, including with serial transplantation (Fig. 4F of Cell Stem Cell paper and Fig. 2I of Cell Reports paper) and limiting dilution experiments (Fig. S2E of Cell Reports paper). The frequency of engraftable LSCs is ~1:5,600 as measured by limiting dilution assay in NSG mice.

To be more accurate with the LSC definition, we can refer to these CD34+ iPSC-derived RAS-MT AML cells as “CD34+ cells” instead of “CD34+ LSCs”.

To further establish that RAS-MT LSCs are VEN resistant, we can provide data from a transplantation experiment of these RAS-MT CD34+ cells followed by **in vivo** VEN treatment, showing that RAS-MT cells are not eliminated by VEN, in contrast survive and expand in the animals and VEN does not extend survival (Figure 4).

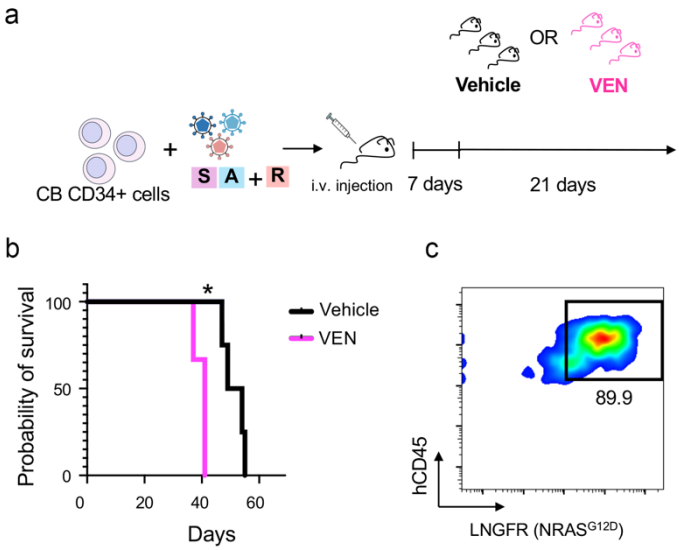


Fig. 4. In vivo resistance of RAS-MT AML to VEN. a, Experimental scheme. CB CD34+ cells transduced with SA+R were transplanted into NSG-3GS mice and treated with VEN or vehicle. **b,** Survival of the mice from a treated with VEN or vehicle. *p<0.05. **c,** 90% of cells at the time of death are NRAS G12D+.

In summary, we can address this point with additional in vivo VEN treatment data and text revisions to substitute “CD34+ LSCs” for “CD34+ cells”.

#9- Lastly, they also wanted to generalise that N/K-Ras confer resistance, they then overexpressed N-Ras on 4 patients derived AML iPS lines with different mutations background. Again, they differentiate these cells and mentioned that LSCs with N-Ras are more resistant. Unclear how LSC were defined. Also they showed before that N-Ras is not able to transform iPS with specific type of mutations like DNMT3A , FLT3 or Runx1 and ASXL1). How do they know that in that case N-Ras is able to transform these iPS lines? Are the ectopic expression of N-Ras in these lines changing their transforming capacity, their phenotype? Are overexpression of N-ras in CD34+ from UCB also provided a more resistant phenotype to normal HSC?

RESPONSE:
“*Unclear how LSC were defined*” The LSCs derived from the AML-iPSC lines used for these experiments, shown in Fig. 5h,i, have previously been characterized in Kotini et al. Blood Cancer Discov 2023, PMID 37067914 (ref 32 in our manuscript) with transplantation experiments, including secondary transplantation (Fig. 4l and Fig. 7 of BCD paper).

“*How do they know that in that case N-Ras is able to transform these iPS lines?*” These iPSC lines are already fully transformed AML (as evidenced in xenograft assays, see Kotini et al. Blood Cancer Discov 2023). The RAS mutation in this set of experiments models a relapse/resistance mutation, as it happens in many AML patients, and is not required for AML transformation.

“*Are the ectopic expression of N-Ras in these lines changing their transforming capacity, their phenotype*?” The RAS mutation is changing their VEN response and BCL2 family protein levels, as we demonstrate in Fig. 5h,i. As mentioned above, these lines are fully transformed. Other phenotypic changes possibly induced by RAS are not relevant to the experiment, which is specifically addressing whether RAS mutation confers VEN resistance and alters the BCL2 family protein expression profile of CD34+ cells. We have examined changes to molecular phenotype that can be attributed to RAS mutation in the experiments presented in Extended Data Fig. 9c,d and Fig. 2k-m.

“Are overexpression of N-ras in CD34+ from UCB also provided a more resistant phenotype to normal HSC?” Whether RAS mutations in CB HSCs confer VEN resistance is not relevant to this study, but we can perform this experiment easily and provide these data.

