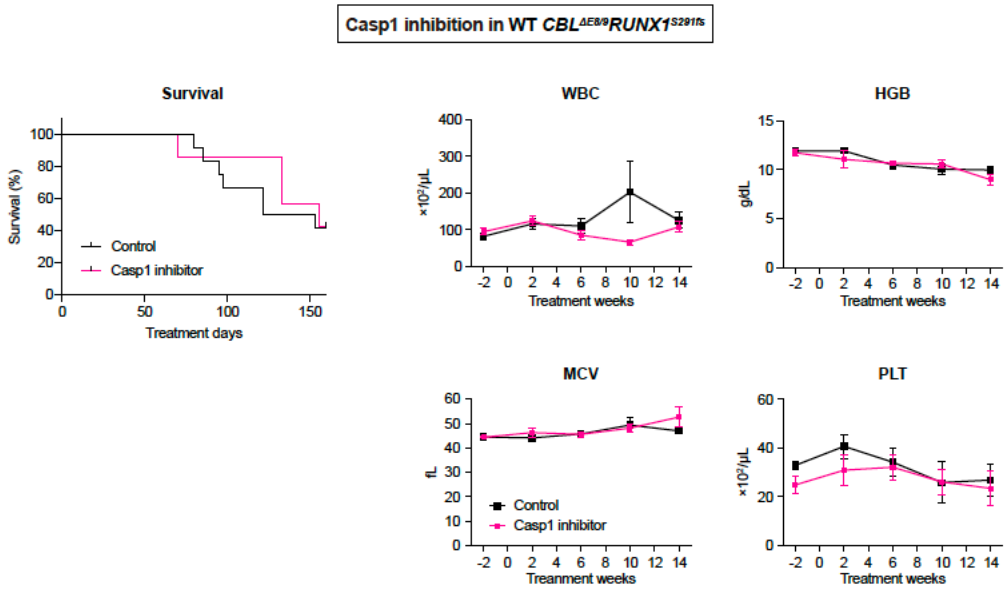
Referee #1 (Comments to the Author):

Andoh et al have submitted a manuscript entitled 'Inflammasome activation promotes the progression of myelodysplastic syndrome'. As motivation for this study, the authors cite literature that has shown elevated levels of pyroptosis in MDS bone marrow and associated cytokines IL-1b and IL-18 in the peripheral blood of patients with MDS. Genetic or pharmacologic studies implicating the inflammasome pathway on MDS pathogenesis are limited. Thus, the authors utilized a previously established transplantation-based model of MDS by lentivirally overexpressing CBL and RUNX1 mutations in cKit+ HSPCs. In this model, mice develop cytopenias and succumb to MDS or transformation to AML within 7 months. The authors were able to establish this model on an Asc+/+ or Asc-/- background and show that while Asc loss does not affect normal hematopoiesis, it does improve survival of mice, though they ultimately succumb to MDS or AML. The authors go on to suggest inflammasome signaling is activated in both progenitor and differentiated hematopoietic cells and show by transcriptional analysis that certain inflammatory signatures are upregulated in an Asc dependent manner. Last, the authors show that Asc loss does not affect normal or stress hematopoiesis, suggesting its relevance is unique to the MDS/AML context.

While there have been numerous analyses reporting inflammasome activation in patients with MDS and AML, functional studies in mouse models in which the inflammasome is genetically or pharmacologically perturbed have naturally been more limited. That said, a few studies have clearly demonstrated that genetic loss of the Nlrp3 sensor can delay AML progression in two different genetic models (Hamarsheh et al, Nat communications and Zhong et al, Frontiers in Immunology). While the survival data presented in figure 5a of this manuscript does clearly substantiate the existing claim that inflammasome activity can promote MDS pathogenesis, the manuscript does little to expand our current understanding of the link between inflammasome activity and MDS. For example, open questions include : when during pathogenesis is the inflammasome activated, what drives its activation and through what sensors, what is its relation to oncogenic signaling, in what cell types is the inflammasome activated and to what extent can pharmacologic inhibition of inflammasome activity ameliorate MDS phenotypes? These questions remain largely unexplored in this manuscript and hence markedly limit its impact.

