MOZ and BMI1 act synergistically to maintain Hematopoietic Stem Cells

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Highlights

1. MOZ and BMI1 synergistically maintain bone marrow HSCs in a cell-intrinsic manner
2. While BMI1 represses senescence, MOZ maintains the quiescent state of HSCs
3. MOZ regulates primary HSCs independently of *Ink4a-Arf*

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Key words

Hematopoiesis, senescence, quiescence, epigenetics, MYST, stem cells

Category

Stem cells

Abstract

Chromatin plays a central role in maintaining hematopoietic stem cells and during their stepwise differentiation. While a large number of histone modifications and chromatin modifying enzymes have been identified, how these act in concert to produce specific phenotypic outcomes remains to be established. MOZ (KAT6A) is a lysine acetyltransferase and enhances transcription at target gene loci. In contrast, the polycomb group protein BMI1 (PCGF4) is part of the transcriptionally repressive PRC1 complex. Despite their opposing effects on transcription, MOZ and BMI1 regulate biological systems in a similar manner. MOZ and BMI1 are required for the development of transplantable HSCs, for restraining cellular senescence, for the proper patterning of the anterior-posterior axis during development, and for the specification and maintenance of the B cell lineage. Thus, we set out to explore the relationship between MOZ and BMI1. We recently established that MOZ and BMI1 have opposing effects on the initiation of *Hox* gene expression during embryonic development and that defects in body segment identity specification observed in single *Moz* and *Bmi1* mutants were rescued in compound mutants. We report here the relationship between MOZ and BMI1 in hematopoiesis. Using $Moz^{+/-}$;$Bmi1^{+/-}$ compound mutant mice, we find that MOZ and BMI1, but not the BMI1-related protein MEL18 (PCGF2), play synergistic roles in maintaining adult HSCs. While BMI1 restrains premature senescence, we establish that MOZ acts to maintain the quiescent state of HSCs. Our work reveals that MOZ and BMI1 regulate HSCs in a synergistic manner by acting on distinct processes required to maintain HSCs.

Highlights

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3. MOZ regulates primary HSCs independently of *Ink4a-Arf*

Introduction

Self-renewing hematopoietic stem cells (HSCs) lie at the top of a hierarchy that gives rise to a diverse array of differentiated blood cells after transplantation [1]. The maintenance of HSCs and their coordinated differentiation is associated with dynamic changes in chromatin structure [2-4], which are important for coordinated gene expression changes required to induce specific cell identities. Chromatin structure is highly dynamic and is controlled by modifications to DNA and histones. Diverse modifications occur on N-terminal histone tails and over 130 different modifications of histones have been identified [5], which are dynamically regulated by "writers" and "erasers". Consistent with the importance of chromatin, numerous chromatin modifiers are mutated or misexpressed in hematopoietic malignancies, while mouse models with mutations in chromatin modifiers typically exhibit anomalies in hematopoiesis [6-8]. While the catalytic activities of individual chromatin modifiers, as well as their roles in hematopoiesis, are well established in many cases, how different chromatin modifiers act in concert to produce particular phenotypic outcomes is not well understood.

MOZ (MYST3, KAT6A) is a lysine acetyltransferase that is critical for the formation of HSCs during embryonic development [9, 10]. Interestingly, when *Moz* is deleted in adult mice there is a rapid loss of HSCs, defined both functionally and by cell surface phenotype, showing that MOZ is required for the maintenance of adult LT-HSCs [11]. During embryonic development, MOZ acetylates histone 3 lysine 9 (H3K9) and positively maintains the transcription of its target genes including *Hox* genes [12], as well as *Tbx1* and *Tbx5* [13, 14]. In contrast, BMI1 is a member of the polycomb repressive complex 1 (PRC1), where it collaborates with the catalytic member RING1B to repress transcription at target loci [15-18].

It is particularly interesting that MOZ and BMI1 have similar functions despite their opposing effects on transcription. For instance, deletion of *Moz* or *Bmi1* in mice leads to a lack of transplantable HSCs [9, 10, 19, 20], to misexpression of *Hox* genes and defects in body segment identity [12, 21-23], as well as to spleen and thymus dysgenesis [9, 22]. Furthermore, loss of a single allele of *Moz* or *Bmi1* in the *Eμ-Myc* lymphoma model leads to a more than 3.5-fold increase in disease-free survival [24, 25]. Consistent with its role in HSCs, translocations involving *MOZ* with *CBP* [26], *p300* [27], *TIF2* [28] and *NCOA3* [29] have been reported in severe cases of human acute myeloid leukemia (AML), while BMI1 is overexpressed in a range of both hematopoietic malignancies including the M0-subtype of AML [30], as well as non-hematopoietic malignancies such as metastatic melanoma [31] and colorectal cancer [32]. In this context, it is particularly interesting that both MOZ [33, 34] and BMI1 [35] restrain senescence via the *Ink4a-Arf* axis in primary embryonic fibroblasts. Since MOZ and BMI1 affect the same biological processes, often in a similar manner, despite having opposing effects of transcription, we set out to explore their relationship. Through the analysis of mice doubly deficient in *Moz* and *Bmi1*, we have recently shown that MOZ and BMI1 play opposing roles during the onset of *Hox* gene expression, such that the anterior and posterior homeotic transformations observed in single $Moz^{-/-}$ and $Bmi1^{-/-}$ mutants, respectively, were rescued in $Moz^{-/-}$;$Bmi1^{-/-}$ mutant animals [36].

In the current study, we explored the functional relationship between MOZ and BMI1 in regulating hematopoiesis. We find here that in contrast to their opposing roles during body segment specification, MOZ and BMI1 act synergistically, by maintaining quiescence and repressing senescence respectively, to preserve adult bone marrow HSCs.

Materials and Methods

Animals

All experiments conformed to guidelines set by the Walter and Eliza Hall Animal Ethics Committee and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. *Moz* [12], *Bmi1* [22], *Mel-18* [37] and *Ink4a-Arf* [38] knockout alleles were maintained on a C57BL/6 background. Mice were kept in a 14 h light, 10 h dark cycle. For transplantation experiments, bone marrow cells from 8 to 12 week old test mice ($CD45.2^+$) were injected alone or in combination with $CD45.1^+$ wild type competitor cells into lethally irradiated hosts ($CD45.1^+$: two doses of 550 rad separated by 2 h). For fetal liver transplants, 400,000 fetal liver test cells ($CD45.2^+$) were mixed with 200,000 competitor $CD45.1^+$ bone marrow cells and transplanted into $CD45.1^+$ lethally irradiated hosts.

Flow cytometry analysis

Cells were isolated from the bone marrow, spleen, thymus and peripheral blood and suspended in a balanced salt solution supplemented with 2% fetal calf serum. Hematopoietic cells were stained with a subset of the following antibodies: B220 (clone 16A), CD3 (clone 17A2), CD4 (clone GK1.5), CD8a (clone 53-6.7), CD11b (clone M1/70), CD19 (clone 1D3), CD25 (clone PC61.5), CD34 (clone RAM34), CD44 (clone IM7), CD45.1 (clone A20), CD45.2 (clone 104,), CD117 (clone 2B8), CD135 (clone A2F10.1), Gr1 (clone RB6-8C5), IgD (clone 11-26c), IgM (clone II/41), CD127 (clone B12-1), Sca1 (clone D7) and Ter-119 (clone TER-119). Antibodies were sourced from BD Pharmingen (USA), eBioscience (USA), Biolegend (USA) or the Walter and Eliza Hall Institute monoclonal facility, and were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll A (PerCP), AlexaFlour700, phycoeryhtrin-cyanine dye (PeCy7) or biotin. For exclusion of lineage positive cells, antibodies against B220, CD3, CD4, CD8,

CD19, Gr1, Mac1 and Ter-119 were used. Cell cycle analyses were carried out using 4'6-diamidino-2-phenylindole dihydrochloride (DAPI) and an anti-Ki67 antibody according to the Cytofix/Cytoperm^{TM} Fixation/Permeabilization kit (BD Biosciences #554714). Cells were analyzed on the LSRII (BD) or Fortessa cytometers (BD).