In summary, we believe that all the concerns here are addressed by previously published data. Although we do not find that the question whether RAS mutation will confer resistance to normal CB HSCs is relevant to our study, we can provide these data.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

One of the most significant advances in AML research over the past decade has been the realization that leukemia cell differentiation states profoundly affect drug sensitivity. The clearest example is Venetoclax. The prevailing view has been that monocytic cell states confer Venetoclax resistance, while some work has also suggested a role for genetics. The work submitted by Sango et al. represents a paradigm shift that integrates both views in a unified model that will advance the field with a profound and lasting impact.

It also deepens our understanding of leukemia stem cells (LSCs) by showing that LSCs exhibit multiple states along disease evolution. Since each state must be targeted to eradicate the disease, this demonstration will redirect targeted therapy investigations. The co-existence of genetically and phenotypically distinct human LSC fractions that propagate leukemia is an original and important innovation of this work.

In short, Sango et al. contribute critical conceptual advances that reconcile and advance the field. This is well-explained in their latest response letter, the bottom paragraphs of page 13.

Regarding the specific concerns described in the latest letter, Table 1 is very helpful in clarifying the models and engraftment parameters. I suggest adding the time to engraftment readout in all rows (under "Engraftment of myeloid-restricted immature human cells"). I agree that the simultaneous induction of NRAS, SRSF2 and ASXL1 will not be more relevant to human disease than the experiments that are already reported. While some models are more established than others, combined with mechanistic data and human cohort analysis, the authors meet the required evidence threshold to propose the unifying model in Extended Data Figure 10.

The minor comments from my previous review should still be addressed.

Referee #2 (Remarks to the Author):

In my opinion, the key novelty of this paper is the proposition that RAS-mutation acquired late in differentiation eg at the GMP phase, gives rise to monocytic disease, potentially from its ability to selectively drive expansion of GMP cells. It is important that this finding is proven separate to VEN resistance, as the latter has previously been reported to show drug-induced selection of cells with high MCL1 expression ie. monocytes.

The next question then becomes, have the authors definitively shown that RAS variants in a GMP cell of

origin AML consistently leads to monocytic disease and is this phenomenon particular to RAS activation, or could other activating variants cause this also eg FLT3-ITD etc.

In Figure 2F, my interpretation is that SA+ control results in monocytic lineage disease and that this is not augmented by SA+R. Therefore, I find these results unhelpful in supporting the hypothesis. This should be clarified

Figure 3j,k,m,n shows a correlation between monocytic AML in patients and presence of a variant affecting the RAS.

Extended Fig 4f shows a monocytic signal from SA + R using ATAC seq reads.

To complete this paper, it would be nice to see
- SA + different types of RAS eg NRAS or KRAS to see how reproducible this monocytic pattern is
- Clear delineation of what is the proposed mechanism of monocytic expansion- is it RASmut driving a transcriptional or epigenetic monocytic program or does RASm drive expansion of a clone already destined to have monocytic lineage.
- If the former, can the authors show that SA + other proliferative drivers fail to drive this monocytic phenyotype.
- If the RASm was reversed, is the monocytic phenotype dependent on RAS activation or not.

Referee #3 (Remarks to the Author):

I would like to thank the authors for providing new clarification on several points I raised during the second revision.
As the revised manuscript as far as I can tell as not be changed since the first revision, I will make this final recommendation.

1- I will indeed recommend that the manuscript should include a definition and criteria for documenting AML in xenograft models at first appearance in the Results as well as documenting the engraftment of “myeloid-restricted immature human cells” by flow cytometry and/or morphology for all models.
2- To further confirm the importance of the order of the mutations in leukemic transformation, they used UCB and induced RAS early or late. Despite clarifying why early was depicted as myeloproliferative instead of AML has been justified because of the cut-off used in clinic, it is not necessarily clear whether this definition is relevant in the context of xenotransplantation as by injected AML patients’ samples in NSG mice, you might not have always engraftment of leukemic cells that are >20%. As noted by the authors, this exp does not necessary addressed the order of the mutation, I will be in favor of removing this UCB experiment.
3- Related to the exp where they show the transduction of SA+R or SAR in GMP, they should indeed add additional FACS data in Extended Data Fig. 5 with more details on the leukemic features of the GMP-

initiated AML (myeloid-restricted and immature, by flow and/or morphology). In my view, it will also be important to provide secondary transplantation data for this experiment as well, as the mutations could possibly only extend the engraftment potential of GMP without transforming them LSC. To show the presence of leukemic initiating cells capacity of these GMP, they should be able to reinitiate leukemia upon transplantation in secondary mice.

4- In response to some of my comments concerning Ven resistance, they provided an interesting new data (figure 4 in their response) of UCB CD34+ being transduced by SA+R and then treated with Ven, where they show that the mice died rapidly even with VEN treatment and that all cells are expressing RAS mutation. I will recommend adding this new data in to paper.