Re: Thank you for your valuable and insightful comments. Regarding pharmacologic inhibition of inflammasome activity, we administrated a caspase-1 inhibitor to MDS mice 3 times a week for 10 weeks, starting from 6 weeks after transplantation; however, we did not obtain significant effects on PB parameters or survival, as shown below. One possible explanation is that the treatment did not achieve sufficient or sustained pharmacological inhibition of caspase-1 in vivo. We plan to investigate

alternative approaches, such as NLRP3 inhibition, in future studies.



As discussed below, IL-1β levels in BM fluid remained very low in the *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} model, whereas IL-18 levels were significantly elevated in an ASC-dependent manner. These findings suggest that IL-1β may not be a major downstream mediator of inflammasome activity in this model and, consequently, that therapeutic inhibition of IL-1β may have limited efficacy.

Given the flexible/somatic nature of the Runx1/Cbl model utilized in this study, it naturally lends itself to exploring the impact of additional relevant alleles beyond Asc to both substantiate some of the claims as well as to add novelty. For example, demonstrating the same phenotype in Casp1 or Casp1/11 double KO could substantiate the presented claims, particularly given the relatively nebulous Casp1 cleavage data presented in figure 6c. Additionally, utilizing knockouts of specific sensors upstream of Asc could allow for a better mechanistic understanding of the drivers of inflammasome activation in this model, which is currently unexplored in this manuscript. Two additional directions could have been informative include (1) an analysis of Asc dependency in additional MDS models in order to assess generalizability, and (2) an attempt to characterize how pharmacological inhibition of inflammasome activation (i.e. IL1b inhibition, CASP1 inhibition, NLRP3 inhibition, etc) can ameliorate MDS phenotypes and phenocopy Asc loss, as this would of course have more direct therapeutic application.

Re: Thank you for your valuable and instructive comments. Due to the limited time available for revision, we were unable to perform rescue experiments using Caspase-1 knockout mice or mice

deficient in molecules upstream of ASC. Instead, we performed the following additional experiments to address this issue.

(1) to assess whether the role of ASC extends to additional MDS models, we examined a *Tet2*-deficient model. BM cells from *Tet2*$^{fl/fl}$*;Vav1-iCre* mice were transplanted into lethally irradiated recipient mice, and disease progression was evaluated in the presence and absence of ASC (revised Figure 7A). Consistent with previous reports, *Tet2*-deficient BM cells developed MDS-like disease, and a subset of mice died during the observation period (revised Figure 7B). These mice exhibited pancytopenia (revised Figure 7C), mild myeloid-biased differentiation (revised Figure 7D), expansion of BM HSPCs (revised Figure 7E), and a mild block in erythroid differentiation at the transition from stage IV to stage V (revised Figure 7F), accompanied by increased cell death of Ter119^{+} erythroid cells (revised Figure 7G). These phenotypes were largely consistent with those observed in the *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} MDS model. Notably, *Asc* deficiency substantially attenuated the MDS phenotypes induced by *Tet2* deficiency. In this model, IL-1β and IL-18 levels were markedly elevated in BM fluid, and these increases were reduced in the absence of ASC, particularly in the case of IL-1β. These findings provide additional evidence supporting the requirement for ASC-dependent inflammasome activation in MDS progression. We described these points on page 19, lines 410-425.

(2) As described above, we administrated a caspase-1 inhibitor into MDS mice 3 times a week for 10 weeks, starting from 6 weeks after transplantation; however, we did not obtain significant effects on PB parameters or survival, as shown above. One possible explanation is that the treatment did not achieve sufficient or sustained pharmacological inhibition of caspase-1 *in vivo*. We plan to investigate alternative approaches, such as NLRP3 inhibition, in future studies. As discussed below, IL-1β levels in BM fluid remained very low in the *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} model, whereas IL-18 levels were significantly elevated in an ASC-dependent manner. These findings suggest that IL-1β may not be a major downstream mediator of inflammasome activity in this model and, consequently, that therapeutic inhibition of IL-1β may have limited efficacy.