Cell Culture and Molecular Biology

Agar colony forming assays were carried out as previously described [39]. For gene expression analysis, RNA was isolated from bone marrow cells using the RNeasy kit (Qiagen) and 1 μg of total RNA was used to prepare cDNA with oligo dT_{20} primer and the Superscript III enzyme (Life Technologies). Gene expression levels were quantified using SYBR green chemistry (SensiMix^{TM}, Bioline) on the LightCycler 480 (Roche) and the primers:

Moz F: 5'CTTACACGGATGCCAAAAGG3' R: 5'GTTTTATCTGTGCCGCCTTC3' *Bmi1* F: 5'GAGCAGATTGGATCGGAAAG3' R: 5'GCA TCACAGTCA TTGCTGCT3' *Ink4a* F: 5'CGTACCCCGATTCAGGTGAT3' R: 5'TTGAGCAGAAGAGCTGCTACGT3'; *Ink4b* F: 5'AGATCCCAACGCCCTGAAC3' R: 5'CCCATCATCATGACCTGGATT3'; *Arf* F: 5'GCCGCACCGGAATCCT3' R: 5'TTGAGCAGAAGAGCTGCTACGT3'; *Gapdh* F: 5'TTCACCACCATGGAGAAGGC3' R: 5'CCCTTTTGGCTCCACCCT3' as originally described by [40], and *Hsp90ab1* F: 5' ACCTGGGAACCATTGCTAAG3' R: 5' AGAATCCGACACCAAACTGC3'

Results

Moz haploinsufficiency leads to a reduction in LSK CD34^{neg} Flt3^{neg} bone marrow cells

Homozygous deletion of *Moz* or *Bmi1* individually leads to a complete lack of transplantable HSCs [9, 10, 19, 20]. Interestingly, *Moz*, but not *Bmi1* heterozygous HSCs have an intermediate defect in the HSC compartment [9, 10, 19, 20]. Given the embryonic lethality of *Moz*$^{-/-}$ and the death of *Bmi1*$^{-/-}$ mutants around the time of weaning, we studied *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ double heterozygous mice to investigate the functional relationship between MOZ and BMI1 in adult hematopoiesis. From a *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ x wild type cross, significantly fewer *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ pups (120) were observed compared to *Moz*$^{+/-}$ (177), *Bmi1*$^{+/-}$ (214) and wild type (230, χ^2-test, $p < 0.001$, Supplementary Table S1), indicating epistasis between *Moz* and *Bmi1* heterozygosities. The surviving *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ mutants did not display any overt phenotypic abnormalities up to 18 months of age (n = 12 mice per genotype). Furthermore, haploinsufficiency for *Moz* did not affect the mRNA levels of *Bmi1* in bone marrow cells, and likewise *Moz* mRNA levels were unchanged in *Bmi1*$^{+/-}$ bone marrow cells (Supplementary Fig. S1).

We initially analyzed HSC and progenitor compartments using cell surface markers. Hematopoietic stem and early progenitors cells are positive for SCA-1 and c-KIT, and do not express mature blood lineage markers (LSK) [41]. The proportion of LSK cells in the bone marrow of *Moz* and *Bmi1* mutants was not different to wild type (Fig. 1A). Stem and progenitor cells can be enriched from LSK cells using FLT3 and CD34, i.e. long-term HSCs (LT-HSC: FLT3$^-$, CD34$^-$), short-term HSCs (ST-HSCs: FLT3$^-$, CD34$^+$) and multipotent progenitors (MPPs: FLT3$^+$, CD34$^+$) [42-44]. Interestingly, loss of just one allele of *Moz* resulted in a 50% decrease in LT-HSCs, whereas ST-HSCs and MPPs were not affected (Fig. 1B-D). In contrast, loss of one allele of *Bmi1* had no affect on the number of LT-HSC, ST-

HSC or MPP cell populations. Together, analyses of cell surface markers suggest that MOZ is particularly important for maintaining the LT-HSC population (Fig. 1E-H).

MOZ and BMI1 maintain functional HSCs in a synergistic manner

To analyze the function of *Moz* and *Bmi1* mutant HSCs, we carried out transplantation assays. Non-competitive transplantation of one million *Moz* and *Bmi1* heterozygous cells into wild type recipients revealed that $Moz^{+/-}$;$Bmi1^{+/-}$ bone marrow cells possessed stem cell activity and were able to give rise to all major hematopoietic lineages (Fig. 2A-B, Supplementary Tables S2,S3). Peripheral blood parameters of recipient mice injected with *Moz*;*Bmi1* mutant bone marrow were similar to wild type four months after transplantation (Supplementary Table S2).

We next quantified the ability of *Moz*;*Bmi1* mutant cells to contribute to hematopoiesis in competitive transplantations. Either 500,000 or 100,000 *Moz*;*Bmi1* mutant bone marrow cells were mixed with the same number of wild type competitor cells and injected into lethally irradiated recipients (Fig. 2C,E). Four months after transplantation of 500,000 *Moz*;*Bmi1* and 500,000 competitor cells, recipients of $Bmi1^{+/-}$ cells showed similar chimerism in peripheral blood and bone marrow LSK cells as wild type. In contrast, $Moz^{+/-}$ bone marrow cells showed a 3-fold reduction in their ability to contribute to hematopoiesis (Fig. 2D). Intriguingly, the ability of $Moz^{+/-}$;$Bmi1^{+/-}$ bone marrow cells to contribute to hematopoiesis was not only less than wild type ($p < 0.001$), but also inferior to $Moz^{+/-}$ donor bone marrow (Fig. 2D, $p < 0.05$). This suggests that MOZ and BMI1 act synergistically in maintaining HSCs. Six months after transplantation of 100,000 *Moz*;*Bmi1* test and 100,000 competitor cells, we observed significantly more variation due to the small number of transplanted cells. Nevertheless, the normal dose of *Moz* was critical for the ability of bone marrow cells to compete (Fig. 2F).

Both *Moz*$^{+/-}$ and *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ donor cells contributed significantly less than wild type and *Bmi1*$^{+/-}$ bone marrow to the reconstitution (Fig. 2E-F, $p < 0.05$). No defects in homing to the bone marrow (Supplementary Fig. S2 and S3) or the ability of *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ bone marrow to support HSC expansion were observed (Supplementary Fig. S3; Supplementary Tables S4,S5).

Together, our data revealed that deletion of one copy of *Bmi1* alone had no adverse effect on HSC activity, but when added to *Moz* haploinsufficiency, *Bmi1* heterozygosity exacerbated the *Moz* haploinsufficient defects in HSC function after transplantation.

Normal dose of *Moz* and *Bmi1* are required to maintain self-renewal of HSCs

We next analyzed the self-renewal ability of *Moz*;*Bmi1* HSCs through serial transplantation. On day 0, we transplanted 900,000 *Moz*;*Bmi1* bone marrow cells along with 100,000 competitor bone marrow cells into lethally irradiated wild type recipients, and carried out secondary and tertiary transplantations every 120 days (Fig. 3A). While *Bmi1*$^{+/-}$ donor cells showed no defects in self-renewal, deletion of just one allele of *Moz* lead to a significant reduction in the self-renewal of HSCs as determined by chimerism in the peripheral blood and bone marrow as well as individual cell lineages including B cells, T cells and myeloid cells (Fig. 3). Interestingly, when haploinsufficiency of *Moz* was combined with *Bmi1* heterozygosity, *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ HSCs performed worse in this self-renewal assay compared to wild type, and tended to exacerbate the effects of single *Moz*$^{+/-}$ heterozygosity ($p < 0.05$). These data suggest that the normal dose of both MOZ and BMI1 is required to maintain the self-renewal of HSCs.

***Mel-18* and *Moz* heterozygosities do not synergise to maintain bone marrow HSCs**

BMI1 and MEL-18 are homologous PRC1 proteins and in embryonic development act in a functionally redundant manner [45]. While the *Mel-18* gene appears to be expressed at low levels in HSCs (http://haemosphere.org), mouse models have shown that deletion of *Mel-18* results in either a mild decrease in HSC reconstitution ability [20], or a significant increase in HSC self-renewal both in vivo and in vitro [46]. Although its effects on HSCs appears to be opposite to the effects of BMI1 [46], MEL-18, like BMI1, has been shown to localize to the *Ink4a-Arf* locus and maintain it in a repressed state [35, 47]. To investigate the relationship between MOZ and MEL-18 in regulating HSCs, we mixed 900,000 *Moz*;*Mel-18* bone marrow cells with 100,000 wild type competitor cells and injected them into lethally irradiated hosts (Fig. 4A). A secondary transplant was carried out 120 days later. Both in the primary (Fig. 4B) and secondary recipients (Fig. 4C), $Moz^{+/-}$ lead to a reduction in the ability of donor cells to compete ($p < 0.05$). Interestingly, *Mel-18* heterozygosity had no effect alone, or in combination with $Moz^{+/-}$ on HSC activity as assessed via the competitive transplantation assay.

MOZ and BMI1 regulate early hematopoietic progenitors in a synergistic manner

We have established that MOZ and BMI1 work together to maintain functional HSCs as determined by transplantation assays. In addition to homing to the bone marrow, early uncommitted and some lineage restricted progenitors home to the spleen and form clonogenic colonies after transplantation [44, 48, 49]. To analyze the effects of *Moz*;*Bmi1* on spleen colony formation, we transplanted 100,000 *Moz*;*Bmi1* bone marrow cells into lethally irradiated wild type recipients and scored colony forming units 12 days later (CFU-S_{d12}, Fig. 5A). On average, recipients of wild type bone marrow had 14.4 spleen colonies (Fig. 5B). The numbers of colonies were not significantly different in recipients of $Bmi1^{+/-}$ or $Moz^{+/-}$ bone marrow. Interestingly however, recipients of $Moz^{+/-}$;$Bmi1^{+/-}$ bone marrow showed reduced

number of spleen colonies, as well as reduced spleen weight compared to all other genotypes (Fig. 5B-C, $p < 0.001$). These data suggest that MOZ and BMI1 act synergistically in regulating early hematopoietic progenitors that possess the ability to form colonies in the spleen.