Author Rebuttals to Second Revision:

We thank all Referees for their continued commitment to this Review, which has significantly strengthened and improved our study. We have made further revisions and included additional data that we hope should address all remaining Referees' concerns.

A detailed point-by-point response is provided below. The Referees' comments are in *Italics*. All changes in the manuscript text are marked in blue font.

Referee #1:

Sango et al have substantially improved the manuscript by increasing replicates, adding experiments, and clarifying the text. It's interesting and supportive that sorted CMPs do not transform upon RAS transduction but GMPs do. The clear distinction between the ancestral cell-of-origin and the subclonal RAS-mutated LSC is appreciated. My remaining minor comments and suggestions are as follows:

Sometimes, the authors include PTPN11 as an NRAS/KRAS pathway gene; sometimes, they do not. It would be helpful to be consistent, especially going forward if the presented insights are going to be used for treatment decisions.

RESPONSE: The only part of the manuscript where we have included data on PTPN11 as a RAS-pathway mutation are the AML patient cohort analyses showing correlation of these mutations with monocytic phenotype, presented in Fig. 3j,k. Of note, in these analyses, we include data on mutational status of each individual gene of the pathway separately (NRAS, KRAS and PTPN11), which clearly allow the reader to appreciate each individual gene's contribution to this phenotype. We have not included PTPN11 in any other experiments in the manuscript, and refer to "*N/KRAS*" mutations in the experiments that address VEN resistance (in vitro, in vivo or in clinical data). We appreciate the Referee's suggestion to be consistent, for which purpose we would have to just remove the "PTPN11" group from panels 3j and 3k (which are the only data that include PTPN11). We think that these data are more useful than distracting, as they show that PTPN11 mutations also associate with monocytic disease in patients, something that to our knowledge has not been shown before. To avoid any confusion, to clearly state that our findings only definitively implicate *N/KRAS* (and not *PTPN11*) mutations in VEN resistance we have changed the title of the Results paragraph "**RAS-mutant AML LSCs are resistant to Venetoclax** to "***N/KRAS*-mutant AML LSCs are resistant to Venetoclax"**. We also changed "RAS-MT" to "*N/KRAS*-MT" in instances of the Discussion that refer to VEN resistance and implications for clinical practice: "The resistance of *N/KRAS*-MT LSCs to VEN implies that combination therapy with VEN may have limited benefit for patients with preexisting

N/KRAS mutations and may even accelerate disease progression by promoting the growth of the *N/KRAS*-MT subclone."

In several places, the authors should add "n=…" to the legends.

RESPONSE: We added information on number of replicates in the legends of Figs 1b, 1d, 1e, 1i, 1j, 1k, 2d, 2g, 2i, 4g, 4l, 5e, 5h, and Extended Data Figs. 1c-h, 2e, 3b,c, 5f,g,h, 9e. Additionally, information on n numbers for panels j-n of Figure 3 is displayed on the figure.

Some of the genes in Supplementary Table 3 are interesting. I suggest highlighting a few on the right of Figure 2m (ETS1, ID2, IL1B…author's discretion).
RESPONSE: We agree and highlighted the genes ETS1, ID2, IL1B, IL2RG, ETV4, TRAF1, DUSP6 and CBL in Fig. 2m, following the Referee's suggestion.

Page 10: "Furthermore, this effect of RAS mutations…" – please clarify the effect being referred to. Also, check the referral to Fig. 5e at the end of the sentence.

RESPONSE: We rephrased "this effect of RAS mutations" to "VEN resistance endowed by RAS mutations" to clarify this sentence, which now reads "this VEN resistance endowed by RAS mutations does not seem to be restricted to GMPs, but occurs across HSPC types". The referral to Fig. 5e shows that RAS mutations (SA+R) confer resistance to all sorted cell types tested (CMPs, GMPs and HSC/MPPs).

In my opinion, given the limitations of the model systems, the word "must" should be removed on Page 3 in the following context: "N/KRAS mutations MUST occur late in AML pathogenesis."

RESPONSE: We removed the word "must" from this sentence.

The authors do not discuss why some patients develop Venetoclax resistance in the absence of RAS mutations. This point might be of sufficient interest to include.

RESPONSE: We have included the following sentence in our Discussion: “The emergence of VEN resistance has also been associated with other progression mutations in AML, such as FLT3 and TP53 mutations[53,54]. FLT3-ITD has been shown to induce higher expression of MCL1 and BCL-xL[55,56]. It would be interesting to investigate whether LSCs harboring other progression mutations are, similarly to RAS-MT LSCs, resistant to VEN and have altered BCL2 family expression profiles. Consistent with our proposition that VEN resistance and monocytic differentiation are independent processes, FLT3-ITD mutations are not associated with monocytic differentiation, and, on the contrary, appear to give rise to more primitive leukemic blasts[16,32,57].”