Based on the above, I believe the manuscript as currently written is not suitable for publication in Leukemia. That said, further experimental work along any of the directions outlined above could add substantial impact to this work and be informative for the field. Below are my more specific comments.

- Figure 1: While the data is clear, dedicating the entire figure 1 to validating the known biology of Asc in inflammasome signaling is overkill and distracts from the message of the paper. I'd move this to the supplemental section.

Re: Thank you for your suggestion. We have moved these data to revised Supplementary Figure 1

- Figure 2: Misspelling of transplantation throughout the figure. Also, please specify timepoints of analysis in the figure legends.

Re: Thank you for pointing out misspelling. We edited them in revised Figure 1. We also specified timepoints of analysis in the legends.

- Figure 3: In figure 3d, the authors attempt to assess for evidence of spontaneous pyroptosis vs apoptosis in vitro but acknowledge in the text that PI/Annexin staining is not sufficient to distinguish these two. Hence, a more rigorous attempt to assess for ongoing inflammasome activation is necessary and certainly possible in a purified in vitro setting. The authors should assess for spontaneous Il1b or Il18 cleavage/secretion by western blot or ELISA from the media or ASC spec formation by IF. Alternatively, the authors can perform inflammasome assays in the in vitro culture system to determine if CBL/RUNX1 mutant cells are more sensitized to pyroptosis. These can be done with standard stimuli of NLRP3, NLRP4 or AIM2 inflammasome coupled to IL-1b-release or LDH-release as a readout of pyroptosis.

Re: We agree that the assessment of pyrotosis is very important. Following the reviewer's suggestion, we performed cell death assays and measured IL-1β levels in culture supernatants. Briefly, we transduced c-kit positive HSPCs from WT and *Asc*$^{-/-}$ mice with *CBL*$^{\Delta E8/9}$ and *RUNX1*^{S291fs} retroviruses. Cells co-expressing *CBL*$^{\Delta E8/9}$ and *RUNX1*^{S291fs} (*CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} or double positive (DP) cells) were purified by sorting and cultured (revised Figure 2E). On day 8 of culture, cells were challenged with LPS and nigericin to activate the inflammasome, followed by cell death assays using Annexin V and PI. No significant differences in Annexin V^{+}PI^{-} apoptotic cell death were observed between WT and *Asc*$^{-/-}$ control cells or between WT and *Asc*$^{-/-}$ *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells. In contrast, *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells showed significantly increased Annexin V^{+}PI^{+} necrotic cell death compared with WT counterparts, and this increase was further enhanced by inflammasome activation with LPS and nigericin. Importantly, this effect was significantly attenuated by loss of ASC (revised Figure 2F and 2G). We next measured IL-1β levels in the culture supernatants. Although IL-1β levels were below the detection limit under basal conditions, LPS and nigericin treatment markedly increased IL-1β levels in both control and *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells. This induction was completely abolished in *Asc*-deficient cells (revised Figure 2H). In contrast, induction of TNFa was not affected in *Asc*-deficient cells (revised Figure 2H). These data indicate that *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells are more susceptible to inflammasome-dependent pyroptotic cell death and support a role for inflammasome activation in these cells. We described these points on page 14, lines 283-297.

Figure 4C: A more quantitative assessment of dysplasia in the bone marrow would be convincing across all genotypes in the study, as opposed to depiction of a single hpf with a dysplastic cell. Histopathologic analysis across the femur should be performed.