We analyzed more committed progenitors and mature blood populations at steady state using agar cultures of bone marrow cells and flow cytometry (Supplementary Fig. S4). In semi-solid agar cultures, 25,000 bone marrow cells were plated in the presence of cytokines IL-3, EPO and SCF, which promote growth of myeloid and megakaryocyte lineages. We found that *Moz* and *Bmi1* heterozygosity did not affect the formation of blast, granulocyte, mixed granulocyte-macrophage, machrophage, eosinophil or megakaryocyte colonies (Fig. 5D-I).

Undertaking flow cytometry analysis, we found that the numbers of bone marrow progenitors of the myeloid, erythrocyte and megakaryocyte lineages were not affected by the loss of one allele each of *Moz* and *Bmi1* (Supplementary Fig. S4, Supplementary Table S6). In the T cell lineage, *Moz* haploinsufficiency lead to a significant reduction in thymic double negative progenitors, while numbers of more mature T cell populations was normal (Supplementary Table S7). In contrast, loss of one allele of $Bmi1^{+/-}$ alone did not adversely affect T cell development and did not change the effects of *Moz* heterozygosity. Similarly, loss of one allele of *Moz* lead to a reduction in B cell lineage, particularly in the spleen, while *Bmi1* heterozygosity had no observable effect on B cell development (Supplementary Table S8).

These data suggest that MOZ and BMI1 act synergistically in maintaining primitive HSCs and early progenitors, but do not synergistically regulate more restricted cell lineages or differentiated cell populations.

MOZ regulates HSC function independently of *Ink4a-Arf*

BMI1 directly inhibits the *Ink4a-Arf* locus [47], and restrains senescence in HSCs [19, 50]. Homozygous deletion of the *Ink4a-Arf* locus restores functional HSCs in *Bmi1*$^{-/-}$ mice [50]. We and others have recently shown that MOZ inhibits senescence in primary fibroblasts via the *Ink4a-Arf* pathway [33, 34]. However, in contrast to BMI1 [35], MOZ inhibits senescence through activation of *Ink4a-Arf* repressors including *Cdc6* and *Ezh2* [36]. Recently, through the analysis of mice possessing a catalytically inactive form of MOZ, it has been suggested that MOZ may also regulate HSCs in the fetal liver via the *Ink4a-Arf* axis [34].

To determine if MOZ and BMI1 both regulate HSCs through the *Ink4a-Arf* axis, we crossed *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ mice to *Ink4a-Arf*$^{-/-}$ mice. We then undertook competitive transplantation and serial transplantation assays with 500,000 *Moz*;*Bmi1*;*Ink4a-Arf*$^{-/-}$ bone marrow cells and 500,000 wild type competitor cells (Fig. 6A,C). In contrast to *Ink4a-Arf*$^{-/-}$ bone marrow and *Bmi1*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ bone marrow, which showed no reconstitution defects, *Moz*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ and *Moz*$^{+/-}$;*Bmi1*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ bone marrow exhibited significantly decreased contribution to peripheral blood four months after transplantation when compared to *Ink4a-Arf*$^{-/-}$ donors (Fig. 6B). Interestingly, the contribution of *Moz*$^{+/-}$;*Bmi1*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ donors was not significantly different to *Moz*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ donors. Similarly, in serial transplantation assays that test for self-renewal, both *Moz*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ and *Moz*$^{+/-}$;*Bmi1*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ donor cells were deficient in their ability to contribute to hematopoiesis compared to *Ink4a-Arf*$^{-/-}$ controls (Fig. 6D). However, the activity of *Moz*$^{+/-}$;*Bmi1*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ donors was not different to *Moz*$^{+/-}$;*Ink4a-Arf*$^{-/-}$ bone marrow cells (Fig. 6D). Together, these data suggest that the *Bmi1*$^{+/-}$ component, but not the *Moz*$^{+/-}$ component of the defect observed in *Moz*$^{+/-}$;*Bmi1*$^{+/-}$ HSCs (Fig. 2 and 3) can be rescued with the concurrent deletion of *Ink4a-Arf* (Fig. 6A-D). Thus, it

appears that while BMI1 acts via repression of the *Ink4a-Arf* locus, MOZ is likely to act through an alternative mechanism to maintain adult HSCs.

MOZ does not regulate HSC function through the *Ink4a-Arf* axis

Given the previous report that fetal liver cells lacking the catalytic activity of MOZ could be partially rescued by the concurrent deletion of *Ink4a-Arf* [34], we tested whether the same held true for *Moz* knockout fetal liver cells. We transplanted 400,000 fetal liver cells from wild type, $Moz^{+/-}$ and $Moz^{-/-}$ embryos and 200,000 wild type competitor bone marrow cells (Fig. 6E). Consistent with previous reports [9, 10], $Moz^{-/-}$ fetal liver cells failed to contribute to hematopoiesis while $Moz^{+/-}$ cells showed reduced functional capacity to contribute to hematopoiesis (Fig. 6F). Interestingly, the same relationship was maintained on the *Ink4a-Arf*$^{-/-}$ background. $Moz^{-/-}$;*Ink4a-Arf*$^{-/-}$ fetal livers failed to contribute to hematopoiesis 100 days after transplantation, while $Moz^{+/-}$;*Ink4a-Arf*$^{-/-}$ donors showed an intermediate phenotype (Fig. 6G). Consistent with very low levels of *Ink4a* mRNA in wild type HSCs [51], *Ink4a* mRNA was expressed at very low levels in wild type and $Moz^{+/-}$ bone marrow cells (Fig. 6H). In $Moz^{+/-}$ bone marrow cells, *Arf* and *Ink4b* mRNA were expressed at similar levels as wild type (Fig. 6H). In contrast, loss of just one allele of *Bmi1* lead to a significant derepression of the *Ink4a-Arf-Ink4b* locus (Fig. 6H). Together, these data suggest that in contrast to BMI1 [19, 50], and in contrast to MOZ in primary fibroblasts [33], MOZ does not maintain HSCs via repression of the *Ink4a-Arf* locus.

MOZ maintains the quiescence of HSCs

While senescence is one mechanism that ensures longevity of HSCs, maintenance of HSCs in a quiescent state is also critical [52]. Indeed, HSCs are highly quiescent and are thought to divide only once every 145 days in the mouse [53]. Quiescent HSCs can be detected as they

are maintained in the G_0 phase of the cell cycle. To determine if heterozygosity for *Moz* or *Bmi1* lead to a loss of quiescence, we analyzed the cell cycle profile of *Moz*;*Bmi1* HSCs and progenitors at steady state by flow cytometry (Fig.7A).

No defects in the cell cycle profile were observed when all bone marrow cells from *Moz*;*Bmi1* mice were analyzed as a single population (Fig.7B). In contrast however, in the progenitor enriched LSK population, as well as in MPPs, ST-HSCs and LT-HSCs, a decline in the G_0 population was observed in $Moz^{+/-}$ and $Moz^{+/-}$;$Bmi1^{+/-}$ animals with a concurrent increase in the G_1 cell population (Fig.7B,C-F), showing the specific requirement for MOZ in the stem cell and early progenitor compartments. In particular, there was a two-fold increase in the G_1 population in $Moz^{+/-}$;$Bmi1^{+/-}$ LSK, MPP and ST-HSC populations compared to wild type controls (Fig.7C-E). The number of $Moz^{+/-}$;$Bmi1^{+/-}$ LT-HSCs in the G_1 phase was similarly increased by 2.6-fold compared to wild type controls (Fig.7F). The cell cycle profiles of $Bmi1^{+/-}$ hematopoietic stem and progenitor cell populations were not statistically significantly different from wild type, although there was a trend towards a reduction in G0 and an increase in G1, which may indicate that the gene dosage of *Bmi1* could become important under some circumstances (Fig.7C-F). Together, these findings suggest that in contrast to BMI1, which is required to suppress the premature onset of senescence, MOZ acts by maintaining HSCs and early hematopoietic progenitors in a quiescent state.

Discussion

In this study, we set out to explore the relationship between MOZ and BMI1 in hematopoiesis. Through the use of transplantation assays, we show here that MOZ and BMI1 act synergistically, in a cell-intrinsic manner, to maintain adult HSCs. We find that while

BMI1 is required to repress senescence, MOZ is required to maintain the quiescent state of HSCs.

