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Dual Mechanisms by which MiR-125b Represses IRF4 to Induce Myeloid and B cell Leukemias

Running Title: MiR-125b and IRF4 in leukemia

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Key points

- MiR-125b induces tumorigenesis in myeloid cells by repressing the expression of IRF4 at the mRNA and protein level
- MiR-125b promotes oncogenesis in B cells that involves selection of cells that acquire genetic deletion of the gene encoding IRF4

Abstract

The oncomir microRNA-125b (miR-125b) is up-regulated in a variety of human neoplastic blood disorders and constitutive up-regulation of miR-125b in mice can promote myeloid and B cell leukemia. We found that miR-125b promotes myeloid and B cell neoplasm by inducing tumorigenesis in hematopoietic progenitor cells. Our study demonstrates that miR-125b induces myeloid leukemia by enhancing myeloid progenitor output from stem cells as well as inducing immortality, self-renewal, and tumorigenesis in myeloid progenitors. Through functional and genetic analyses, we demonstrated that miR-125b induces myeloid and B cell leukemia by inhibiting IRF4 but through distinct mechanisms; it induces myeloid leukemia through repressing IRF4 at the mRNA level without altering the genomic DNA and induces B cell leukemia via genetic deletion of the gene encoding IRF4.

Introduction

MicroRNAs have been found to be dysregulated in several types of human and mouse cancers, including carcinomas and leukemias. As happens with protein-coding oncogenes, non-coding oncomirs can provoke cancers by dysregulating developmental, signaling and cell survival pathways in different cell types. For the majority of oncomirs, it is not clear how a single microRNA can induce cancer development in various cell types. Potentially, an oncomir can suppress the same target(s) in different cell types to promote tumorigenesis, or it can inhibit distinct cell-specific targets to induce cancer development.

The oncomir microRNA-125b (miR-125b) is up-regulated in a myriad of neoplastic blood disorders, including acute myeloid leukemia (AML) and B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [1-3]. Importantly, we and other researchers showed that enforced constitutive over-expression of miR-125b in mice induces myeloid, B cell, and T cell leukemia [4-7], indicating that miR-125b can provoke the oncogenic state in a range of hematopoietic cells. Interestingly, we found that miR-125b over-expression initially impairs the development of B cells whereas others found that it induces B cell leukemia [5, 6]. This suggests that miR-125b might initially repress the development of B cells but that these cells might acquire secondary mutational or epigenetic events that transform them into cancer cells. To date, the mechanism by which miR-125b induces tumorigenesis in different hematopoietic lineages is unknown.

Previously, we found that down-regulating the expression of the direct miR-125b target IRF4 was sufficient to recapitulate the activated phenotype observed upon over-expressing miR-125b in bone marrow-derived macrophages [8]. Relevant to leukemia, the expression of Irf4 is down-regulated in a range of hematopoietic cancer cell lines [9] as well as in human patients with AML, chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL) [10, 11]. Also, deletion of IRF4 in mice exacerbates the development of myeloid leukemia and

leads to development of B cell leukemia [12-14]. However, whether repression of IRF4 plays a functional role in miR-125b-induced myeloid and B cell leukemia remains to be tested.

In this study, we have investigated the cellular and molecular mechanisms by which miR-125b induces the development of myeloid and B cell leukemia. We found that miR-125b induces cancer development by initiating tumorigenesis in myeloid and B precursor cells. Our data also indicates that in both cases miR-125b induces myeloid and B cell leukemia by inhibiting IRF4 expression. Whereas miR-125b induces tumorigenesis in myeloid cells by repressing the expression of IRF4 at the mRNA and protein level, it promotes oncogenesis in B cells by provoking genetic deletion of IRF4. Thus, miR-125b represents a novel paradigm by which an oncomir induces cancer development in multiple cell lineages by modulating the same signaling pathway but via distinct mechanisms.

Methods

DNA Constructs

pMG, pMSCV-IRES-GFP (MIG), pMG-miR-125b, and pMiR-report IRF4 3'UTR vectors have been described [5, 8]. pMIG-miR-125b co-expresses GFP and miR-125b. pMIG-IRF4 co-expresses GFP and IRF4. pHcRed-miR125b and pmCherry-miR125b co-express miR-125b and HcRed or mCherry, respectively (**Supp. Table 1** for cloning primers).

Infection of BMCs, bone marrow reconstitution, and *in vitro* cell proliferation assays

To generate MG and MG-125b mice, lethally irradiated C57bl/6 recipient mice were injected with virally transduced bone marrow cells (BMCs). Briefly, donor C57bl/6 BMCs were transduced with miR-125b over-expressing vector (pMG-miR-125b or pMIG-miR-125b) through two to four rounds of spin infection, which achieved 25- and 186-fold higher miR-125b over-expression (**Supp. Fig. S7**). The expression levels are within range of the level of miR-125b over-expression observed in human patients with leukemia or myelodysplastic syndromes, which range from several to 262-fold above normal [1, 6, 15]. For the *in vitro* proliferation assays, BMCs and sorted Lin^- $cKit^+$ $Sca1^-$ MPs were cultured and passaged in 50ng/mL SCF, and sorted Lin^- $cKit^+$ $Sca1^+$ HSPCs were cultured in media containing 50ng/mL SCF, 50ng/mL IL6, and 25ng/mL IL3. All animal studies were approved by the IACUC.

Transplantation of miR-125b-induced cancer cells and fluorescent activated cell sorting

Splenic cells, BMCs, sorted GFP^+ $Lin^-cKit^+Sca1^-$ cells, and sorted GFP^+ $CD19^+$ cells were harvested from MG-125b mice when they developed leukemia and transplanted into sublethally irradiated C57bl/6 recipients. For transplantation of common myeloid progenitors, 3.5K Lin^-

cKit^{+}Sca1^{-} cells (Lin = ILR7a, Thy1, Cd11b, Cd11c, B220, Ter119, CD3e, NK1.1, GR-1) were sorted from MG-125b mice when they were moribund. Cells were sorted using the FACSAria (BD Biosciences) or iCyte (Sony Biotech) instrument. Flow cytometric antibodies were purchased from eBioscience or Biolegend.

Single cell analysis and cell differentiation assay

MiR-125b over-expressing Lin^{-} cKit^{+} Sca1^{-} MPs were passaged six times in 50ng/mL SCF media. Single cells were plated. One month later, an aliquot of the expanded cells were assayed for granulocytes formation and some cells were cultured in 20ng/mL MCSF or J558L-GMCSF conditioned media to differentiate into macrophages and dendritic cells, respectively.

***In vitro* proliferation assay with rescued expression of IRF4**

For *in vitro* proliferation assays, BMCs were first infected with pHcRed-miR-125b retroviruses that express miR-125b and HcRed. Subsequently, cells were infected with pMIG or pMIG-IRF4 retroviruses. HcRed^{+} GFP^{+} cells were sorted and cultured in 50ng/mL SCF.

Competitive repopulation of miR-125b over-expressing cells with rescued IRF4 expression.

Donor BMCs were infected with mCherry^{+} miR-125b over-expressing virus (pmCherry-miR-125b vector). The cells were then infected with GFP^{+} virus (pMIG vector) or GFP^{+} IRF4 over-expressing virus (pMIG-IRF4 vector) and transplanted into irradiated C57bl/6 mice.

Transplantation of sorted miR-125b over-expressing cells with restored IRF4 expression

BMCs were infected with GFP^+ miR-125b over-expressing retroviruses (pMG-miR-125b vector) or co-infected with $HcRed^+$ miR-125b over-expressing (pHcRed-miR-125b vector) and GFP^+ IRF4 over-expressing retroviruses (pMIG-IRF4 vector). GFP^+ cells or GFP^+ $HcRed^+$ cells were sorted from the former and latter group of cells, respectively. The sorted BMCs were transplanted into irradiated C57bl/6 mice.

Exome sequencing and comparative genomic hybridization microarray (aCGH)

See supplemental methods for detailed experimental setup and data analysis. The microarray GEO accession number is: GSE58900.

Results

MiR-125b over-expressing leukemic cells are serially transplantable

To examine the ability of miR-125b-induced myeloid cancer cells to serially propagate, we over-expressed miR-125b through bone-marrow reconstitution experiments using the MSCV-GFP$^+$ (MG)-based retroviral system in mice (hereon denoted as 'MG-125b') [5]. When the MG-125b mice developed frank myeloid leukemia, cells from the spleen or bone marrow were transferred into sub-lethally irradiated secondary recipients. These recipients were moribund or died within 70 days (**Fig. 1A**) and developed leukemia indicated by increased white blood cell count (**Fig. S1A**) and splenomegaly (**Fig. S1B**). Recipient mice that received cells from control 'MG' mice did not develop cancer and remained healthy through the duration of the study (**Fig. 1A**). Remarkably, transplantation of miR-125b-induced cancer cells from secondary recipients into tertiary non-irradiated immuno-competent recipients also induced myeloid leukemia in the mice (**Fig. 1A, Fig. S1C-D**). Collectively, our data indicates that the myeloid cancer induced by miR-125b over-expression is serially transplantable.