Re: Thank you for your suggestion. We performed histological analyses and observed a significant reduction in the number of mature megakaryocytes in the BM of *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} mice (revised Supplementary Figure 4), indicating impaired maturation of megakaryocytes. In contrast, no significant differences were observed between WT and *Asc*$^{-/-}$ *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} mice. We described these points on page 15, lines 313-317.

- Figure 4E: Discrepancy between the flow cytometric findings and lack of clear Hgb difference between genotypes is notable. More traditional methods to assess erythroid maturation utilize CD44, cell size and Ter119 staining (Liu et al PNAS 2013 PMID 23287863). Is there a maturation defect using this alternative flow cytometric approach?

Re: Following your suggestion, we performed flow cytometric analysis and detected a mild but significant block in erythroid differentiation at the transition from stage IV to stage V (revised Figure 3E and F), which is consistent with the mild anemia shown in revised Figure 3B. We described this point on page 15, lines 319-322.

Figure 6C: I have never seen flow cytometric assessment of cleaved caspase 1 as a measure of inflammasome activation. There is no convincing signal that I see in these flow plots for CASP1 cleavage, particularly given the lack of significant loss in signal in Asc-/- mice. Although inflammasome activation can be challenging to track in vivo, there are certainly a number of more rigorous methods out there. First, tracking serum ELISA for IL1b and IL18 would be helpful in this model in order to determine when inflammasome activity is occurring during MDS pathogenesis. Second, to determine which cells are engaging the inflammasome, immunofluorescence in the bone marrow or on sorted subpopulations of cells for Asc specs or casp1 may be informative. Alternatively, western blots for Casp1 cleavage or Il1b cleavage may be an additional approach.

Re: Thank you for your valuable suggestion. We measured IL-1β and IL-18 levels in BM fluid from MDS mice. While IL-1β levels remained very low, IL-18 levels were significantly elevated in *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} MDS mice, and this increase was abolished by *Asc* deficiency (Figure 5C). In contrast, as shown in Figure 7, *Tet2*-deficient MDS mice showed elevated levels of both IL-1β and IL-18. These increases were attenuated in the absence of ASC, particularly for IL-1β (revised Figure 7H). These findings support inflammasome activation in both MDS models, but the downstream cytokine responses differ between them. The differential ASC dependency of IL-1β and IL-18

production further suggest that distinct mechanisms may contribute to inflammasome activation and downstream signaling in these two disease models. We described these points on page 17, lines 366-369 and page 19, lines 421-425, and in the discussion (pages 20-21, lines 450-462).

- Figure 7C: The heatmap is not particularly informative to the reader and should be moved to the supplement or removed.

Re: We moved the heatmap to revised Supplementary Figure 7B.

- Figure 7: RNA-seq signatures are notoriously not meaningful for inflammasome activity as its largely regulated in a post-translational manner and the active molecules are secreted. The gene signatures are highly overlapping and generally seem to suggest decreased inflammation in Asc-/- cells which I guess is nice to see but does not add much. As opposed to figure 7E, perhaps it would be informative to present a focused heatmap of inflammatory genes, depicting how their expression changes as a function of oncogene expression and Asc status (i.e. similar to 7C but focused on inflammatory gene expression).

Re: Thank you for your suggestion. We have included a heatmap of inflammatory genes in the revised Figure 6E. As suggested, a heatmap of inflammatory- and MDS-related DEGs in WT *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} MDS cells demonstrated that the majority of these transcriptional changes were attenuated in *Asc*$^{-/-}$ *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} MDS cells (Figure 6E) (page 18, lines 399-401).

Referee #2 (Comments to the Author):

The manuscript by Andoh et al investigates the role of the inflammasome adaptor protein, ASC, in the development of MDS in a CBL/RUNX1 mutant mouse model. Overall, the manuscript is well written and clearly presented but there are several issues with the data presented.