HSCs have been vigorously studied since their identification, particularly as a single HSC is able to restore the entire hematopoietic system in mouse [54] and humans [55]. Not surprisingly, a large number of factors that regulate HSC function have been identified. Amongst these, cell cycle regulators are particularly interesting, as HSCs are normally maintained in a quiescent non-dividing state [53]. Cell cycle progression is restricted by cyclin-dependent kinase (CDK) inhibitors [56], which include the CDNK2 (INK4) family. The *Ink4a-Arf* mRNA is alternatively spliced to produce two distinct proteins, $p16^{INK4A}$ and $p19^{ARF}$ [57]. These proteins act synergistically to inhibit cell cycle progression through inhibition of CDK4/6 and cyclin D (via $p16^{INK4A}$) and through activation of p53 (via $p19^{ARF}$). Genetic models show that repression of *Ink4a-Arf* leads to increased tumorigenic activity [58, 59] and the retention of HSC proliferation, especially in ageing animals [60, 61]. While we find that BMI1 represses the *Ink4a-Arf* locus, which is consistent with previous studies [19, 50], we establish that MOZ is required to maintain the quiescent state of HSCs independently of $p16^{INK4A}$ and $p19^{ARF}$.

The importance of MOZ in regulating HSCs is well established [9, 10]. Interestingly, the catalytic activity of MOZ is required for the complete maintenance of HSC function [62]. Using mice with a catalytically inactive form of MOZ, it was recently shown that MOZ maintains HSCs, at least in part, by directly repressing the *Ink4a-Arf* locus and inhibiting senescence [34]. While we have recently found a role for MOZ in primary mouse embryonic fibroblasts showing that MOZ inhibits *Ink4a-Arf* expression indirectly via CDC6 and EZH2 [33], we were unable to find a relationship between MOZ and *Ink4a-Arf* in regulating HSCs.

These differences may reflect non-catalytic functions of MOZ. MOZ is a large protein possessing 2004 amino acids and a number of functionally diverse domains [63], which in turn mediate the interaction of MOZ with its non-catalytic complex members ING5, BRPF1/2/3 and EAF6 [64, 65]. In addition, MOZ associates with other transcription factors including Runx1 [66] and PU.1 [10], which are both important for hematopoiesis. MOZ is also a unique chromatin modifier, in that the catalytic domain of MOZ can directly bind to DNA in contrast to other KATs including the *Drosophila* MOF, human PCAF and yeast gcn5 [67]. Thus, it is likely that in addition to its catalytic activity promoting gene expression via H3K9 acetylation, the presence of the MOZ complex at target loci may have a structural role in maintaining chromatin in a permissive state. Consistent with this hypothesis, mice with a catalytically inactive form of MOZ [62] show significantly greater survival compared to *Moz* knockout mice [9, 10].

The relationship between different chromatin modifying complexes is only just starting to be thoroughly studied, especially in a systemic manner. However, there is evidence that cross talk between different histone modifications and chromatin-modifying complexes is necessary for determining cellular phenotypes. For instance, mice deficient in *Moz* [9, 10], *Mll1* [68] or *Mll5* [69] all show severe defects in the HSC compartment. Interestingly, MOZ and MLL collaborate in human CD34+ cord blood cells to maintain normal levels of *HOX* gene expression [70]. Indeed, in the absence of MOZ, MLL occupancy at *Hox* loci is reduced [12]. *Hox* genes are important in maintaining HSC self-renewal, whereby overexpression of *Hoxa9* [71] or *Hoxb4* [72-75] leads to an expansion in the HSC compartment, while deletion of *Hoxa9* leads to reduced HSC activity [76]. Overexpression of *Meis1* along with *HoxA* cluster genes is common in leukemia arising from MLL [77, 78] as well as MOZ gene rearrangements [79, 80].

We have recently shown that MOZ and BMI1 play opposing roles during the activation of *Hox* genes in patterning the anterior-posterior axis of the body [36]. The absence of MOZ leads to an anterior homeotic transformation, whereas the absence of BMI1 leads to a posterior homeotic transformation. Remarkably mutation of both *Moz and Bmi1* leads to a morphologically normal axial skeleton [36]. In contrast, we find here that MOZ and BMI1 action synergizes to maintain HSCs, albeit through different mechanisms. While BMI1 primarily acts through regulation of the *Ink4a-Arf* locus, MOZ acts independently to maintain stem cell quiescence. The BMI1-containing PRC1 complex [21, 22, 81], as well as the PRC2 complex that consists of EZH2, SUZ12, EED and RBAP48 proteins [82] and methylates H3K27, maintain gene silencing [83, 84] at developmentally important loci including *Hox* genes during embryonic development. Interestingly, while PRC1 and PRC2 collaborate to repress *Hox* gene expression during development, which is particularly well established in *Drosophila* [83, 85], their relationship in hematopoiesis is much more complex. Knockout mouse models have shown that reduction in expression levels of PRC2 members such as *Ezh2* [86, 87] leads to an increase in HSC activity, while complete deletion of family member *Suz12* adversely reduces HSC function in conditional models [86]. The PRC1 family members show similarly contrasting phenotypes. For instance, deletion of *Bmi1* [9, 10, 19, 20] leads to loss of HSC activity, while depletion of the closely related family member *Mel-18* has been reported to result in both a mild reduction in HSC activity [20] or an increase [46]. These studies suggest that the interaction between the PRC1 and PRC2 complexes, together with complexes containing enzyme subunits that activate gene expression, such as MOZ, is multi-dimensional and likely to be context-dependent.

Conclusions

Here, we investigated the relationship between MOZ and BMI in maintaining HSCs. We found that MOZ and BMI1 act synergistically in regulating hematopoiesis. While BMI1 represses senescence via silencing *Ink4a-Arf*, we establish that MOZ regulates HSC function not via the *Ink4a-Arf* axis, but rather by maintaining HSCs in a quiescent state. Together with our previous work, the current study highlights that chromatin-modifying complexes have unique relationships that vary in a context dependent manner.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 – Cells with a cell surface phenotype of LT-HSCs are reduced in *Moz*$^{+/-}$ heterozygous mice

(**A-D**) Percentage of bone marrow cells positive for cell surface markers of (**A**) LSK cells (lineage negative, SCA-1 positive, c-KIT positive), (**B**) LT-HSCs (LSK, FLT3 negative, CD34 negative), (**C**) ST-HSCs (LSK, FLT3 negative, CD34 positive) and (**D**) multipotent progenitors (MPPs; LSK, FLT3 positive, CD34 positive). (**E-H**) Flow cytometry plots showing the LSK population (left panels), along with LT-HSC, ST-HSC and MPP (right panels). A representative plot for each genotype as indicated is provided. Percentages in the LSK plots depict the proportion of bone marrow cells that possess the LSK cell surface phenotype, while the FLT3-CD34 plots depict breakdown of the LSK population into the LT-HSC, ST-HSC and MPP populations.

Data in (**A-D**) are presented as mean ± s.e.m. Data were analyzed using a Student *t*-test between each of the four genotypes. Asterisks show significance at $*p < 0.05$ between the indicated genotype and wild type.

Figure 2 – Compound *Moz* and *Bmi1* heterozygosity severely reduces HSC activity

(**A**) Experimental design to functionally test long-term hematopoietic stem cells through non-competitive bone marrow transplantion. One million *Moz*;*Bmi1* bone marrow cells were injected into lethally irradiated wild type recipients. (**B**) Chimerism in peripheral blood and bone marrow of recipient mice four months after non-competitive bone marrow transplantation. (**C**) Experimental overview of competitive transplantation of 500,000 nucleated *Moz;Bmi1* bone marrow and 500,000 wild type competitor cells into lethally irradiated recipients, to quantify HSC activity. (**D**) Chimerism in the peripheral blood and bone marrow of CD45.1^{+} wild type recipients 120 days after transplantation with 500,000

Moz;*Bmi1* and 500,000 competitor bone marrow cells. (**E**) Experimental overview depicting competitive transplantation of 100,000 nucleated *Moz*;*Bmi1* against 100,000 wild type bone marrow cells to quantify HSC activity. (**F**) Chimerism in the peripheral blood and bone marrow of $CD45.1^+$ wild type recipients 120 days after transplantation with 100,000 *Moz*;*Bmi1* and 100,000 competitor bone marrow cells.

Data are presented as mean ± s.e.m. Data were analyzed using a Student *t*-test between each of the four genotypes. Asterisks show significance at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ between the indicated genotype and wild type. Hash tags indicate statistical significance between the indicated genotypes at $\#p < 0.05$.