Referee #1 comments to Rebuttal:

One of the most significant advances in AML research over the past decade has been the realization that leukemia cell differentiation states profoundly affect drug sensitivity. The clearest example is Venetoclax. The prevailing view has been that monocytic cell states confer Venetoclax resistance, while some work has also suggested a role for genetics. The work submitted by Sango et al. represents a paradigm shift that integrates both views in a unified model that will advance the field with a profound and lasting impact.

It also deepens our understanding of leukemia stem cells (LSCs) by showing that LSCs exhibit multiple states along disease evolution. Since each state must be targeted to eradicate the disease, this demonstration will redirect targeted therapy investigations. The co-existence of genetically and phenotypically distinct human LSC fractions that propagate leukemia is an original and important innovation of this work.

In short, Sango et al. contribute critical conceptual advances that reconcile and advance the field. This is well-explained in their latest response letter, the bottom paragraphs of page 13. Regarding the specific concerns described in the latest letter, Table 1 is very helpful in clarifying the models and engraftment parameters. I suggest adding the time to engraftment readout in all rows (under "Engraftment of myeloid-restricted immature human cells"). I agree that the simultaneous induction of NRAS, SRSF2 and ASXL1 will not be more relevant to human disease than the experiments that are already reported. While some models are more established than others, combined with mechanistic data and human cohort analysis, the authors meet the required evidence threshold to propose the unifying model in Extended Data

Figure 10.

RESPONSE: We are grateful to the Referee for their appreciation of our work' impact in the field. We added time to engraftment readout in all rows (under "Engraftment of myeloid-restricted immature human cells") in the table which is now included in SI as Supplementary Table 1.

The minor comments from my previous review should still be addressed.

RESPONSE: These were addressed above.

Referee #2:

I thank the authors for their comprehensive and detailed responses to my review and the high quality of the added data, which I agree has led to a substantially more impressive manuscript.

In Figure 2a: this looks like transduction by SA, yet there are 3 colors of virus shown. Is the red NRAS G12D virus present in error?

RESPONSE: We thank the Referee for noticing this, indeed this part of the schematic included the red virus in error. It has now been removed.

Regarding the comment:
Strikingly, the LSCs of the RAS-MT clone had reduced BCL2 expression compared to the LSCs of the RASWT clone, as well as potentially higher MCL1 and BCL2L1 expression and lower expression of pro-apoptotic BAX (Fig. 4h, upper panels).
As neither of the MCL1 or BCL2L1 expression changes in LSCs are significant, should this statement be amended?

RESPONSE: Indeed the MCL1 and BCL2L1 expression changes are statistically not significant, but these scRNA-Seq data, comparing LSCs, which is a small cell population, are also limited in statistical power due to dropout. We therefore consider that the trends in gene expression, albeit not reaching statistical significance, are worth commenting on. We previously described them as

"potentially higher", so as not to overstate. We now changed this to "and showed a trend towards" to be more clear.

Fig 4h: What is the expression alteration in MCL1 that is significantly different in monocytic cells? Is it higher MCL1 expression related to WT or KRAS mut cells? By eye it looks higher in the WT population.

RESPONSE: Indeed Fig 4h, second panel in lower row, shows that MCL1 expression is higher in the WT monocytic cells. Regardless, MCL1 expression is very high in both KRAS-MT and WT monocytic cells, which are all consequently VEN resistant (regardless of mutational status), as we show in Fig. 4g and others have shown before (for example Pei et al. Ref 24 of our manuscript). This instance is potentially a consequence of the nature of scRNA-Seq data that creates the reverse problem than the one discussed in the previous comment of the Referee, i.e. here, because monocytic cells are a very abundant population in the sc data, even small differences may reach statistical significance, even though they are not biologically important. In other words, statistical differences in MCL1 expression in the monocytic cells among genotypes are inconsequential, as all monocytic cells express MCL1 at high enough levels to be VEN resistant. This again underscores the value of ascertaining these findings on VEN resistance using diverse orthogonal approaches, combining different cellular models in vitro and in vivo and AML patient-derived datasets, as we employ in this manuscript.

Referee #2 comments to Rebuttal:

In my opinion, the key novelty of this paper is the proposition that RAS-mutation acquired late in differentiation eg at the GMP phase, gives rise to monocytic disease, potentially from its ability to selectively drive expansion of GMP cells. It is important that this finding is proven separate to VEN resistance, as the latter has previously been reported to show drug-induced selection of cells with high MCL1 expression ie. monocytes.

The next question then becomes, have the authors definitively shown that RAS variants in a GMP cell of origin AML consistently leads to monocytic disease and is this phenomenon particular to RAS activation, or could other activating variants cause this also eg FLT3-ITD etc.