Major Critiques

1. The primary conclusion of the manuscript is that inflammasome activation via ASC is involved in MDS progression, which is based largely on data in Figures 5A and 6A. However, it is unclear if the input number and phenotype of CBL/RUNX1 mutant cells were equivalent between WT and Asc knockout at transplant day 0; transplantation of a greater number of CBL/RUNX1 mutant stem or progenitor cells in WT vs. Asc knockout could lead to a difference in disease kinetics. It would be useful to show pre-transplantation flow cytometry data characterizing the number and phenotype (i.e., lineage and differentiation) of cells with and without expression of the CBL/RUNX1 mutant constructs.

Re: Thank you for your valuable suggestion. After transduction, we first confirmed by flow cytometric analysis that the proportion of *RUNX1*^{S291fs}*CBL*$^{\Delta E8/9}$ cells, which are positive for both hNGFR (*RUNX1S291fs*) and GFP (*CBL*$^{\Delta E8/9}$), was greater than 10% of c-kit-positive cells (Supplemental Figure 3). Subsequently, we transplanted bulk c-kit-positive cells containing 6 × 10^6 *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells into congenic recipient mice irradiated with a sublethal dose of 7.0 Gy (revised Figure 3A). We have clarified this point in the Methods (page 9, lines 166-170), Results (pages 14-15, lines 300-306), and Figure 3 legend.

2. Did the authors measure serum or plasma levels of inflammatory cytokines in WT and Asc knockout models? IL1b and IL18 quantification should be prioritized but others (e.g., TNFa, IL6, IFNg, IFNa/b, S100A8/9) could also be informative.

Re: Thank you for your valuable suggestion. We measured IL-1β and IL-18 levels in BM fluid from MDS mice. While IL-1β levels remained very low, IL-18 levels were significantly elevated in *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} MDS mice, and this increase was abolished by *Asc* deficiency (Figure 5C). In contrast, as shown in Figure 7, *Tet2*-deficient MDS mice showed elevated levels of both IL-1β and IL-18. These increases were attenuated in the absence of ASC, particularly for IL-1β (revised Figure 7H). These findings support inflammasome activation in both MDS models, but the downstream cytokine responses differ between them. The differential ASC dependency of IL-1β and IL-18 production further suggest that distinct mechanisms may contribute to inflammasome activation and

downstream signaling in these two disease models. We described these points on page 17, lines 366-369 and page 19, lines 421-425, and in the discussion (pages 20-21, lines 450-462).

3. Do Asc knockout cells show any lineage bias post-transplant? Flow cytometry data characterizing myeloid and lymphoid lineages in Figures 2D and 2E would be useful.

Re: *Asc*$^{-/-}$ mice showed a mild but significant increase in the proportion of B cells in PB under steady-state conditions. This trend was reproduced only in secondary transplantations (revised Figure 1A and E). These data suggest that inhibition of inflammasome activation through ASC loss has a moderate effect on lineage composition of differentiated white blood cells. We described this point on page 12, lines 248-251.