Genetic analyses of miR-125b myeloid cancer

Using deep-sequencing technologies, it has been determined that cancer cells can acquire few to thousands of secondary mutations in their exomes [16-20]. Thus, we sorted GFP$^+$ Cd11b$^+$ myeloid cancer cells induced by miR-125b over-expression and subjected them to exome deep-sequencing analysis. The first sample (sample #1) was harvested from a mouse two months after bone marrow reconstitution, which we validated as cancerous cells as demonstrated by the ability of these cells to be transplanted into a non-irradiated recipient mouse (data not shown). The second sample (sample #2) was harvested from a miR-125b

over-expressing mouse three months after reconstitution; this mouse developed frank myeloid leukemia as characterized by moribund feature, exacerbated myeloid cell count in the blood (27x higher than normal), and splenomegaly (data not shown). The third sample (sample #3) was harvested from miR-125b-induced myeloid leukemic cells that were serially transplanted into recipient mice to ensure that we were analyzing cancerous cells in our deep-sequencing analysis. The deep-sequencing data sets were filtered based on a 0.1% false discovery rate, and cancer-specific mutations were obtained by subtracting those that were also identified in normal healthy mice. From the three independent experiments, we identified two, four, and thirty-two mutations from samples #1, #2, and #3, respectively (**Fig. 1B, Supp. Table 2**). The variations in the number of mutations from the different samples might be due to the different time at which they were harvest after bone marrow reconstitution. We have not ruled out the possibility that a small fraction of the myeloid cancer cells might have random mutations, which is outside of the deep-sequencing detection limit. However, our data suggests that the majority of miR-125b-driven myeloid cancer cell populations in the samples tested have relatively few nucleotide mutations in the coding regions and do not have widespread genomic instability.

Over-expression of miR-125b in primary BMCs *in vitro* recapitulates myeloid malignancy phenotype

Previously, we showed that induction of myeloid leukemia in mice deleted of the gene for a different microRNA, miR-146a, was in part dependent on extrinsic factors released by miR-146a$^{-/-}$ lymphocytes [21]. Thus**,** we investigated whether miR-125b over-expressing lymphocytes contribute to myeloid cancer development in MG-125b mice. BMCs harvested from B cell-deficient EμMT [22] or lymphocyte-deficient Rag1$^{-/-}$ [23] donor mice were transduced with miR-125b encoding retroviruses and transplanted into normal C57bl/6 recipient mice. These

recipient mice developed myeloid leukemia (**Fig. 1C**) at a similar time scale and rate (100%) compared to those transplanted with miR-125b over-expressing WT BMCs (**Fig. 1D**), indicating that the development of myeloid leukemia provoked by miR-125b does not require miR-125b over-expressing lymphocytes.

Next, we tested whether over-expressing miR-125b in BMCs *in vitro* could induce cellular hyper-proliferation and recapitulate the myeloproliferative phenotype observed in mice. We over-expressed miR-125b in BMCs and cultured them in cytokine-supplemented media (SCF+IL3+IL6, SCF+IL-3 or SCF+IL-6) that supports growth and differentiation of HSCs, myeloid progenitors, and mature myeloid cells. Indeed, over-expression of miR-125b *in vitro* elevated the growth rate of BMCs (**Fig. S2A-C**) and induced hyper-expansion of myeloid cells (**Fig. 2A**). MiR-125b over-expressing BMCs cultured in SCF alone, which supports growth of hematopoietic stem and progenitor cells, also grew faster (**Fig. 2B**), suggesting that miR-125b induces hyper-proliferation in part due to its effect in stem/progenitor cells. Indeed, growth of BMCs with ectopic miR-125b expression generated higher number of Lin^- $cKit^+$ $Sca1^-$ cells (**Fig. 2C**). In normal counterparts, Lin^- $cKit^+$ $Sca1^-$ cells represent myeloid progenitors/MPs. Consistently with elevated number of myeloid progenitors, miR-125b over-expressing BMCs generated more dendritic cells when subjected to a methylcellulose-based colony-forming assay (**Fig. S2D**) or differentiated into dendritic cells in liquid culture (**Fig. 2D**). MiR-125b over-expressing cells also formed more colonies in methylcellulose assay when cultured in present of SCF+IL3+IL6 (**Fig. S2E**) although the cells did not serially re-plate (data not shown). Thus, our *in vitro* assay recapitulated the phenotype observed with over-expressing miR-125b in mice with higher numbers of myeloid progenitors and differentiated $Cd11b^+$ myeloid cells [5].

Moreover, we investigated whether miR-125b over-expression is also sufficient to transform and immortalize BMCs *in vitro*. Indeed, whereas control cells died after several

passages, miR-125b over-expressing cells continued to grow indefinitely (**Fig. 2E**). When we injected miR-125b over-expressing BMCs that had been cultured *in vitro* into sublethally irradiated recipient mice, the cells dominated the blood of these mice and produced myeloid leukemia (**Fig. 2F)** marked by splenomegaly (**Fig. 2G**) and bone marrow pallor (**Fig 2H**). These data suggest that the cultured miR-125b over-expressing BMCs were transformed *in vitro* into cancer cells capable of inducing malignancy *in vivo.*

Effect of over-expressing miR-125b in hematopoietic stem and progenitor cells

Next, we tested whether miR-125b over-expression elevates the myeloid progenitor pool by enhancing the hematopoietic output of cells upstream of MPs, such as hematopoietic stem and progenitor cells (HSPCs). We cultured sorted MG and miR-125b $Lin^-cKit^+Sca1^+$ (LKS^+) cells (**Fig. S3A**) and found that those with miR-125b-over-expression generated higher number of cells overall (**Fig. 3A**) with a moderate increase in absolute LKS^+ cell numbers (**Fig. 3B**) after six days of growth. When we instead looked at the $Lin^-cKit^+Sca1^-$ (LKS^-) population, the cellular output was dramatically higher in the miR-125b over-expressing samples (**Fig. 3C**) with exaggerated ratio of LKS^- to LKS^+ cells (**Fig. 3D**). Overall, our data suggests that miR-125b functions in HSPCs and increases the output of downstream MPs. Next, we tested whether miR-125b also functions intrinsically in MPs to accelerate the proliferation of these cells. We cultured LKS^- cells sorted from BMCs transduced with MG or miR-125b encoding retroviruses. We found that the cells with ectopic miR-125b expression grew much faster (**Fig. 3E**) and generated more myeloid cells ($Cd11b^+$) of both granulocytic ($GR1^+$) and non-granulocytic lineages ($Cd11b^+$ $GR1^-$) (**Fig. 3F**). In addition, the miR-125b-overexpressing cells were also more resistant to cell death as indicated by propidium iodide staining (**Fig. S3B**). Thus, miR-125b induces hyper-proliferation and resistance to cell death of MPs *in vitro*.

Moreover, we found that whereas control LKS$^-$ cells died after several passages, miR-125b over-expressing cells continued to grow indefinitely, suggesting that miR-125b MPs were immortalized (**Fig. 3G**). We also tested whether cultured miR-125b over-expressing LKS$^-$ retain their ability to differentiate and whether a single cell can re-populate the myeloid repertoire. After passaging miR-125b-over-expressing MPs six times, single cells were plated into individual wells. Of the 96 single cells plated, 11 clones expanded beyond one month and were able differentiate into GR1$^+$ granulocytes, F4/80$^+$ macrophages, and Cd11c$^+$ dendritic cells (**Fig. S3C**). Collectively, this data indicates that miR-125b over-expression induces self-renewal of MPs *in vitro*.

To test whether miR-125b over-expression induces tumorigenesis in myeloid progenitors, we transplanted sorted MPs from MG-125b mice into sublethally irradiated C57bl/6 recipients. Indeed, the recipient mice were moribund within 70 days and developed frank myeloid leukemia characterized by large excess of myeloid and myeloblast cells in the peripheral blood (**Fig. 3H**), splenomegaly (**Fig. 3I**) and bone marrow pallor (**Fig. 3J**), and dissemination of cancer cells into non-lymphoid tissues (liver, lung, kidney) (data not shown). In further support that miR-125b induces myeloid leukemia through progenitor cells, injection of terminally differentiated miR-125b over-expressing dendritic cells into mice did not induce leukemia (**Fig. S3D-F**), indicating the requirement of progenitor state for miR-125b to initiate tumorigenesis.