In Figure 2F, my interpretation is that SA+ control results in monocytic lineage disease and that this is not augmented by SA+R. Therefore, I find these results unhelpful in supporting the hypothesis. This should be clarified
Figure 3j,k,m,n shows a correlation between monocytic AML in patients and presence of a variant affecting the RAS.
Extended Fig 4f shows a monocytic signal from SA + R using ATAC seq reads.

To complete this paper, it would be nice to see
- SA + different types of RAS eg NRAS or KRAS to see how reproducible this monocytic pattern is
- Clear delineation of what is the proposed mechanism of monocytic expansion- is it RASmut driving a transcriptional or epigenetic monocytic program or does RASm drive expansion of a clone already destined to have monocytic lineage.
- If the former, can the authors show that SA + other proliferative drivers fail to drive this monocytic phenotype.

- If the RASm was reversed, is the monocytic phenotype dependent on RAS activation or not.

RESPONSE: Here the Referee is proposing that perhaps the SRSF2 and/or ASXL1 mutations play a role in the monocytic bias and that RAS mutations augment this by possibly driving expansion of monocytic-biased cells. This is an interesting proposition and a reasonable scenario to consider given the frequent presence of SRSF2 and ASXL1 mutations in other myeloid malignancies with predominant monocytic features, such as CMML and JMML.

However, our data provide strong support for a monocytic lineage-defining role of RAS mutations, and, importantly, in the context of a variety of ancestral mutations (other than SRSF2 or ASXL1). Specifically:

1. Our scRNA-Seq data presented in Extended Data Fig. 6i and Extended Data Fig. 9c show that RAS-mutant GMPs upregulate monocytic lineage genes (such as CD14, S100A8, S100A9 etc) and reciprocally downregulate granulocytic genes (such as MPO, AZU1, ELANE). This is not the case with SA alone. SA mutations alone (without R) do not upregulate monocytic lineage genes in CB GMPs (Extended Data Fig. 6i and Extended Data Fig. 9b,c). This is consistent with RAS mutation driving monocytic differentiation of GMPs at the expense of granulocytic differentiation. We further show upregulation of inflammatory pathways, NFkB and TNFa signaling in GMPs with RAS mutation that could account for this skewing (Fig. 2k,l, Extended Data Fig. 9d).

2. By examining a large patient cohort of ~500 AML patients we found strong association between RAS pathway mutations (including NRAS, KRAS and PTPN11) with both a higher fraction of CD14+ monocytic blasts and higher frequency of FAB M4 and M5 AML (Fig. 3j,k). In contrast, we found no association between *ASXL1* mutations, *SRSF2* mutations or their combination and monocytic features (CD14+ monocytic blasts or FAB M4 and M5 AML) (Fig. 3l,m).

3. As the Referee suggested previously, we analyzed all 399 patients with CMML (classified based on WHO 2016 guidelines) from the IWG cohort of 3328 MDS patients from *Bernard et al. 2022 NEJM Evidence* and found that RAS mutations frequently co-exist with mutations of ASXL1 and SRSF2 in human CMML (Extended Data Fig. 6g).

In addition, data by several other groups – recently published or close to publication – provide independent support for the association between RAS mutations and monocytic disease in AML (albeit without direct experimental evidence that we provide here and without addressing the GMP target cell of transformation aspect like we do). Specifically:

1. The *Miles, Bowman et al. Nature 2020* paper by Ross Levine's group (PMID 33116311) using single-cell DNA sequencing coupled with cell surface markers found very strong positive association between *NRAS*, *KRAS* and *PTPN11* mutations and CD11b expression and reciprocally negative association with CD34 expression. Please see Fig. 4b of that paper: https://www.nature.com/articles/s41586-020-2864-x/figures/4

2. The *Zeng et al. Nat Med 2022* paper by John Dick's group (PMID 35618837) also reported very strong association between *N/KRAS* mutations and mature AML phenotype. Please see Fig. 2e of that paper: https://www.nature.com/articles/s41591-022-01819-x/figures/2