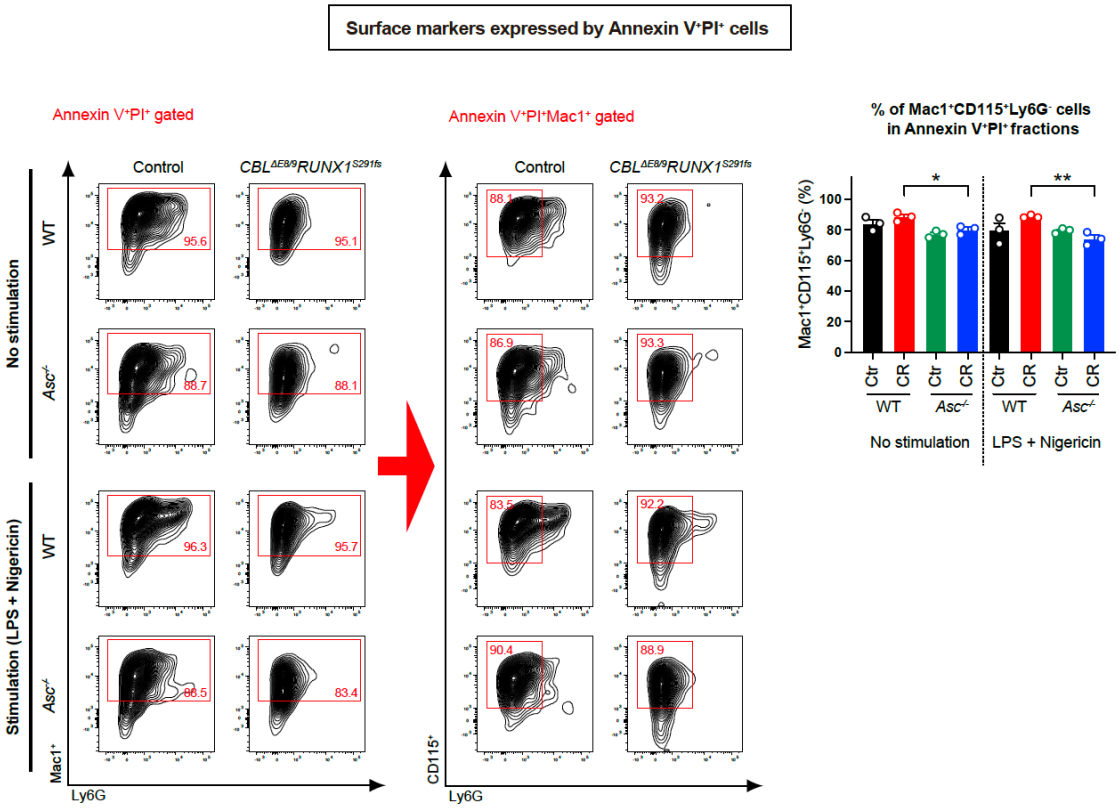
4. Does Asc knockout affect lineage and/or differentiation in the CBL/RUNX1 mutant model? In many cases, it would be useful to include flow cytometry data tracking CBL/RUNX1 mutant cells (by GFP/hNGFR as shown in Figure 3A).
a. Figure 3C: Is there a difference between WT and Asc knockouts in the myeloid differentiation or myeloid subset distribution? What fraction of the cells expressed the CBL/RUNX1 mutant constructs?

Re: In this study, now shown in the revised Figure 2, we purified cells co-expressing *CBL*$^{\Delta E8/9}$ and *RUNX1*^{S291fs} (*CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells) by cell sorting (revised Figure 2B), and plated equal numbers of sorted cells for subsequent analyses. On day 15 of culture, control and *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cultures contained approximately the same proportion of differentiated Mac-1^{+} myeloid cells. Among the myeloid cells, while Ly6G^{+} neutrophils predominated in control culture, CD115^{+} monocytes predominated in *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cultures. However, none of these phenotypic changes was significantly altered by loss of ASC (Supplementary Figure 2A, B). We described these points on pages 13-14, lines 265-269, and lines 275-279.

b. Figure 3D: Which myeloid subsets are in the apoptotic and pyroptotic fractions in each condition? What fraction of the cells expressed the CBL/RUNX1 mutant constructs?

Re: In this study, we purified cells co-expressing *CBL*$^{\Delta E8/9}$ and *RUNX1*^{S291fs} (*CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells) by cell sorting (revised Figure 2A, B and 2E) and plated equal numbers of sorted cells for subsequent analyses. At day 8 of culture, cells were challenged with LPS and nigericin, followed by cell death assays using Annexin V and PI. No significant difference was observed in Annexin V^{+}PI^{-} apoptotic cell death between WT and *Asc*$^{-/-}$ control cells or between WT and *Asc*$^{-/-}$ *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells. In contrast, *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells showed significantly increased Annexin V^{+}PI^{+} necrotic cell death compared with WT counterparts, and this increase was further

enhanced by inflammasome activation with LPS and nigericin. Importantly, this effect was significantly attenuated by loss of ASC (Figures 2F and G). Most of the Annexin V^+PI^+ cells was found in $Mac1^+CD115^+Ly6G^-$ monocytic cells (data shown below). These data indicate that $CBL^{\Delta E8/9}RUNX1^{S291fs}$ cells are more susceptible to pyroptosis, implicating inflammasome activation in these cells. We described these points on pages 13-14, lines 264-269, and lines 283-290.