Figure 3 – Combined *Moz* and *Bmi1* heterozygosity leads a decrease in HSC self-renewal

(**A**) Experimental outline to test long-term self-renewal of HSCs through serial transplantation. *Moz*;*Bmi1* bone marrow cells were mixed with wild type competitor cells in a ratio of 9:1 and injected into wild type lethally irradiated hosts. Every four months, chimerism in the peripheral blood and bone marrow was analyzed and 1 million bone marrow cells transplanted into new irradiated hosts. (**B-G**) Chimerism in (**B**) leukocytes, (**C**) B cells, (**D**) T cells as well as (**E**) myeloid cells in the peripheral blood, in addition to (**F**) all bone marrow and (**G**) LSK cells in wild type recipients after each round of serial transplantation.

Data are presented as mean ± s.e.m. Data were analyzed using a Student *t*-test between each of the four genotypes. Asterisks show significance at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ between the indicated genotype and wild type. Hash tags indicate statistical significance between the indicated genotypes at $\#p < 0.05$.

Figure 4 – *Mel-18* heterozygosity does not affect HSC activity

(**A**) Experimental outline to test HSC activity and long-term self-renewal of HSCs through serial transplantation. Bone marrow cells from *Moz*;*Mel-18* mice and wild type competitors were mixed in a ratio of 9:1 and injected into wild type lethally irradiated hosts. Recipient mice were analyzed four months after transplantation. (**B-C**) Chimerism in blood and bone marrow 120 days after the (**B**) primary and 120 days after the (**C**) secondary transplants. Data are presented as mean ± s.e.m. Data were analyzed using a Student *t*-test between each of the four genotypes. Asterisks show significance at $*p < 0.05$, and $**p < 0.01$ between the indicated group and wild type.

Figure 5 – *Moz* and *Bmi1* synergistically regulate progenitor activity

(**A**) Experimental design outlining the colony-forming unit - spleen (CFU-S) assay. 100,000 nucleated bone marrow cells were injected into lethally irradiated recipients, and spleens were analyzed 12 days after injection. (**B**) Number of colonies per spleen in recipient mice 12 days after transplantation. (**C**) Weight of recipient spleens, 12 days after transplantation. (**D-I**) Agar colony forming assays detecting the formation of (**D**) blast, (**E**) granulocyte, (**F**) mixed granulocyte-macrophage, (**G**) macrophage, (**H**) eosinophil and (**I**) megakaryocyte colonies. Data are presented as mean ± s.e.m. Data were analyzed by a Student *t*-test between each of the four genotypes. Asterisks show significance at $***p < 0.001$ between the indicated groups and wild type. Hash tags represent a statistical difference between the marked genotypes at $###p < 0.001$.

Figure 6 – MOZ maintains HSCs independently of the *Ink4a-Arf* pathway

(**A**) Experimental design of competitive transplantation assay of 500,000 nucleated *Moz*;*Bmi1* bone marrow cells on the *Ink4a-Arf*$^{-/-}$ background and 500,000 wild type competitor cells to

quantify HSC activity. (**B**) Chimerism in peripheral blood and bone marrow of wild type recipient mice 120 days after transplantation with *Moz*;*Bmi1;Ink4a-Arf*$^{-/-}$ cells. (**C**) Experimental design to test long-term self-renewal of *Moz*;*Bmi1* HSCs on the *Ink4a-Arf*$^{-/-}$ background through serial transplantation. *Moz*;*Bmi1;Ink4a-Arf*$^{-/-}$ bone marrow cells were mixed with wild type competitor cells in a 9:1 ratio and injected into lethally irradiated wild type hosts. Every four months, chimerism in the peripheral blood and bone marrow was analyzed and one million bone marrow cells transplanted into new irradiated hosts. (**D**) Chimerism in the peripheral blood and bone marrow of wild type recipients after each round of serial transplantation. (**E**) Experimental outline of fetal liver transplants using 400,000 wild type, *Moz*$^{+/-}$ and *Moz*$^{-/-}$ test cells and 200,000 wild type competitor bone marrow cells. (**F**) Chimerism in recipients of *Moz* fetal liver versus wild type bone marrow after competitive transplantation. The test cells were on an *Ink4a-Arf* wild type background. (**G**) Chimerism in recipients of *Moz;Ink4a-Arf*$^{-/-}$ fetal liver versus wild type bone marrow competitive transplantation. The test cells were on an *Ink4a-Arf* $^{-/-}$ background. (**H**) mRNA levels of *Ink4a*, *Arf* and *Ink4b* in *Moz*;*Bmi1* bone marrow cells. Gene expression levels were standardized to the expression of housekeeping genes *Hsp90ab1* and *Gapdh*.

Data are presented as mean ± s.e.m. Data were analyzed using a Student *t*-test between each of the four genotypes. Asterisks show significance at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ between the indicated groups and wild type, while hash tags depict significance between the marked genotypes at ###$p < 0.001$.

Figure 7 – MOZ and BMI1 maintain HSC quiescence

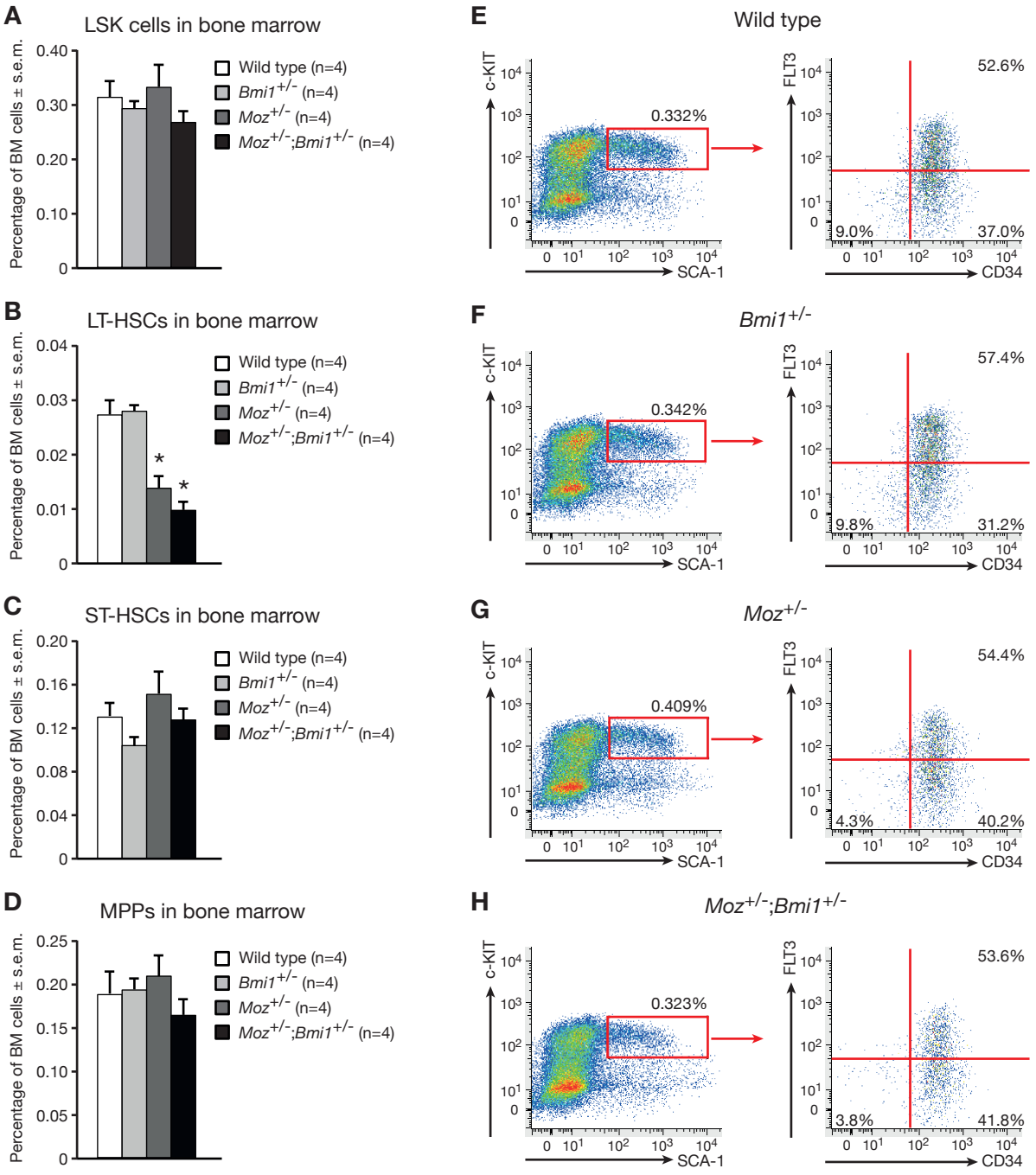
(**A**) Gating strategy for analyzing the cell cycle profile of HSC-enriched populations. LSK cells were defined as lineage negative, SCA-1 positive and c-KIT positive. The LSK population was further subdivided into LT-HSC, ST-HSC and MPP enriched populations

based on FLT3 and CD34 expression. The different phases of the cell cycle were detected based on DNA content (DAPI staining) and KI67 expression. (**B**-**F**) Quantification of *Moz*;*Bmi1* cells in different phases of the cell cycle in (**B**) all bone marrow cells, (**C**) LSK cells, (**D**) MPPs, (**E**) ST-HSCs and (**F**) LT-HSCs. The G_0 phase marks the quiescent cell population (2N DNA and KI67 negative).