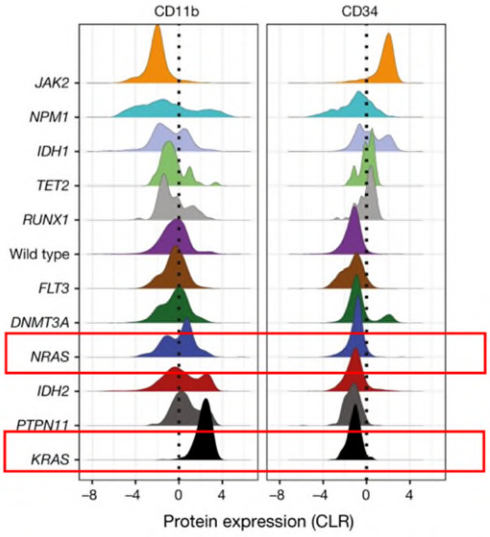


Fig. 4b from Miles, Bowman et al. *Nature 2020*

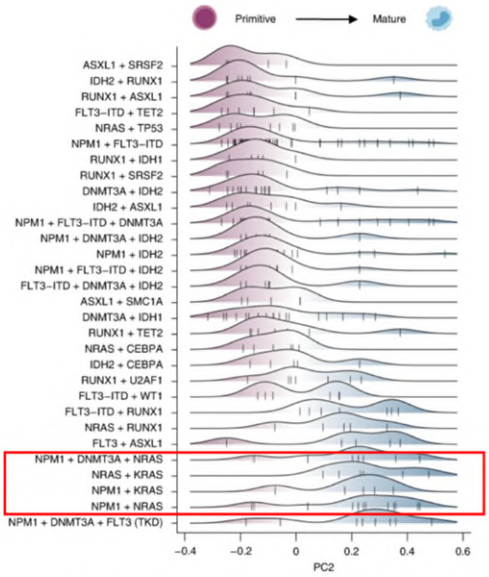


Fig. 2e from Zeng et al. *Nat Med 2022*

3. A manuscript accepted in *Blood* by co-author Elli Papaemmanuil (*Bernard et al.*) also reports strong association between RAS pathway mutations and monocytosis in patients with MDS. Interestingly, this study also found a weaker, but statistically significant association between SRSF2 mutations and monocytosis and no association between ASXL1 mutations and monocytosis in MDS patients.

Leukocytosis

Mutation	N.	Odds ratio	p
RAS.pathway	500	6.34 (4.59, 8.77)	<0.001
EZH2	201	2.90 (1.74, 4.76)	<0.001
SRSF2	526	1.90 (1.26, 2.85)	0.002
TET2.biallelic	463	1.86 (1.24, 2.79)	0.003
ASXL1	812	1.78 (1.25, 2.54)	0.001
RUNX1	413	1.57 (1.06, 2.31)	0.023
IDH2	136	1.30 (0.63, 2.54)	0.457
TET2.other	509	1.05 (0.65, 1.65)	0.845
U2AF1	262	0.86 (0.47, 1.49)	0.611
SETBP1	108	0.76 (0.37, 1.47)	0.438
SF3B1	708	0.55 (0.32, 0.91)	0.026
STAG2	272	0.34 (0.18, 0.59)	<0.001
ZRSR2	163	0.33 (0.12, 0.76)	0.016

0.2 0.5 1 2 5

Monocytosis

Mutation	N.	Odds ratio	p
RAS.pathway	500	3.96 (3.10, 5.05)	<0.001
EZH2	201	0.95 (0.60, 1.47)	0.82
SRSF2	526	1.75 (1.30, 2.35)	<0.001
TET2.biallelic	463	4.66 (3.54, 6.14)	<0.001
ASXL1	812	1.35 (1.04, 1.75)	0.03
RUNX1	413	1.26 (0.92, 1.70)	0.14
IDH2	136	1.57 (0.92, 2.59)	0.09
TET2.other	509	1.14 (0.83, 1.57)	0.41
U2AF1	262	1.00 (0.65, 1.48)	0.98
SETBP1	108	0.95 (0.54, 1.60)	0.85
SF3B1	708	0.89 (0.66, 1.19)	0.43
STAG2	272	0.20 (0.11, 0.33)	<0.001
ZRSR2	163	1.36 (0.88, 2.06)	0.16

0.2 0.5 1 2 5

From Bernard et al. In Press in *Blood*

4. Finally Craig Jordan's group recently described a so-called "monocytic LSC" (*Pei et al. Cancer Discov 2023*), transcriptionally resembling a GMP/Promonocyte, that is associated with monocytic AML and purported to drive resistance to VEN. While the mutational status of this "mLSC" was not investigated in that study, we think that it is very likely that at least some cases of these mLSCs correspond to the RAS-mutant GMP/Promono-type LSCs that we report in the present manuscript.

On the other hand, there is strong published evidence that FLT3-ITD mutations do not cause monocytic differentiation but the opposite, they associate with more primitive AML phenotype. We have shown this in our recent *Kotini et al. BCD, 2023* paper by comparing xenografts from a pair of isogenic AML-iPSC lines with and without FLT3-ITD using scRNA-Seq (https://aacrjournals.org/view-large/figure/15259321/318fig7.jpeg).