IRF4 expression rescues miR-125b-induced myeloid leukemia

Next, we investigated signaling pathways regulated by miR-125b to induce cancer development. Previously, we and other labs showed that miR-125b directly inhibits the expression of the transcription factor IRF4 [8, 24, 25]. We first confirmed that miR-125b inhibits

IRF4 expression in primary mouse sorted LKS^+ and LKS^- cells (**Fig. S4A-B**) and represses a luciferase reporter linked to the 3'UTR of IRF4 (**Fig. S4C**). Next, we tested and found that re-introduction of IRF4 without its 3'UTR (**Fig. S4D-E**) into miR-125b-over-expressing BMCs was sufficient to reverse the hyper-proliferative phenotype induced by miR-125b (**Fig. 4A**) and prevented miR-125b-mediated myeloid expansion *in vitro* (**Fig. S4F**).

Moreover, we tested the effect of uncontrolled IRF4 expression on miR-125b-over-expressing mice. To this end, we retrovirally transduced BMCs with a $mCherry^+$ miR-125b-over-expressing vector and then co-infected these cells with retroviruses expressing both GFP and IRF4 (lacking its UTR) or with GFP controls (**Fig. 4B**). These unsorted retrovirally transduced BMCs were then transplanted into C57Bl/6 recipient animals. After reconstitution, we found that mice transplanted with cells co-infected with IRF4-GFP and miR125b-mCherry vectors were deficient of cells expressing both miR-125b and IRF4 ($mCherry^+GFP^+$ population) (**Fig. 4C, Fig. S4G**). They did, however, have a prominent population of $mCherry^+GFP^-$ cells (**Fig. 4C**), demonstrating that the cells that persisted in these mice were those expressing only miR-125b but not ones co-expressing IRF4. In contrast, recipient mice transplanted with cells transduced with 125b-mCherry and GFP control vector contained prominent amounts of $mCherry^+GFP^+$ cells (**Fig. 4C, Fig. S4G**). Collectively, our data indicates that hematopoietic cells that over-express miR-125b alone robustly outcompete those with uncontrolled expression of IRF4, suggesting that IRF4 inhibits the enhanced proliferative capacity induced by miR-125b *in vivo*.

Finally, we examined whether rescued expression of IRF4 could inhibit myeloid leukemia provoked by miR-125b over-expression in mice. To this end, we transplanted into recipient mice GFP^+ BMCs that over-express miR-125b alone or along with restored IRF4 expression. Both populations of BMCs engrafted in the recipients as demonstrated by GFP positivity in the blood of these mice. Whereas the recipient mice transplanted with the former group of cells exhibited

severe myeloid hyperplasia after three months, the animals that received cells with restored IRF4 expression had normal myeloid counts (**Fig. 4D)**. As expected, the mice transplanted with miR-125b over-expressing cells had exaggerated development of myeloid in their spleens (**Fig. 4E**) accompanied by bone marrow pallor (**Fig. 4F**), which are pathognomonic features of miR-125b-mediated myeloid leukemia, while those with restored IRF4 expression had normal splenic myeloipoiesis and displayed normal reddish bone marrow (**Fig. 4E-F**). Collectively, our data indicates that suppression of IRF4 is important for miR-125b to induce myeloid leukemia and restoration of IRF4 can inhibit disease development.

MiR-125b over-expression induces B cell cancer harboring IRF4 deletion

We confirmed our previous finding that MG-125b mice have impaired B cell development (**Fig. 5A**) [5]. Curiously, others have shown that B cell-restricted over-expression of miR-125b induces lymphoblastic leukemia in mice [6]. We posited that our experimental mice might be dying from complications associated with myeloid leukemia before manifesting features of B cell leukemia. To examine whether miR-125b promotes B cell leukemia in our experimental setting, we sorted the few GFP^+ $CD19^+$ B cells from MG-125b mice when they developed myeloid leukemia and transplanted these B cells into C57bl/6 recipients. The cells were able to engraft in the secondary recipients (**Fig. 5B**) and proved lethal within two months, generating a fulminant $CD19^+$ cancer (**Fig. 5C**). Moreover, the resultant GFP^+ $CD19^+$ cancer cells were serially transplantable into sublethally irradiated or non-irradiated recipient mice for up to 11 generations (**Fig. S5A**), inducing B cell leukemia (**Fig. 5D**) and in some cases B cell lymphoma (**Fig. 5E**) in the recipients. The development of B cell leukemia in these mice was also marked by splenomegaly (**Fig. 5F**) and dissemination of GFP^+ $CD19^+$ into non-hematopoietic organs,

including the lung, liver, and kidney (**Fig. S5B**). Thus, miR-125b over-expression promotes tumorigenesis in B cells in addition to myeloid cells.

Next, we examined the stage of B cell development at which cells are sensitive to miR-125b induced transformation; specifically, we investigated whether B cell maturation is required for miR-125b to induce transformation. To this end, we over-expressed GFP^+ miR-125b in donor BMCs from EμMT mice, which do not develop mature B cells but produce $CD19^+$ pre-B cells [22]. C57Bl/6 mice were reconstituted with these cells and three months after transplantation, sorted GFP^+ $CD19^+$ cells were injected into sublethally irradiated mice. These cells became leukemic in the recipient mice, as indicated by domination of GFP^+ $CD19^+$ cells in the bone marrow (**Fig. 5G**) and induction of splenomegaly (**Fig. 5H**). Thus, miR-125b can initiate tumorigenesis at or earlier than the pre-B cell stage of development. Potentially, miR-125b can directly transform pre-B cells, or miR-125b can initiate tumorigenesis at earlier stages of cell development with complete transformation occurring at the B cell progenitor stage. Nevertheless, our experiment indicates that B cell maturation is not required for miR-125b to induce lymphomagenesis.

In our experiments, miR-125b ectopic over-expression initially repressed B cell genesis (**Fig. 5A**) but some cells of this lineage became cancerous later (**Fig. 5B-F**), leading to the possibility that induction of B cell leukemia may result from the accumulation of secondary genetic mutations involved in cellular transformation. To test this possibility, we performed comparative genomic hybridization microarray experiments (aCGH) of miR-125b B cancer cells to identify genomic alterations that might have occurred. In two independent experiments, we found that the B cancer cells induced by over-expression of miR-125b had an extra copy of chromosome 11 (**Fig. 6A**, **Fig. S6A**). This characteristic was specific to miR-125b-induced B cancer cells and was not found in the myeloid cancer cells (**Fig. S6A**). G-band karyotyping

analysis confirmed that the miR-125b-induced B cancer cells indeed harbor trisomy 11 (**Fig. 6B**). No obvious inversions or translocations were identified through karyotyping. Further, the aCGH experiments indicated that the IgK (chromosome 6) and IgH (chromosome 12) loci had suffered deletions in the cancerous B cells harvested from two separate groups of mice (**Fig. 6A, Fig. S6B**), suggesting that these loci had been undergo VDJ rearrangement. Thus, we performed flow cytometric analyses to characterize these cancer B cells further and found that they were $CD43^+$ $CD24^+$ IgM^- $cKit^-$ (data not shown) with one group of cells being IgK^+ $IgG1^+$ (group #1) and another being predominantly IgK^- $IgG1^-$ (**Fig. 6C**), indicating the former group of cells have productively rearranged their immunoglobulin loci whereas the latter did so non-productively.

Strikingly, in both of our independent aCGH experiments, we found that cancer B cells induced by miR-125b over-expression harbored genetic deficiency at the tumor suppressor IRF4 locus (**Fig. 6A, Fig. S6A**). The IRF4 genetic deletion was specific to the miR-125b-induced B cancer cells and was not observed in miR-125b-induced myeloid cancer cells (data not shown). Quantitative PCR analysis confirmed that the translational start site of both copies of IRF4 was deleted in the B cell cancer samples (**Fig. 6D-E**), and genotyping analyses revealed that almost the entire IRF4 coding region (exon2-exon7, amino acids 1-366 of 450 amino acids full length protein) was deleted (**Fig. 6D-F, Fig. S6C**). Interestingly, the population of cancer B cells was found to be heterogeneous with some expressing CD47 whereas others did not (**Fig. S6D**); also, individual cancer B cell samples exhibited different IRF4 genetic breaks (**Fig. S6E**), indicating that although there were different cancer clones, IRF4 deletion is ubiquitous. The strong selective pressure for genetically eliminating both copies of IRF4 suggests a functional role of IRF4 in miR-125b-induced B cell cancer. Indeed, multiple studies have recently

demonstrated that deletion of IRF4 in mice leads to the development of B cell leukemia [12-14, 26-29].