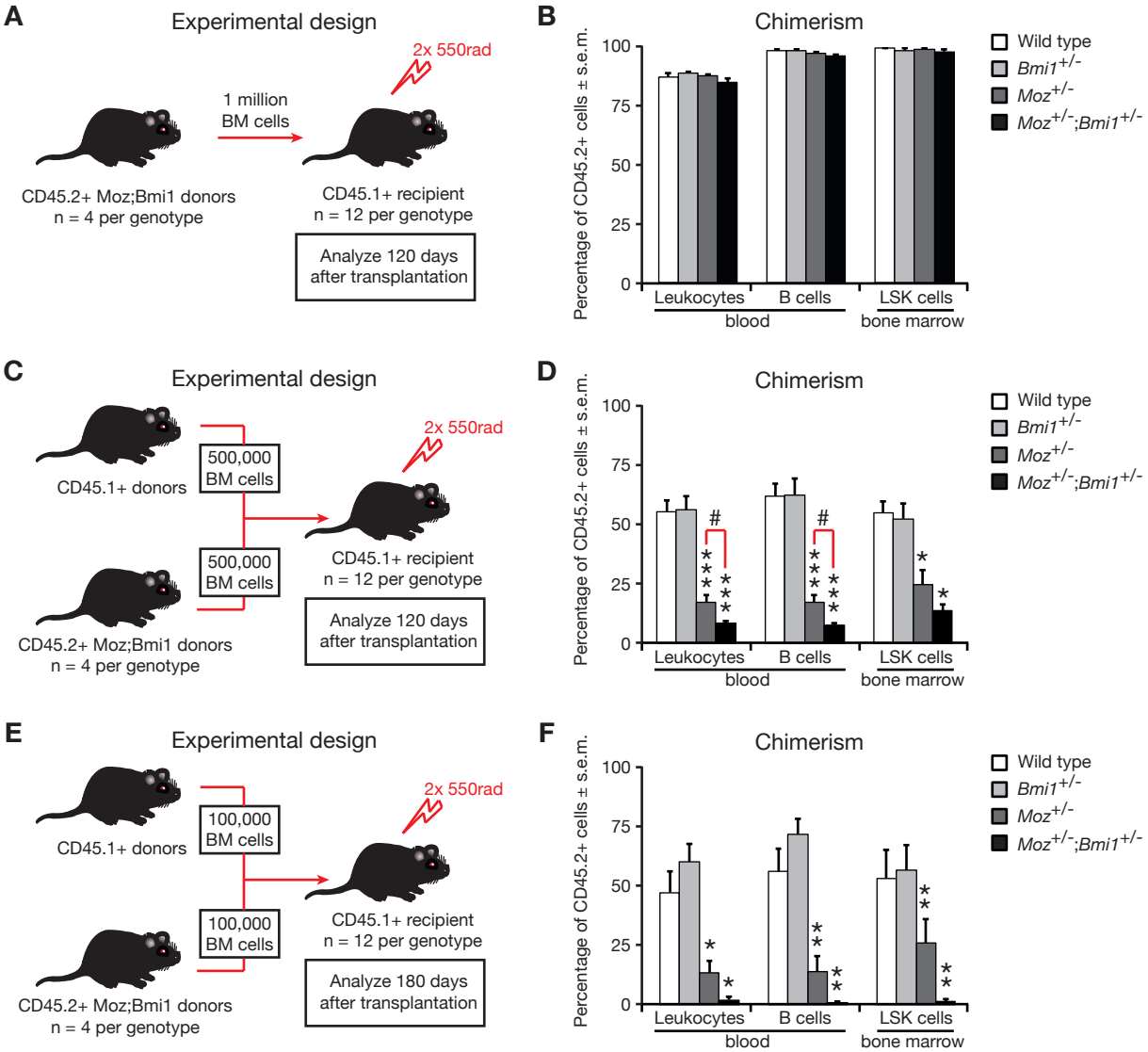
Data are presented as mean ± s.e.m. Data were analyzed using a Student *t*-test between each of the four genotypes. Asterisks show significance at $*p < 0.05$, and $**p < 0.01$ between the indicated groups and wild type.