Association between FLT3-ITD and primitive AML phenotype is also reported in *van Galen et al. Cell, 2019* (shown in Fig. 5E:

https://www.cell.com/cms/attachment/2fd99f16-4f8f-4b97-ae4c-2e09539572fc/gr5.jpg)

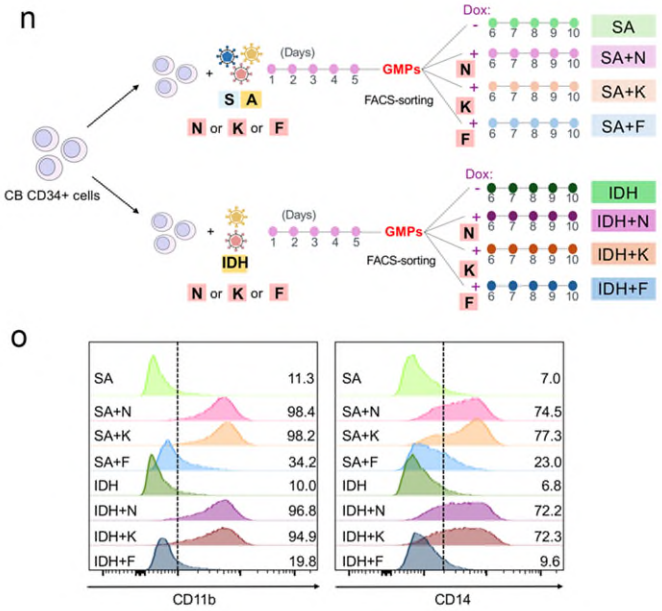
and in *Zeng et al. Nat Med, 2022* (in Fig. 2e: https://www.nature.com/articles/s41591-022-01819-x/figures/2).

All this evidence withstanding, to further address the Referee's concern, we performed new experiments and provide new experimental data that we now present in new Fig. 3n,o and Extended Fig. 6g,h and describe in the Results as follows:

"Furthermore, lentiviral expression of either $NRAS^{G12D}$ or $KRAS^{G12D}$ in CD34+/CD45+ cells from 4 patient-derived AML-iPSC lines of different genetic groups: splicing factor (SF)-mutated (AML-47.1); core binding factor (CBF, AML-37.1); del7 (AML-4.24, see also Fig. 3a) and MLL-rearranged (MLLr, AML-9.9)[32] induced myeloid maturation in vitro (Extended Data Fig. 6f)."

And:

"To test a potential contribution of the *SRSF2* and *ASXL1* mutations to the *N/KRAS* mutation-driven monocytic phenotype, sorted CB GMPs with *SRSF2* and *ASXL1* (SA) or an IDH1^{R132H} transgene were transduced with NRAS^{G12D}, KRAS^{G12D} or FLT3-ITD (Fig. 3n and Extended Data Fig. 6h). The *NRAS* and *KRAS* mutations potently drove monocytic differentiation of GMPs regardless of the initiating mutations (*SRSF2+ASXL1* or *IDH1*) (Fig. 3o). SA or IDH1 mutations



alone did not cause monocytic differentiation and neither did the FLT3-ITD mutation (Fig. 3o)."

We believe that all the above (our previous data, our new data, and data by others) collectively provide very strong evidence to support that indeed both NRAS and KRAS mutations drive monocytic differentiation of GMP-type LSCs with a broad range of ancestral mutations, while the other prominent AML signaling mutation, FLT3-ITD, does not have this effect.

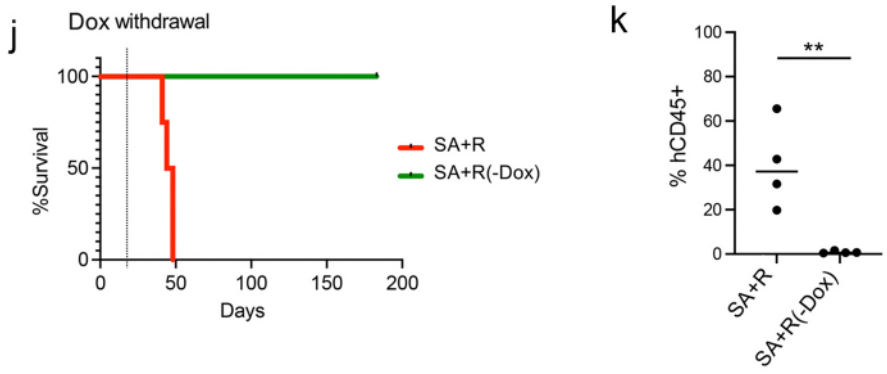
While it is possible that some interactions may exist between initiating mutations and RAS mutations (or other progression mutations) that modify these effects that future studies can interrogate, all the data by us and others point to RAS mutations being a very strong driver of monocytic differentiation in AML and our new data clearly show that they do so by driving a monocytic program in mutant GMPs (new Fig. 3o).

From Revised Fig. 3:

n, Experimental scheme.

o. Flow cytometry for the indicated myelomonocytic markers in GMPs transduced with various lentiviral

Finally, we also included a new experiment in which we reversed the *NRAS* G12D mutation in CB SA+R cells after transplantation. This experiment, shown in new Fig. panels Fig. 1j,k, shows that reversal of the *NRAS* mutation completely abrogates the leukemia.