Discussion

We set out to determine the mechanisms by which miR-125b induces development of leukemia. Relating to myeloid leukemia, we found that: (1) Myeloid cancer cells induced by miR-125b exhibit relatively few mutational alterations; (2) miR-125b is able to promote tumorigenesis *in vitro* in a cell intrinsic manner; (3) miR-125b causes myeloproliferation by enhancing output of MPs from HSPC as well as promoting hyper-proliferation of MPs; (4) constitutive over-expression of miR-125b promotes self-renewal of MPs *in vitro* and induces the transformed state in these cells; (5) miR-125b represses IRF4 expression to induce myeloid leukemia. In relation to miR-125b-induced B cell leukemia we found that: 1) Over-expression of miR-125b initially represses B cell development but constitutive long-term up-regulated expression provokes a highly aggressive serially transplantable B cell leukemia; 2) miR-125b can promote B cell leukemia by inducing tumorigenesis in cells at or earlier than the pre-B cell stage; 3) The miR-125b-induced B cancer cells are capable of immunoglobulin recombination; 4) Leukemic B cells induced by miR-125b acquire common genetic deletion of the tumor suppressor IRF4.

Our deep-sequencing analysis indicates that miR-125b-driven myeloid cancer cells acquire relatively few nucleotide mutations in their exomes, suggesting that the majority of these cells have not acquired permanent common genetic mutations. Thus, we speculate that reducing miR-125b expression might produce a regression of the myeloid tumors. In support of this notion, it was recently shown that survival of myeloid leukemic cells induced by miR-125a requires continual over-expression of this microRNA and removal of miR-125a after cancer development was accompanied by regression of the leukemia [30]. Further, we established that miR-125b functions within both HSPCs and MPs to drive a myeloproliferative disorder *in vitro*. Indeed, transplanting miR-125b over-expressing MPs could induce leukemia in recipient mice. These data along with the fact that miR-125b over-expressing MPs could grow indefinitely and

also retain their ability to generate the entire myeloid lineage from a single cell suggests that miR-125b induces an abnormal self-renewal program in MPs *in vitro*.

Our data suggests that in both cases miR-125b induces myeloid and B cell leukemia by modulating IRF4 but through distinct mechanisms. Whereas miR-125b induces tumorigenesis in myeloid cells by repressing the expression of IRF4 at the mRNA and protein levels without an affect on its genomic content, it promotes oncogenesis in B cells by a process involving genetic deletion of IRF4. Thus, miR-125b represents a novel paradigm by which an oncomir might induce cancer development in multiple cell lineages by modulating the same signaling pathway but involving distinct mechanisms. In one case, the oncomir works directly to yield tumor cells; in the other, it blocks differentiation but a presumably spontaneous deletion then appears to lead to the oncogenic state. It is likely that the miR-125b-induced block of B cell differentiation is then overcome by the effect of the IRF4 deletion, leading to uncontrolled growth of the cells. It has been shown that $IRF4^{-/-}$ but not $IRF4^{+/-}$ mice develop B cell cancer [14], indicating that complete ablation of IRF4 is sufficient but reduced expression of IRF4 is insufficient to drive tumorigenesis in B cells. Thus, we speculate that although miR-125b over-expression represses IRF4 expression at the mRNA level, the magnitude is not sufficient to promote tumorigenesis in B cells and thus require selection of cells that acquire genetic deletion of IRF4. In addition to IRF4 deletion, other genes may play a role in this process as indicated by the occurrence of trisomy 11.

It has also been shown that human patients with B cell leukemia have elevated expression of miR-125b [1, 2, 31]. There are also reports of patients that acquire genetic mutations at the IRF4 locus [32-35]. The increased expression of miR-125b could be caused by a chromosomal translocation that joins the miR-125b locus to, for instance, the immunoglobulin heavy chain gene regulatory elements [1, 6, 31] or it could be epigenetic. Because of our

finding that over-expression of miR-125b associates with genetic aberration at the IRF4 gene, we speculate that miR-125b up-regulation in human cancer patients might result in a selection of IRF4 genetic mutations and consequently induce lymphoblastic leukemia. In support of this idea, it has already been established that IRF4 deletion in mice leads to the development of B cell leukemia, and over-expression of IRF4 inhibits the development of B cell leukemia in mice using cancer models induced by c-Myc and BCR/ABL [26, 28]. Of note, our finding that the IRF4 tumor suppressor gene is deleted in miR-125b-induced B cancer cells suggests that persistent up-regulation of miR-125b might no longer be required for maintaining oncogenicity of these cancer cells. Although additional experiments will be required to test this point, we speculate that inhibiting miR-125b will not be a suitable therapy for treating B cell leukemia invoked by this microRNA because permanent genetic lesions have occurred.

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Authorship Contributions

AYS, AAC, RS, AM, DC, CX, JK, ELL, YGF, SJ, CK, and PR performed the experiments and analyzed the data; AYS and DB conceived the study. AYS, AAC, RS, AM, DC, CX, JK, ELL, SJ provided crucial reagents; AYS and DB wrote the manuscript; and all authors provided input for the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

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Figure Legends

Figure 1: Features of miR-125b-induced myeloid cancer cells.

A) Leukemic cells from MG-125b are serially transplantable. Cells from MG and MG-125b mice were harvested three to six months after bone marrow reconstitution. 200K GFP^+ splenic cells (black dotted line) or 400-1000K GFP^+ BMCs (red line) from MG-125b mice were transferred into secondary sublethally irradiated C57bl/6 recipients. Two million GFP^+ BMCs from the secondary mice were injected into tertiary non-irradiated C57bl/6 recipients (orange line indicated as 'no irradiation'). Death point of the recipients (at least four mice per group) were recorded when found dead or moribund. **B**) Myeloid cancer cells induced by miR-125b over-expression were harvested and subjected to exome deep-sequencing analysis. Every exon was sequenced an average of 50 times. The sequencing data sets were filtered on a 0.1% false positive discovery rate and cancer-associated mutations were obtained by excluding those that were identified in normal non-cancerous mice. Plot shows the number of cancer-associated exonic mutations identified in each of the three independent experiments. **C**) Donor BMCs from C57bl/6, $Rag1^{-/-}$, and EμMT (B6.129S2-Ighmtm1Cgn/J) mice were transduced with MG-125b retroviruses, which encode for miR-125b and GFP expression. As control, donor BMCs from C57bl/6 animals were transduced with empty MG retroviruses, which encode for GFP. BMCs were transplanted into lethally irradiated C57bl/6 recipients, and the blood of the mice was subjected to flow cytometric analyses three months after reconstitution. **D**) The survival curve of the mice described in panel C is shown (three mice per group). Mice were considered dead when they became moribund or found dead.

Figure 2: Over-expressing miR-125b in BMCs induces myelo-proliferative disorder *in vitro.* **A**) Hematopoietic-stem and progenitor cell (HSPC)-enriched BMCs, which were obtained

by injecting C57bl/6 mice with 5-fluorouracil, were transduced with MG control or MG-125b retroviruses. Equal numbers of BMCs were cultured in 50ng/mL SCF, 50ng/mL IL-6, and 25ng/mL IL-3. After 5 days, the total number of myeloid cells ($CD11b^+$) cells was determined by flow cytometry. **B)** MG control or miR-125b over-expressing HSPC-enriched cells were expanded at same starting density in 50ng/mL SCF. Cells were counted by flow cytometry. **C)** Equal numbers of HSPC-enriched cells transduced with MG or miR-125b over-expression cassette were expanded in 50ng/mL SCF. The density of $Lin^- Sca1^- cKit^+$ cells after four days was determined by flow cytometry. **D**) Equal numbers of MG control or miR-125b over-expressing BMCs were cultured in 20ng/mL GMCSF to induce differentiation into dendritic cells. The number of dendritic cells was determined by flow cytometry six days after culture. **E)** MG or miR-125b transduced BMCs were cultured in 50ng/mL SCF. When the miR-125b over-expressing cells were confluent between 4-8 days after plating, the cells were re-seeded at a starting density of 20K per mL. The X-axis represents the passage number, and the Y-axis represents the cell density. Panels A-E are representative of at least two independent experiments. **F)** MiR-125b over-expressing BMCs were cultured and expanded *in vitro*. One million GFP^+ cells were injected into sublethally irradiated C57bl/6 mice. Two months post-transplantation, recipient blood was subjected to flow cytometric analysis to determine engraftment of GFP^+ cells. The pictures of **G**) spleen and **H**) femur are representative examples of these organs taken from moribund miR-125b transduced mice (right panel) and age-matched C57bl/6 controls (left panel). Representative of six mice.