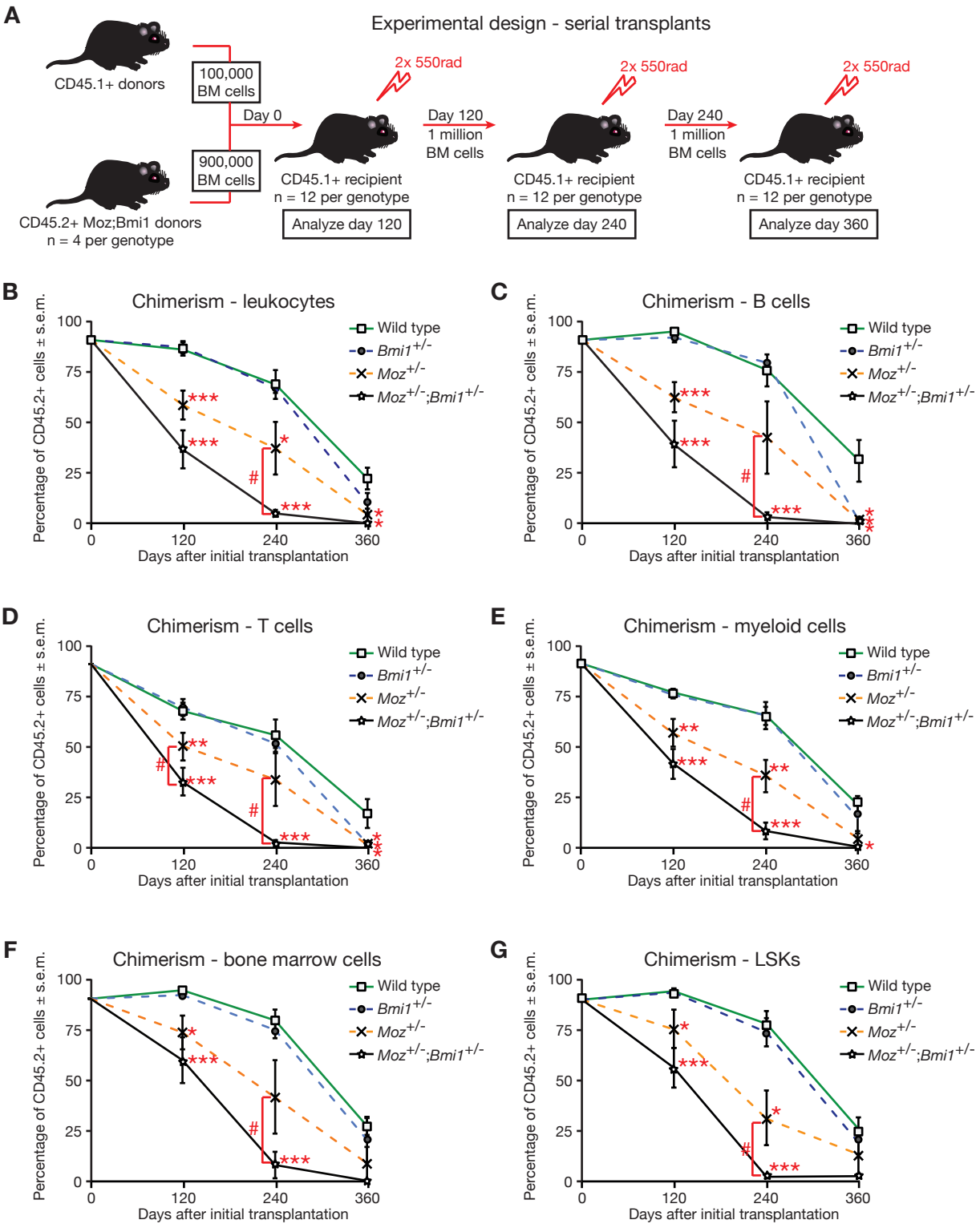
Figure

Sheikh Figure 1

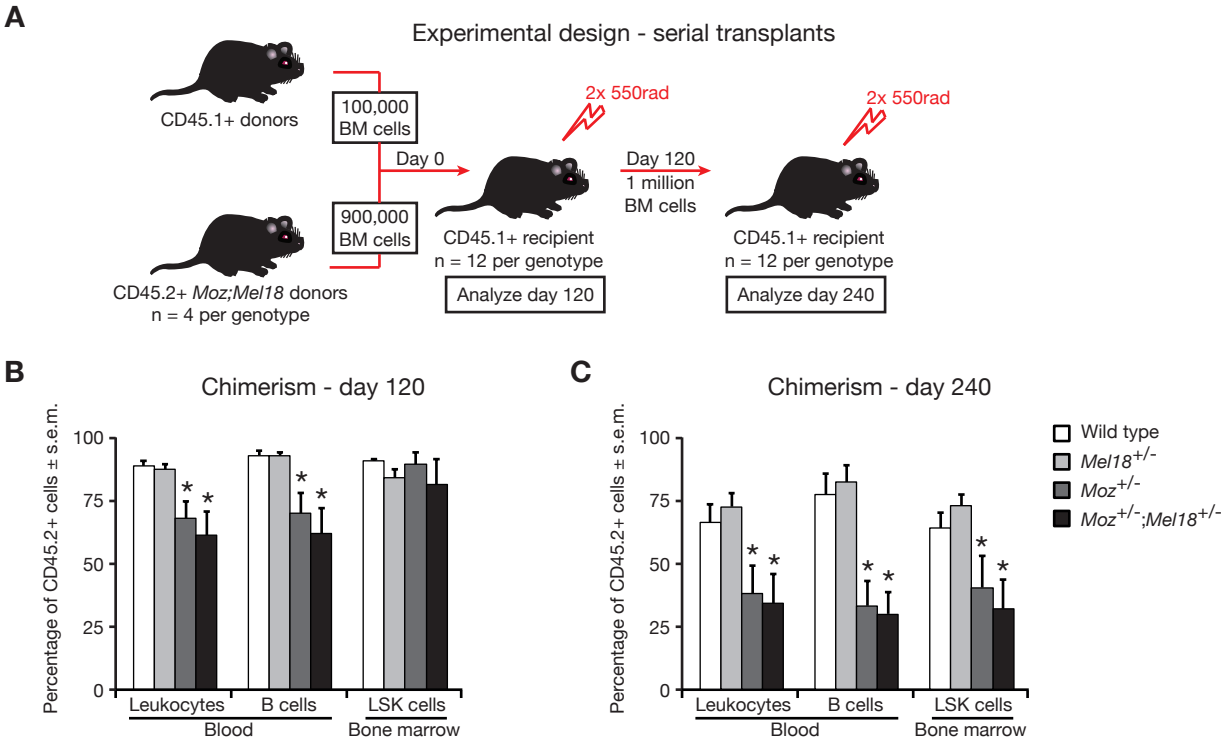


Sheikh Figure 2

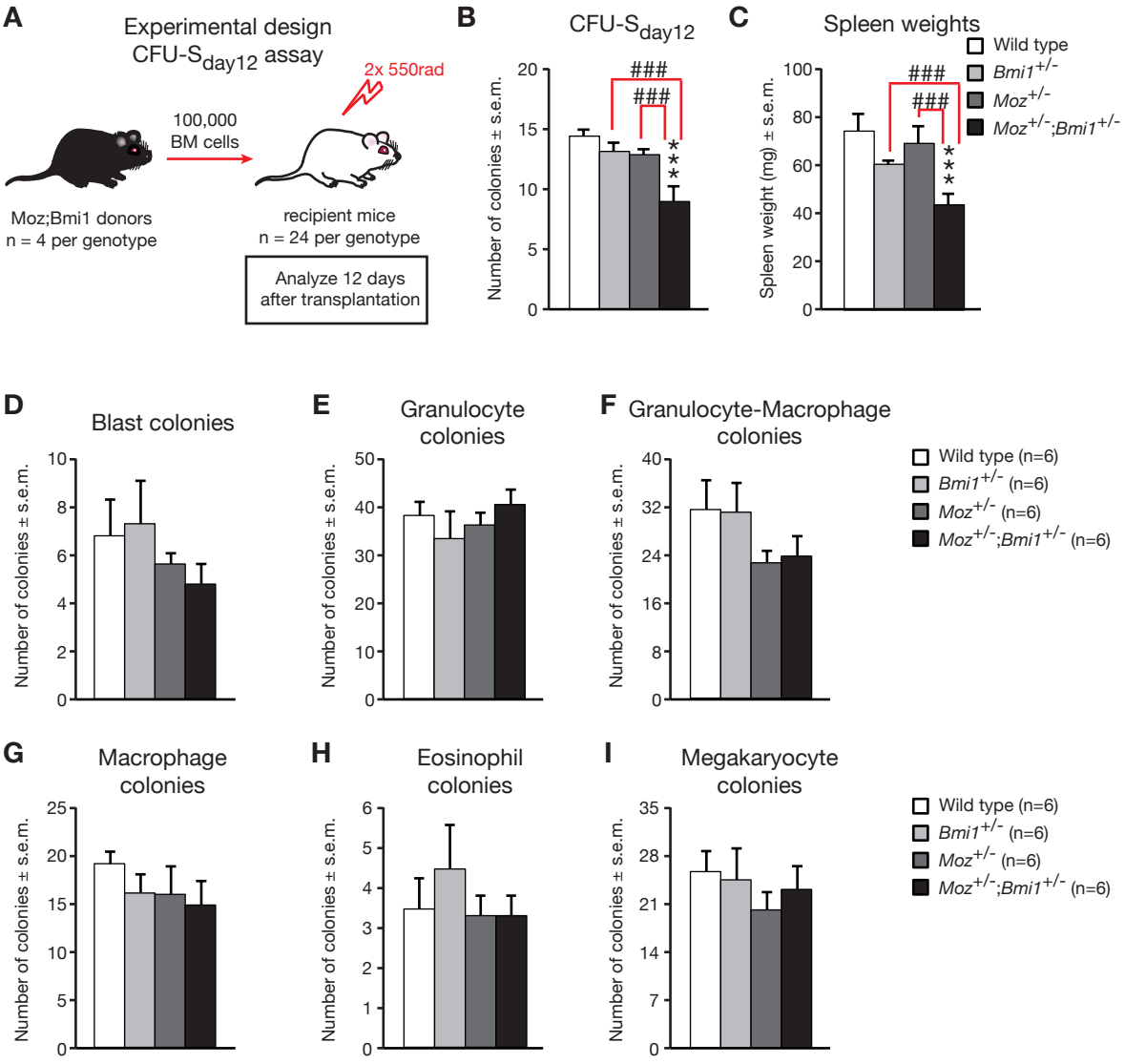


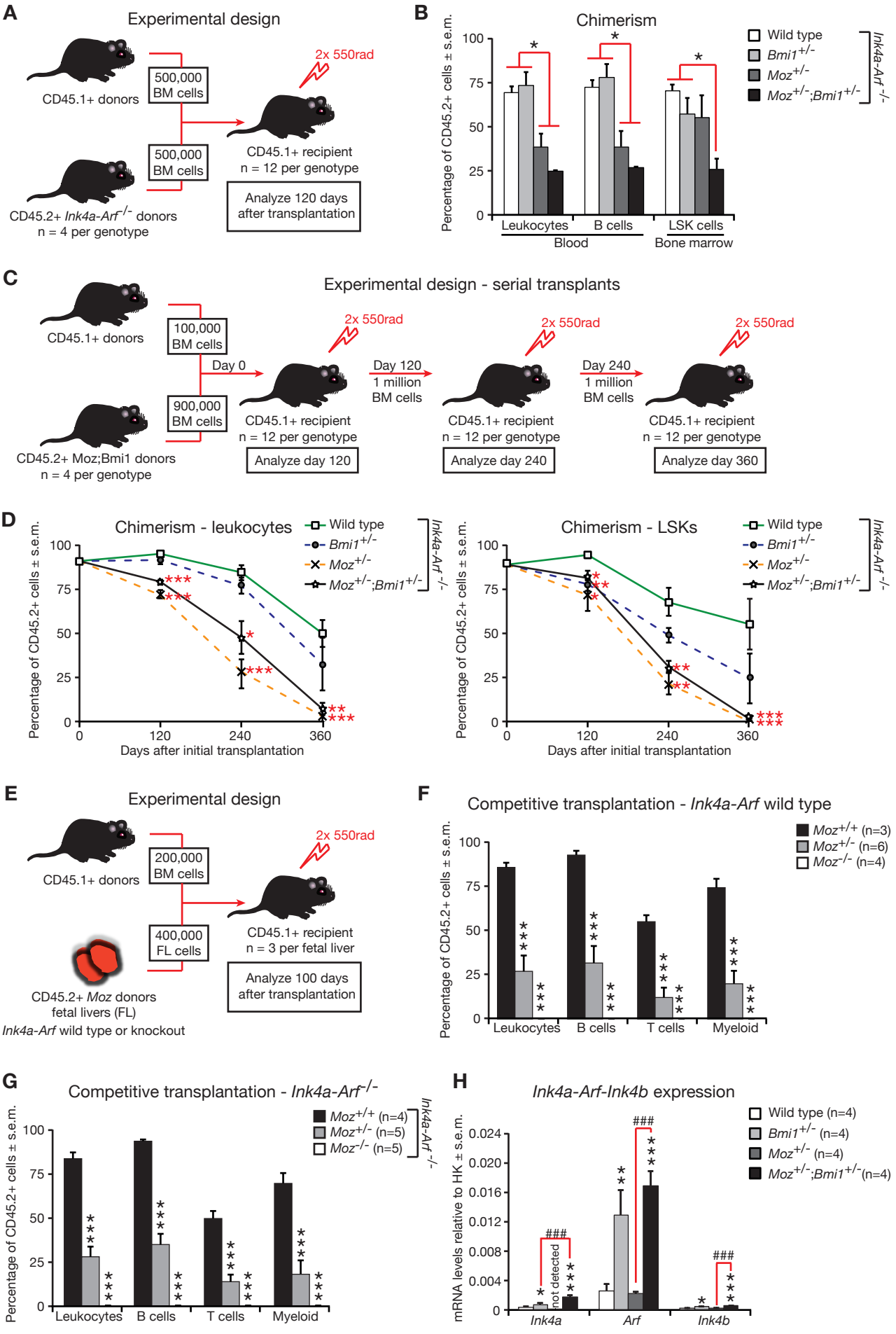

A
Experimental design - serial transplants
CD45.1+ donors
100,000 BM cells
Day 0
900,000 BM cells
CD45.2+ Moz;Bmi1 donors n = 4 per genotype
2x 550rad
CD45.1+ recipient n = 12 per genotype
Analyze day 120
Day 120 1 million BM cells
2x 550rad
CD45.1+ recipient n = 12 per genotype
Analyze day 240
Day 240 1 million BM cells
2x 550rad
CD45.1+ recipient n = 12 per genotype
Analyze day 360
B
Chimerism - leukocytes
C
Chimerism - B cells
D
Chimerism - T cells
E
Chimerism - myeloid cells
F
Chimerism - bone marrow cells
G
Chimerism - LSKs
Percentage of CD45.2+ cells ± s.e.m.
Days after initial transplantation
Wild type
Bmi1+/-
Moz+/-
Moz+/-;Bmi1+/-

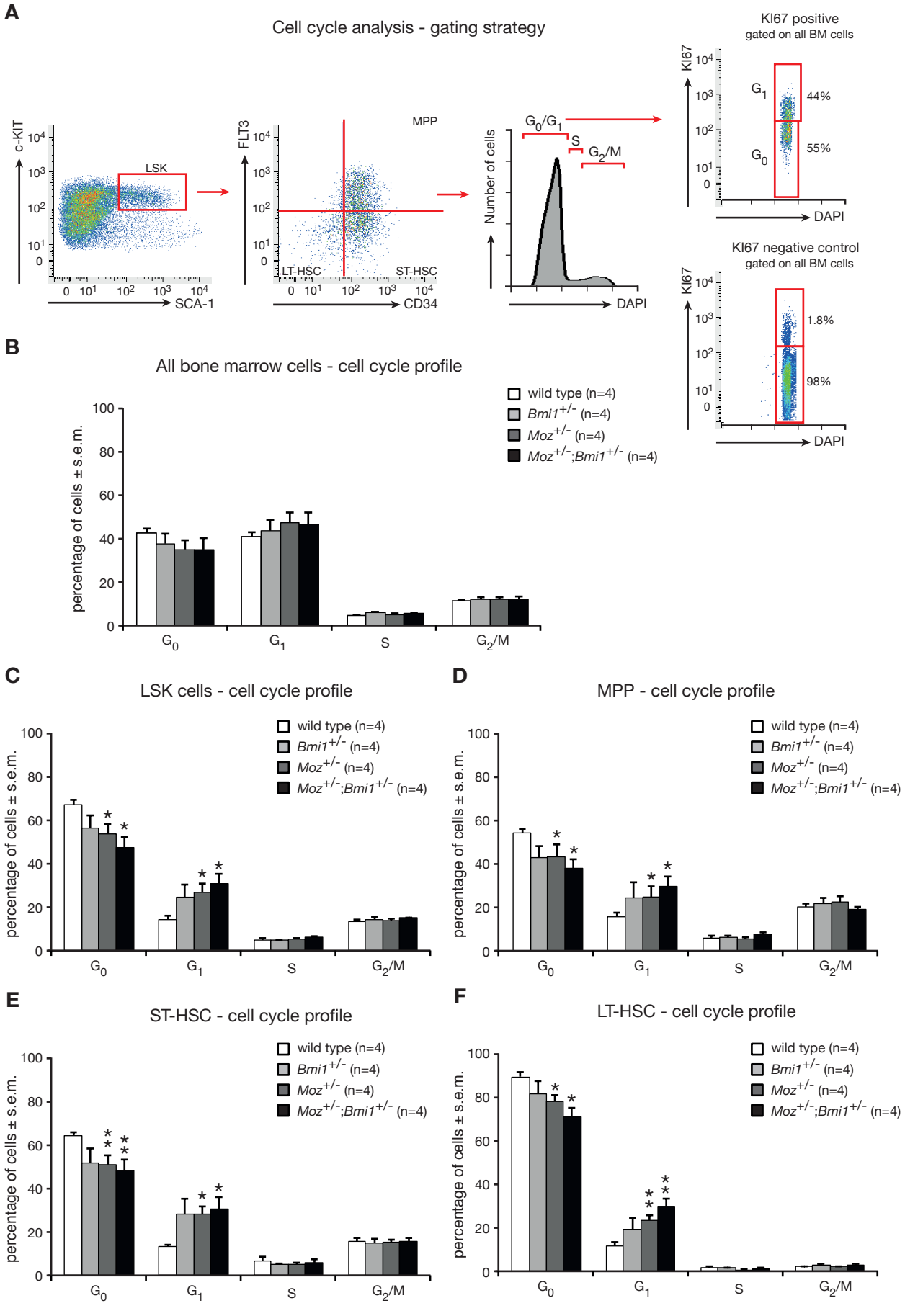
Sheikh Figure 4



Sheikh Figure 5




A
Experimental design
CD45.1+ donors
500,000 BM cells
500,000 BM cells
CD45.2+ Ink4a-Arf-/- donors n = 4 per genotype
2x 550rad
CD45.1+ recipient n = 12 per genotype
Analyze 120 days after transplantation
B
Chimerism
Percentage of CD45.2+ cells ± s.e.m.
Leukocytes
B cells
LSK cells
Blood
Bone marrow
Wild type
Bmi1+/-
Moz+/-
Moz+/-;Bmi1+/-
Ink4a-Arf -/-
C
Experimental design - serial transplants
CD45.1+ donors
100,000 BM cells
900,000 BM cells