c. Figure 4B: Were the numbers of myeloid and lymphoid cells measured at additional timepoints (similar to CBC data presented in Figure 6A)? What fraction of myeloid, B, and T cells expressed the CBL/RUNX1 mutant constructs?

Re: Original Figure 4B (revised as Figure 3B) shows the numbers of myeloid, B, and T cells in PB at 3 months post-transplantation. We have now included those of MDS mice at their moribund stages and control mice at 7 months post-transplantation, whose CBC is shown in the revised Figure 5B, in the revised Supplementary Figure 6. We also presented the proportions of GFP/hNGFR-double positive $CBL^{\Delta E8/9}RUNX1^{S291fs}$ cells within myeloid, B, and T cell populations in PB in the revised Figure 3B and Supplementary Figure 6. The proportion of $CBL^{\Delta E8/9}RUNX1^{S291fs}$ cells was high in myeloid cells and non-MyBT cells, but was significantly lower in B and T cells. As the disease progressed to the moribund stage, $CBL^{\Delta E8/9}RUNX1^{S291fs}$ expression became increasingly concentrated in myeloid cells and/or non-MyBT cells.

d. Figures 4D/E: What fraction of each cell population expressed the CBL/RUNX1 mutant constructs?

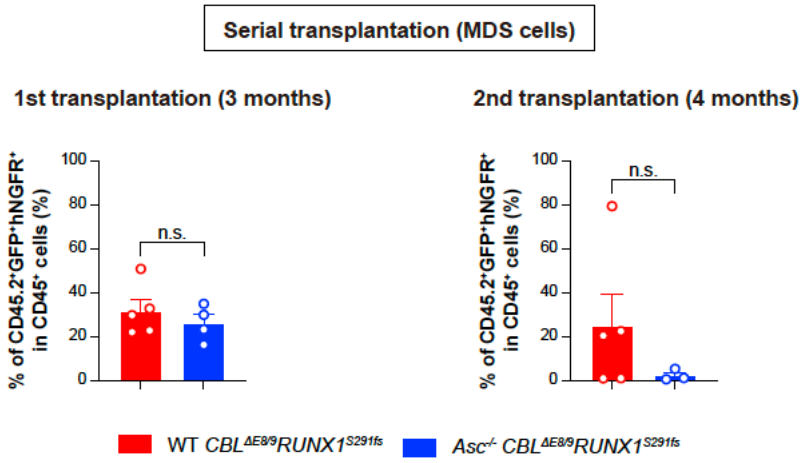
Re: We showed the proportion of GFP/hNGFR-double positive *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells in revised Figure 3D and F.

5. For all CBL/RUNX1 mutant transplanted mice that died from MDS or AML, can the authors present quantitative and phenotypic flow cytometry data? Similar to what is presented in Figures 5B/C but also including quantification of cells expressing the CBL/RUNX1 mutant constructs?

Re: We showed the absolute numbers of myeloid cells, B cells, T cells and non-MyBT cells in PB at moribund stage of MDS and AML mice in Supplementary Figure 6A, as well as the proportion of *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} DP cells in each lineage (Supplementary Figure 6B). Both WT and *Asc*$^{-/-}$ MDS mice that developed AML showed a significant expansion of non-MyBT immature cell populations in the PB at the moribund stage (Supplementary Figure 6A). The proportion of *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells was high in myeloid cells and non-MyBT cells but significantly lower in B and T cells, regardless of *Asc* status (Supplementary Figure 6A).