Figure 3: Effect of over-expressing miR-125b in HSPCs and MPs. **A**) Equal numbers of MG or MG-125b transduced $Lin^- cKit^+ Sca1^+$ HSPCs were cultured, and the cell density was determined using flow cytometry. Two-way ANOVA was used to obtain P values. The number of

B) Lin^- $cKit^+$ $Sca1^+$ HSPCs and **C**) Lin^- $cKit^+$ $Sca1^-$ MPs was determined by flow cytometry six days after culture. **D**) The ratio of MPs to HSPCs was calculated and plotted. Two independent experiments were performed. **F**) The density of myeloids ($Cd11b^+$), granulocytes ($GR1^+$) or non-granulocytic myeloids ($CD11b^+$ $GR1^-$) cells after growing miR-125b over-expressing MPs was determined by flow cytometry ten days later. Two independent experiments performed. **G**) Sorted MG or miR-125b transduced Lin^- $cKit^+$ $Sca1^-$ MPs were cultured and passaged similarly as described in Fig. 2E legend. **H**) Sublethally irradiated C57bl/6 recipients were injected with common myeloid progenitors sorted from MG-125b mice. Wright stain of the blood was performed when the recipients were moribund. **I**) The spleen and **J**) femur were harvested and imaged when the mice were moribund and sacrificed. Representative of four mice. Control mice represent recipient mice injected with BMCs from MG mice.

Figure 4: Rescued expression of IRF4 inhibits miR-125b-induced myeloid leukemia. A) IRF4 inhibits miR-125b-induced hyper-proliferation *in vitro*. Control cells correspond to MG infected BMCs. BMCs were transduced with retroviruses that encode miR-125b and HcRed. These cells were then transduced with MG or MIG-IRF4 retroviruses, which co-expresses GFP and IRF4. $HcRed^+$ GFP^+ cells were sorted, and 20K cells per mL of cells were plated. The cell number was determined three days later. Representative of three experiments. **B**) BMCs were transduced with retroviruses that encode miR-125b and mCherry. These cells were then infected with MG or MIG-IRF4 viruses. Shown are flow cytometric plots of these cells and infection efficiency before transplantation into mice. Plots show cells over-expressing miR-125b in the X-axis ($mCherry^+$) and either GFP^+ or GFP^+ IRF4-over-expressing cells in the Y-axis. **C**) BMCs described in panel B were transplanted into recipient C57bl/6 mice. One month after transplantation, the peripheral blood of the recipient mice was analyzed by flow cytometry. The

left and right panel represents the blood of recipient mice transplanted with BMCs co-infected with miR125b-mCherry with GFP vector and miR125b-mCherry with IRF4-GFP vector, respectively. Representative of four mice. **D**) Recipient mice were transplanted with donor BMCs that over-express miR-125b alone or along with restored IRF4 expression mice. The peripheral blood of the recipient mice (greater than six mice per group) was analyzed by flow cytometry. Plot displays the $Cd11b^+$ myeloid cell count three months after transplantation of donor cells. Control mice represent normal healthy C57bl/6 mice. P value obtained through Mann Whitney T test. **E**) The percent splenic $Cd11b^+$ myeloid was determined using flow cytometry ~3 months after cell transplantation. **G**) Representative images of the femur and tibia of the corresponding recipient mice are shown.

Figure 5: MiR-125b induces tumorigenesis in pre-B cells. **A**) MG and MG-125b were bled 12 weeks after bone marrow reconstitution. The figure represents the percent of B cells ($CD19^+$) within the GFP^+ gated population in the peripheral blood of these mice (at least three mice per group). P value was calculated by Student's T test. **B**) GFP^+ $CD19^+$ B cells were sorted from MG-125b mice 4-6 months after bone marrow reconstitution. 35-52K $CD19^+$ B cells were transplanted into sublethally irradiated mice. The figure shows the flow cytometric analysis of the secondary recipient mice (bone marrow) six weeks after transplantation. Representative of eight mice. **C**) The figure shows the survival of curve of the secondary recipient mice transplanted with GFP^+ $CD19^+$ cells from MG-125b mice (eight mice) or total BMCs from MG mice (six mice). **D**) Wright stain was performed from the blood of recipient mice injected with miR-125b over-expressing $CD19^+$ cells. The dark purple cells represent leukocytes. The smaller cells with central pallor are red blood cells. **E**) Some recipients of miR-125b over-expressing $CD19^+$ cells develop lymphomas. Lymphomas shown at the superficial cervical (top blue arrow) and inguinal

lymph node sites (side blue arrow). The normal mouse shown is a healthy C57bl/6 mouse. **F**) Left panel: The spleen weight of the recipient mice transplanted with GFP^+ $CD19^+$ cells (denoted as "miR-125b $CD19^+$) from MG-125b mice or total BMCs from MG animals were obtained 4-6 months after transplantation. Six mice per group. Right panel: Representative images of spleens harvested from normal C57bl/6 control and miR-125b CD19 transplanted mice 6-months post transplant. **G**) MiR-125b induces pre-B cell cancer. 10K sorted GFP^+ $CD19^+$ cells harvested from mice reconstituted with miR-125b over-expressing EμMT BMCs were injected into sublethally irradiated secondary C57bl/6 recipients. Bone marrow of the secondary recipient harvested when the mice became moribund, and figure shows flow cytometric plot of the leukocyte population of the bone marrow. **H**) Spleen weights of the secondary recipients described in panel H are shown (four mice). The controls signify spleen weight from secondary recipients injected with cells from MG reconstituted mice (eight mice). P value was calculated by Student's T test.

Figure 6: Deletion of IRF4 in miR-125b cells. A) Analysis of genetic mutations in cancer B cells through comparative genomic hybridization microarray (aCGH). Genomic DNA harvested from sorted miR-125b over-expressing GFP^+ $CD19^+$ cancer B cells was subjected to aCGH analysis. Genomic DNA from normal C57bl/6 mice of the same gender (female) was used as controls. The figure represents the relative amount of genetic content of the cancer sample versus the C57bl/6 control (plotted as ratio in Y axis). A genomic region was considered different between the samples if 3 consecutive probes exhibited different signal intensities in the microarray. Regions highlighted in green and orange indicate genomic areas in which the cancer cells have higher and lower DNA content, respectively. The other aCGH analysis is displayed in **Fig. S6A**. **B**) G-band karyotyping of miR-125b-induced cancer B cells. Trisomy 11 is highlighted in red box. **C**) Flow cytometric analysis of cancer B cells. Sorted GFP^+ $CD19^+$ cells isolated from

independent groups of MG-125b mice were transplanted into recipient mice. Upon cancer development, the bone marrow of these mice (highlight in red) was analyzed by flow cytometry. The plot shows the samples within the $CD19^+$ gated population. The sample overlaid in black represents total BMCs harvested from healthy control C57bl/6 mice. Group #1 and group #2 corresponds to the aCGH samples displayed in **Fig. 6A** and **Fig. S6A** respectively. **D**) IRF4 locus and genotyping primer sequences. IRF4 locus is shown with exons represented as solid bars. 'Start,' 'ex,' and 'int' signifies translation start site, exon, and intron respectively. The red bars represent the PCR product amplified by the primers used for genotyping in panel E-F and **Fig. S6E**. **E**) Quantitative PCR analysis of IRF4 locus. The relative amount of genomic DNA from miR-125b-induced cancer B cells were quantified by qPCR and normalized to control Hsp70 locus. The control samples are genomic DNA harvested from normal C57bl/6 mice. The PCR reaction amplifies a region spanning the translational start site (denoted as 'start' in panel C). **F**) Genomic DNA from miR-125b-induced cancer B cells was subjected to genotyping analysis. Exon2 (ex2), exon4 (ex4), and exon6 (ex6) of IRF4 were assessed. Bach1 locus was used as positive control. Samples #3-4 and #5-6 correspond to miR-125b-induced cancer B cells harvested from mice originating from group #1 and group #2 described in **Fig. 5G**, respectively. The agarose gel images of the PCR reactions are displayed.