From Revised Fig. 1:

j, Survival of mice injected with CB CD34+ cells transduced with SA+R. Red line shows survival of animals maintained in Dox throughout i.e. with continuous $NRAS^{G12D}$ expression (n=4); green line shows survival of mice in which Dox was withdrawn 14 days after transplantation (n=4).

Referee #3:

I would like to thank the authors for providing new clarification on several points I raised during the second revision.
As the revised manuscript as far as I can tell as not be changed since the first revision, I will make this final recommendation.

RESPONSE: We sincerely thank the Referee for accepting our suggested revisions. We have made all requested changes and added all the new data requested to the revised manuscript, as detailed below.

1- I will indeed recommend that the manuscript should include a definition and criteria for documenting AML in xenograft models at first appearance in the Results as well as documenting the engraftment of ???myeloid-restricted immature human cells??? by flow cytometry and/or morphology for all models.

RESPONSE: We rewrote the relevant parts of the Results to better explain the results in the xenografts regarding leukemic engraftment, included a new Supplementary Table

(Supplementary Table 1) with the criteria and detailed descriptions of the characteristics of each model and added new figure panels that document engraftment of myeloid-restricted immature blast-like human cells harboring AML driver mutations for all models. The new panels are: Fig. 1d, and f-k, Fig. 2j-l, Extended Data Fig. 3a-c and Extended Data Fig. 5f-h. Additional control groups have now been added to the experiments shown in the second half of Fig. 1, namely mCherry/GFP and SA-only controls (in addition to the SA+R, R+SA groups), showing lack of or very minimal engraftment of CB CD34+ cells from these groups after the period of in vitro culture used in these experiments (Fig. 1c,d).

2- To further confirm the importance of the order of the mutations in leukemic transformation, they used UCB and induced RAS early or late. Despite clarifying why early was depicted as myeloproliferative instead of AML has been justified because of the cut-off used in clinic, it is not necessarily clear whether this definition is relevant in the context of xenotransplantation as by injected AML patients??? samples in NSG mice, you might not have always engraftment of leukemic cells that are >20%. As noted by the authors, this exp does not necessary addressed the order of the mutation, I will be in favor of removing this UCB experiment.

RESPONSE: This experiment has been removed (previous panels Fig. 1c-g).

3- Related to the exp where they show the transduction of SA+R or SAR in GMP, they should indeed add additional FACS data in Extended Data Fig. 5 with more details on the leukemic features of the GMP-initiated AML (myeloid-restricted and immature, by flow and/or morphology). In my view, it will also be important to provide secondary transplantation data for this experiment as well, as the mutations could possibly only extend the engraftment potential of GMP without transforming them LSC. To show the presence of leukemic initiating cells capacity of these GMP, they should be able to reinitiate leukemia upon transplantation in secondary mice.

RESPONSE: We have added new panels to document engraftment of myeloid-restricted immature blast-like human cells harboring AML driver mutations, including secondary transplantation of the SA+R GMPs. These data are shown in Fig. 2j-l and Extended Data Fig. 5f-h. We have also included additional control data showing lack of engraftment of CMPs or GMPs without RAS mutation (-Dox) (Extended Data Fig. 5f,g).

4- In response to some of my comments concerning Ven resistance, they provided an interesting new data (figure 4 in their response) of UCB CD34+ being transduced by SA+R and then treated with Ven, where they show that the mice died rapidly even with VEN treatment and that all cells are expressing RAS mutation. I will recommend adding this new data in to paper.

RESPONSE: We added this new data to the revised manuscript as new Fig. 4k-m panels and described in the Results section as follows: “To further test if RAS-MT LSCs are VEN-resistant in vivo, we treated mice transplanted with CB SA+R cells with VEN for 3 weeks (Fig. 4k). All mice succumbed to lethal leukemia, which was accelerated in VEN-treated, compared to control (vehicle-treated), animals (Fig. 4l). Almost all leukemic cells retrieved from these mice expressed the $NRAS^{G12D}$ transgene (Fig. 4m).”

Reviewer Reports on the Third Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have appropriately addressed all my concerns.

Referee #2 (Remarks to the Author):

I thank the authors for their responses. My concerns have been adequately addressed, particularly the nice new data showing that SA or IDHm-->KRAS both cause monocytic phenotype and that FLT3-ITD as the final "hit" does not. I feel this additional data has positively improved the paper's strength in making this claim. The only thing missing perhaps is the negative control showing that WT RAS does not cause the monocytic phenotype as further proof that the phenotype is dependent on the kinase function of the protein.

Referee #3 (Remarks to the Author):

In this second revision the authors have addressed all my comments and I believe the quality of the paper has been improved compared to the original version.