6. Is it possible to serially transplant MDS or AML cells expressing the CBL/RUNX1 mutant constructs to further evaluate the potential kinetic and phenotypic differences between WT and Asc knockout disease models?

Re: Thank you for your valuable suggestion. We performed serial transplantation of MDS. Three months after the primary transplantation, 5×10^6 total BM cells from primary MDS mice were transplanted into lethally irradiated secondary recipient mice. The graphs below show the proportions of CD45.2$^+$GFP$^+$hNGFR$^+$ cells in the PB of primary recipients at 3 months after transplantation (left) and of secondary recipients at 4 months after the secondary transplantation (right). In primary recipients, CD45.2$^+$GFP$^+$hNGFR$^+$ cells accounted for approximately 20–40% of CD45$^+$cells. However, these cells showed poor engraftment after the secondary transplantation, with donor-derived cells becoming nearly undetectable by 4 months, particularly in the *Asc*$^{-/-}$ *CBL*$^{\Delta E8/9}$*RUNX1*^{S291f} group. In contrast, one mouse in the WT *CBL*$^{\Delta E8/9}$*RUNX1*^{S291f} group developed leukemia, as evidenced by a marked expansion of CD45.2$^+$GFP$^+$hNGFR$^+$ cells (approximately 80% chimerism), accompanied by elevated WBC counts and the accumulation of immature cells (non-MyBT cells). Because secondary engraftment of MDS cells was generally poor and one WT recipient underwent leukemic transformation, we were unable to accurately compared the disease kinetics and phenotypic differences between WT and *Asc* knockout MDS cells.



7. For the data presented in Figure 7, were there differences in lineage and differentiation status among KIT-positive cells isolated from the 4 different groups? In addition, what fraction of KIT-positive cells in the blue and red groups expressed the CBL/RUNX1 mutant constructs? It is possible that the transcriptomic differences observed are simply due to differences in lineage representation and/or differentiation status among KIT-positive cells, or differences in the fraction of CBL/RUNX1 mutant-expressing cells.

Re: Thank you for raising this important point. For the RNA-seq analysis (shown as revised Figure 6), we used purified GFP$^+$hNGFR$^+$ *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} cells isolated by cell sorting; therefore, all analyzed cells expressed both *CBL*$^{\Delta E8/9}$ and *RUNX1*^{S291fs}. However, since the GFP$^+$hNGFR$^+$ fraction was very small in the control group, we sorted the GFP$^+$ and/or hNGFR$^+$ fractions among Lin$^-$c-kit$^+$ cells. We described these points in the legend of Figure 6A.

We now presented the composition of the Lin$^-$c-kit$^+$ cells used for RNA-seq (Supplementary Figure 7A). While the proportions of myeloid progenitor populations were comparable between WT and *Asc* KO *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} c-kit-positive cells, clear differences were observed between control and *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} c-kit-positive cells. Therefore, the differential gene expression profiles observed between WT and *Asc* KO *CBL*$^{\Delta E8/9}$*RUNX1*^{S291fs} c-kit-positive cells are likely attributable to the presence or absence of ASC, rather than differences in cellular composition. While we cannot completely exclude subtle differences in population structure, our data suggest that the observed transcriptional changes primarily reflect the biological effects of *Asc* deficiency.

Minor Critiques

1. Figure 1 is validation of the Asc knockout phenotype, which is important but is well established in

the field and has been published before. The authors should consider moving this figure to supplement. It would also be helpful to add molecular weight markers to 1B.

Re: Thank you for your suggestion. We moved these data to revised Supplemental Figure 1 and added molecular weight markers to revised Supplemental Figure 1B.

2. Transplantation is misspelled in Figures 2C-E

Re: Thank you. We edited them.

3. It would be helpful to include scale bars in Figures 4C, 5B, and 5C.

Re: We added scale bars (revised Figure 3C, 4B 4C, and Supplemental Figure 5).