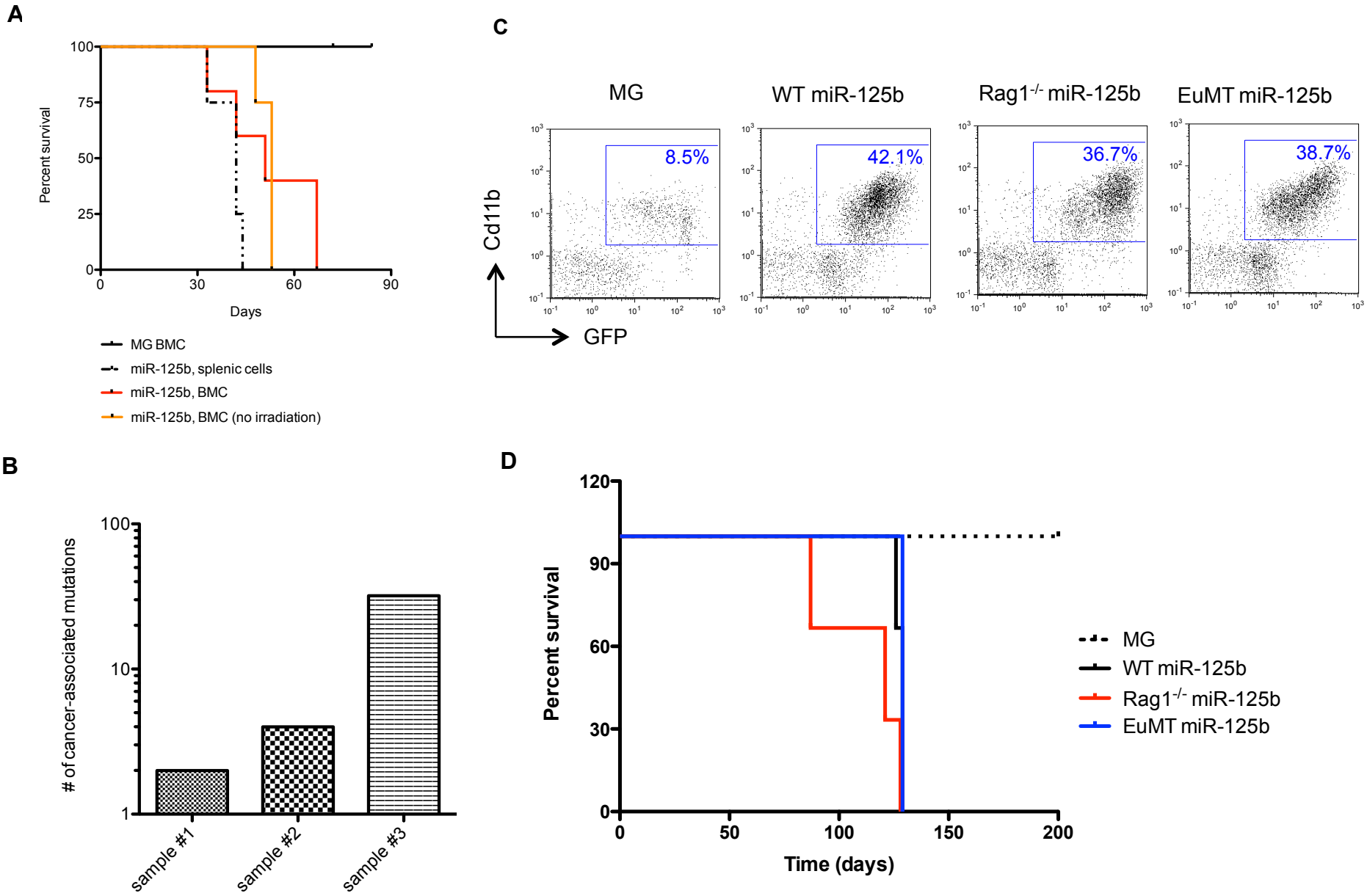
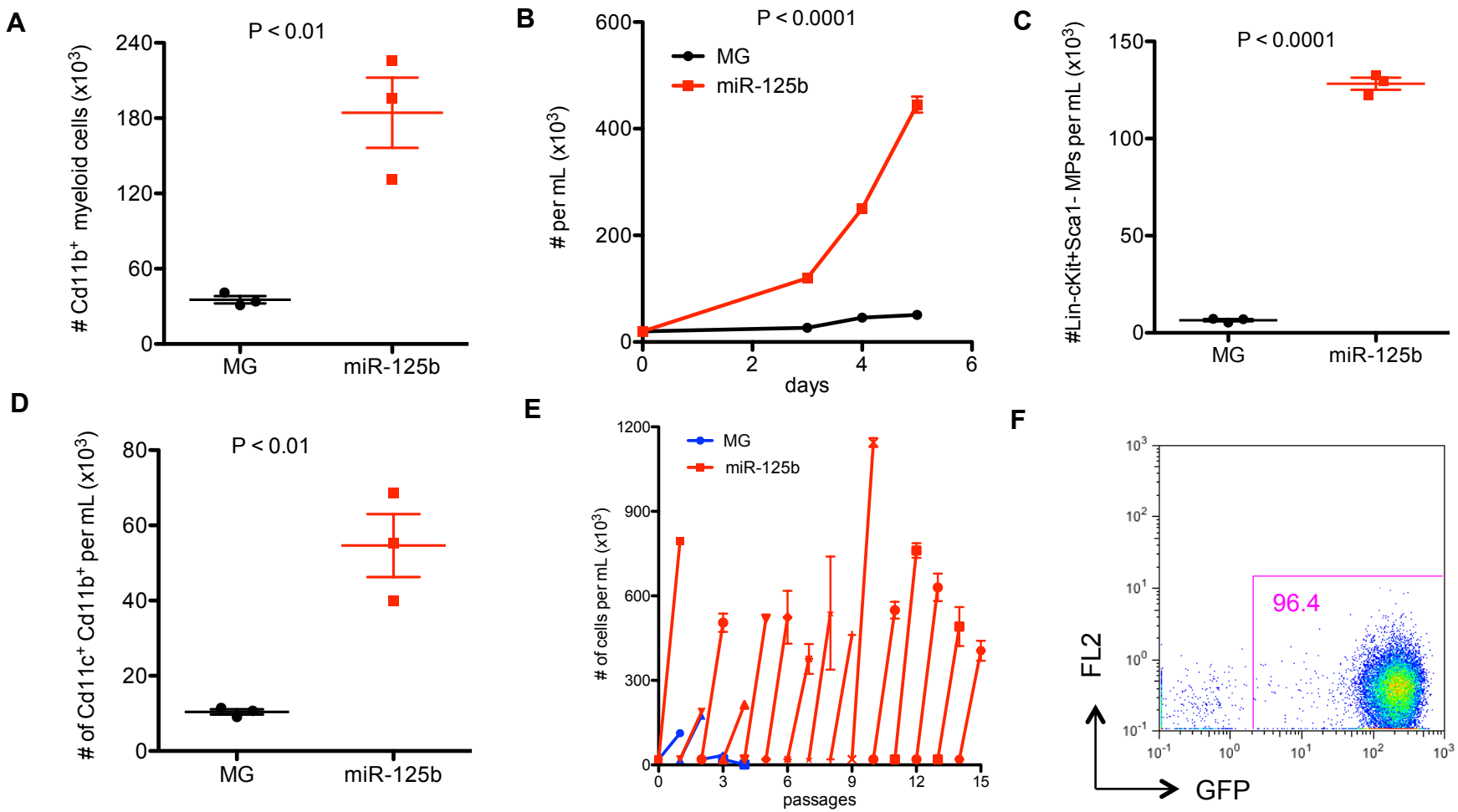

A
Percent survival
Days
MG BMC
miR-125b, splenic cells
miR-125b, BMC
miR-125b, BMC (no irradiation)
B
# of cancer-associated mutations
sample #1
sample #2
sample #3
C
MG
WT miR-125b
Rag1-/- miR-125b
EuMT miR-125b
8.5%
42.1%
36.7%
38.7%
Cd11b
GFP
D
Percent survival
Time (days)
MG
WT miR-125b
Rag1-/- miR-125b
EuMT miR-125b

Figure 1


A
P < 0.01
Cd11b+ myeloid cells (x10^3)
240
180
120
60
0
MG
miR-125b
B
P < 0.0001
MG
miR-125b
per mL (x10^3)
600
400
200
0
0
2
4
6
days
C
P < 0.0001
#Lin-cKit+Sca1- MPs per mL (x10^3)
150
100
50
0
MG
miR-125b
D
P < 0.01
# of Cd11c+ Cd11b+ per mL (x10^3)
80
60
40
20
0
MG
miR-125b
E
MG
miR-125b
# of cells per mL (x10^3)
1200
900
600
300
0
0
3
6
9
12
15
passages
F
96.4
FL2
GFP
10^3
10^2
10^1
10^0
10^-1