CD45.2+ Moz;Bmi1 donors n = 4 per genotype
Day 0
2x 550rad
CD45.1+ recipient n = 12 per genotype
Analyze day 120
Day 120
1 million BM cells
Analyze day 240
Day 240
1 million BM cells
Analyze day 360
D
Chimerism - leukocytes
Chimerism - LSKs
Percentage of CD45.2+ cells ± s.e.m.
Days after initial transplantation
E
Experimental design
CD45.1+ donors
200,000 BM cells
400,000 FL cells
CD45.2+ Moz donors fetal livers (FL)
Ink4a-Arf wild type or knockout
CD45.1+ recipient n = 3 per fetal liver
Analyze 100 days after transplantation
F
Competitive transplantation - Ink4a-Arf wild type
Moz+/+ (n=3)
Moz+/- (n=6)
Moz-/- (n=4)
Leukocytes
B cells
T cells
Myeloid
G
Competitive transplantation - Ink4a-Arf-/-
Moz+/+ (n=4)
Moz+/- (n=5)
Moz-/- (n=5)
H
Ink4a-Arf-Ink4b expression
mRNA levels relative to HK ± s.e.m.
not detected
Ink4a
Arf
Ink4b
Wild type (n=4)
Bmi1+/- (n=4)
Moz+/- (n=4)
Moz+/-;Bmi1+/- (n=4)


A
Cell cycle analysis - gating strategy
c-KIT
SCA-1
LSK
FLT3
CD34
MPP
LT-HSC
ST-HSC
Number of cells
DAPI
G0/G1
S
G2/M
KI67 positive
gated on all BM cells
KI67
G1
G0
44%
55%
KI67 negative control
gated on all BM cells
1.8%
98%
B
All bone marrow cells - cell cycle profile
wild type (n=4)
Bmi1+/- (n=4)
Moz+/- (n=4)
Moz+/-;Bmi1+/- (n=4)
percentage of cells ± s.e.m.
C
LSK cells - cell cycle profile
D
MPP - cell cycle profile
E
ST-HSC - cell cycle profile
F
LT-HSC - cell cycle profile