G


control
miR-125b

H

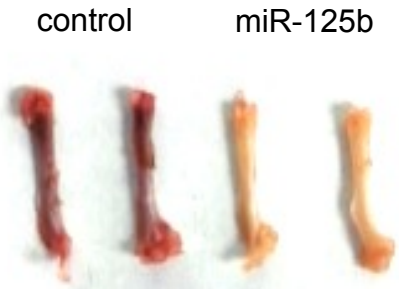

control
miR-125b

Figure 2

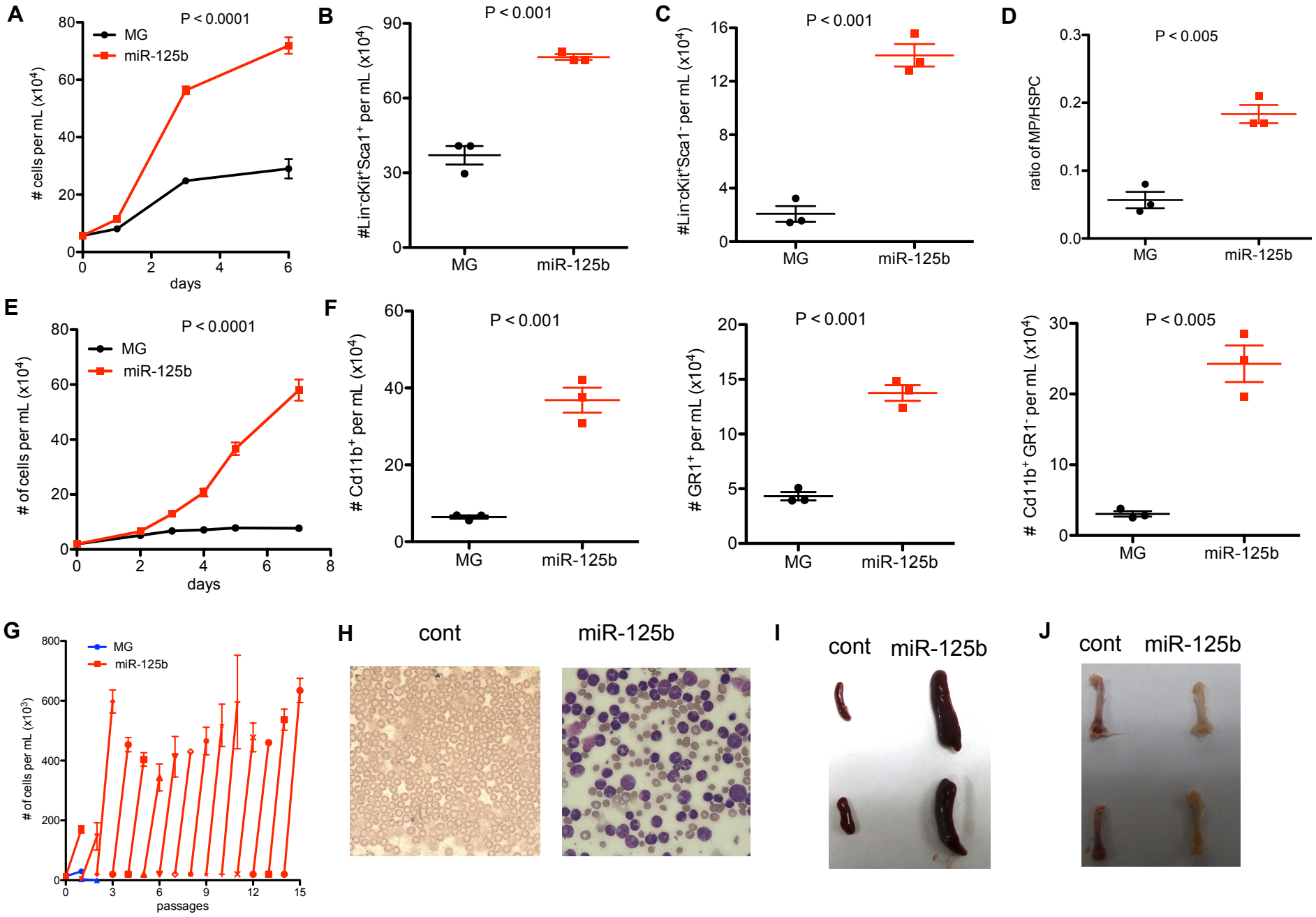

A
P < 0.0001
MG
miR-125b
cells per mL (x10^4)
days
B
P < 0.001
#Lin-cKit+Sca1+ per mL (x10^4)
MG
miR-125b
C
P < 0.001
#Lin-cKit+Sca1- per mL (x10^4)
MG
miR-125b
D
P < 0.005
ratio of MP/HSPC
MG
miR-125b
E
P < 0.0001
MG
miR-125b
# of cells per mL (x10^4)
days
F
P < 0.001
Cd11b+ per mL (x10^4)
MG
miR-125b
P < 0.001
GR1+ per mL (x10^4)
MG
miR-125b
P < 0.005
Cd11b+GR1- per mL (x10^4)
MG
miR-125b
G
MG
miR-125b
# of cells per mL (x10^3)
passages
H
cont
miR-125b
I
cont
miR-125b
J
cont
miR-125b

Figure 3

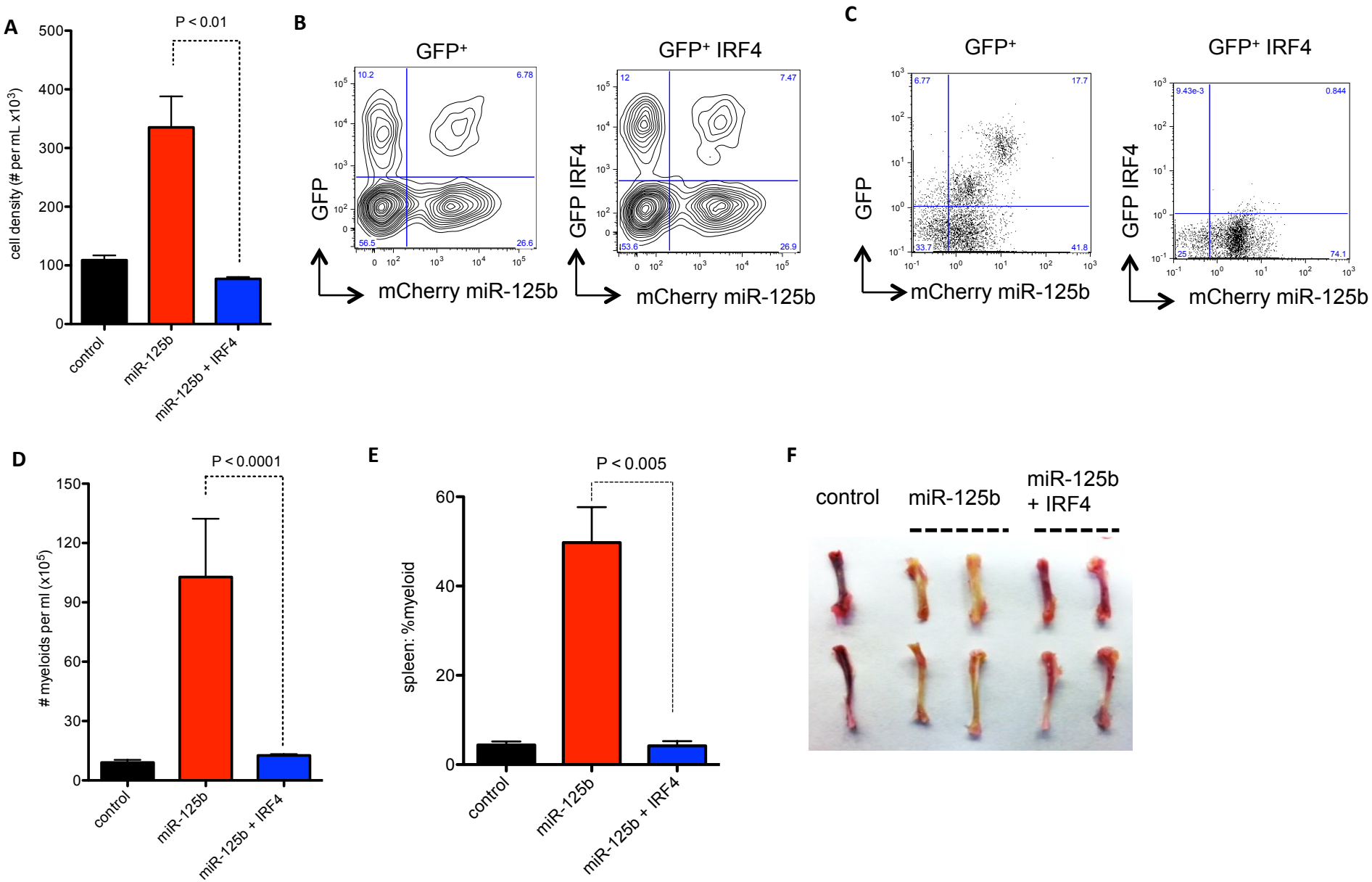

A
P < 0.01
cell density (# per mL x10^3)
500
400
300
200
100
0
control
miR-125b
miR-125b + IRF4
B
GFP+
GFP+ IRF4
GFP
GFP IRF4
mCherry miR-125b
mCherry miR-125b
C
GFP+
GFP+ IRF4
GFP
GFP IRF4
mCherry miR-125b
mCherry miR-125b
D
P < 0.0001
myeloids per ml (x10^5)
150
120
90
60
30
0
control
miR-125b
miR-125b + IRF4
E
P < 0.005
spleen: %myeloid
60
40
20
0
control
miR-125b
miR-125b + IRF4
F
control
miR-125b
miR-125b + IRF4

Figure 4

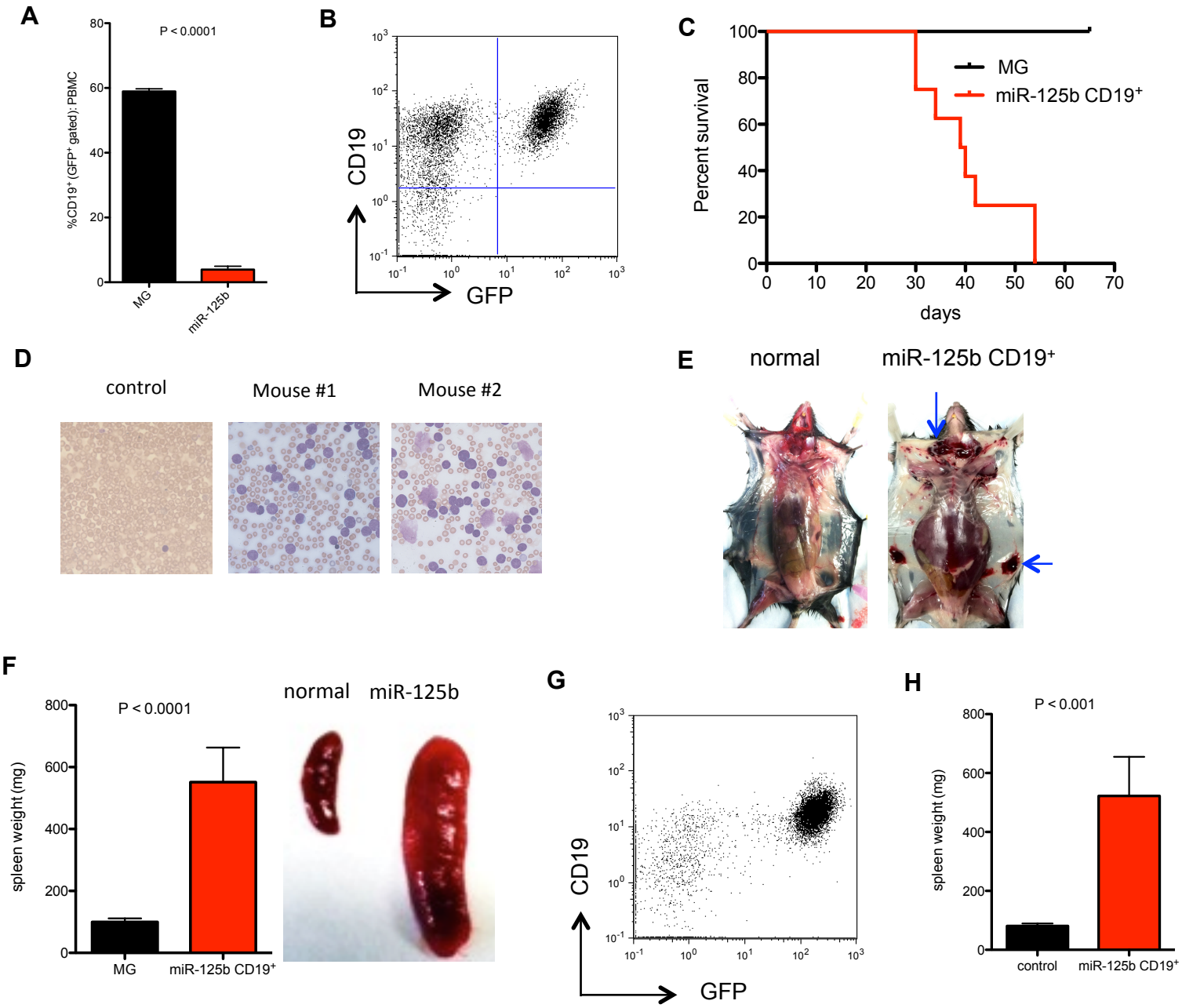

A
P < 0.0001
%CD19+ (GFP+ gated); PBMC
MG
miR-125b
B
CD19
GFP
C
Percent survival
days
MG
miR-125b CD19+
D
control
Mouse #1
Mouse #2
E
normal
miR-125b CD19+
F
P < 0.0001
spleen weight (mg)
MG
miR-125b CD19+
normal
miR-125b
G
CD19
GFP
H
P < 0.001
spleen weight (mg)
control
miR-125b CD19+

Figure 5

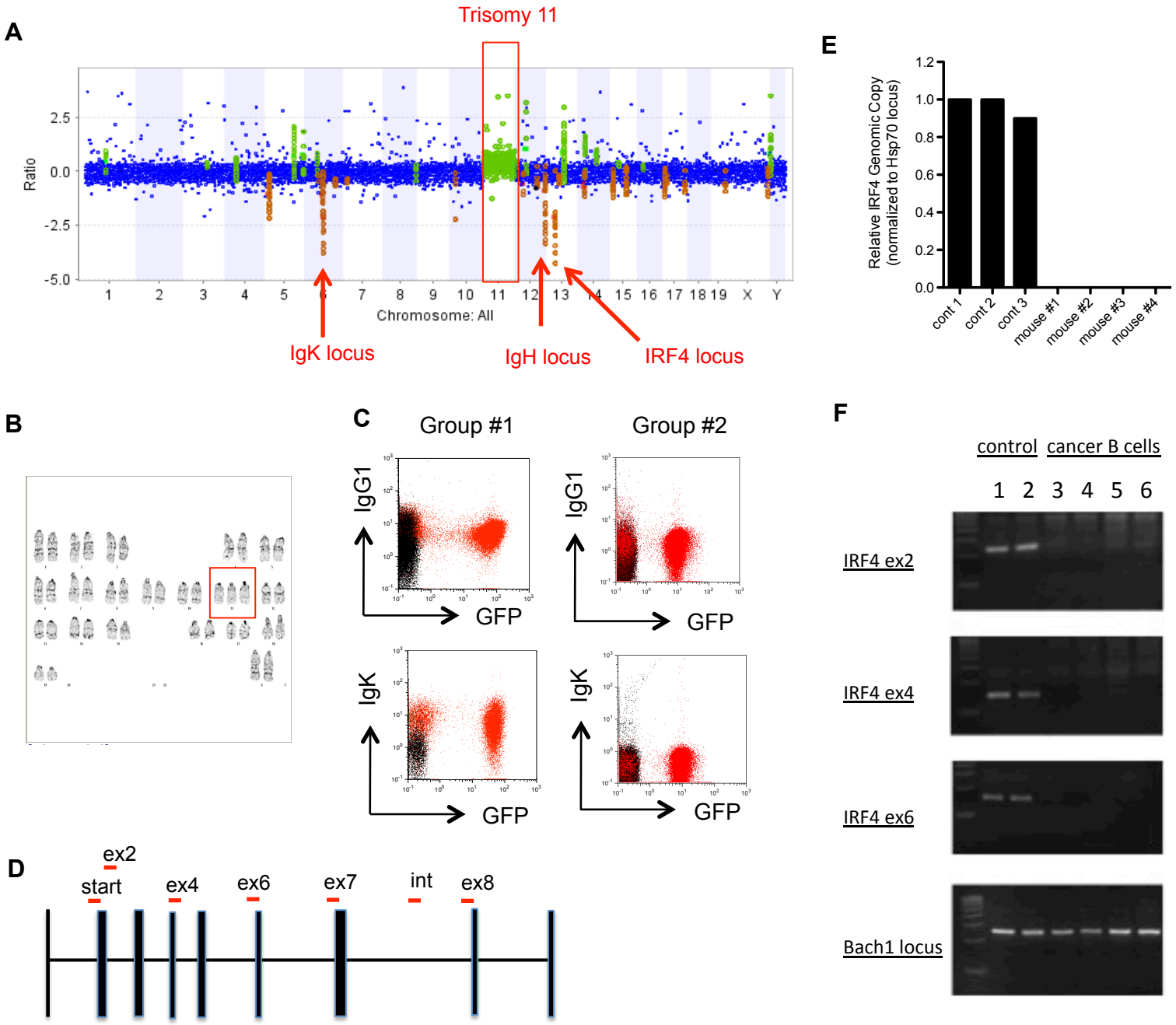

A
Trisomy 11
Ratio
2.5
0.0
-2.5
-5.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X Y
Chromosome: All
IgK locus
IgH locus
IRF4 locus
E
Relative IRF4 Genomic Copy (normalized to Hsp70 locus)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
cont 1
cont 2
cont 3
mouse #1
mouse #2
mouse #3
mouse #4
B
C
Group #1
Group #2
IgG1
GFP
IgK
GFP
D
ex2
start
ex4
ex6
ex7
int
ex8
F
control
cancer B cells
1 2 3 4 5 6
IRF4 ex2
IRF4 ex4
IRF4 ex6
Bach1 locus

Figure 6