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Loss of *Dnmt3a* and endogenous *Kras$^{G12D/+}$* cooperate to regulate hematopoietic stem and progenitor cell functions in leukemogenesis

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Abstract

Oncogenic *NRAS* and *KRAS* mutations are prevalent in human juvenile and chronic myelomonocytic leukemia (JMML/CMML). However, additional genetic mutations cooperating with oncogenic *RAS* in JMML/CMML progression and/or their transformation to acute myeloid leukemia (AML) remain largely unknown. Here, we tested the potential genetic interaction of *DNMT3A* mutations and oncogenic *RAS* mutations in leukemogenesis. We found that *Dnmt3a$^{-/-}$* induces multiple hematopoietic phenotypes after a prolonged latency, including T cell expansion in peripheral blood, stress erythropoiesis in spleen, and myeloid malignancies in liver. *Dnmt3a$^{-/-}$* significantly promoted JMML/CMML progression and shortened the survival of *Kras$^{G12D/+}$* mice in a cell-autonomous manner. Similarly, downregulating Dnmt3a also promoted myeloid malignancies in *Nras$^{G12D/+}$* mice. Further studies show that *Dnmt3a* deficiency rescues *Kras$^{G12D/+}$*-mediated depletion of hematopoietic stem cells and increases self-renewal of *Kras$^{G12D/+}$* myeloid progenitors. Moreover, ~33% of animals developed an AML-like disease, which is driven by *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* myeloid progenitors. Consistent with our result, COSMIC database mining demonstrates that the combination of oncogenic *RAS* and *DNMT3A* mutations exclusively occurred in patients with JMML, CMML, or AML. Our results suggest that *DNMT3A* mutations and oncogenic *RAS* cooperate to regulate hematopoietic stem and progenitor cells and promote myeloid malignancies.

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Conflict of interest:

We declare that no conflict of interest exists.

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Keywords

Dnmt3a; oncogenic Kras; juvenile/chronic myelomonocytic leukemia; acute myeloid leukemia

Introduction

Constitutively active mutations in *NRAS* and *KRAS* genes are identified in human hematopoietic malignancies at significant frequencies (1). In particular, oncogenic *NRAS* and *KRAS* mutations are predominant in juvenile myelomonocytic leukemia (JMML), myeloproliferative variant of chronic myelomonocytic leukemia (MP-CMML), and the M4 and M5 monocytic subtypes of acute myeloid leukemia (AML) (including both de novo AML and secondary AML with antecedent JMML/CMML). Consistently, mice expressing endogenous oncogenic Kras or Nras develop highly penetrant JMML/MP-CMML-like phenotypes (2–8). Although these animals rarely develop AML spontaneously, acquisition of other mutations does promote their malignant transformation to monocytic AML (7). These findings indicate that JMML, MP-CMML, and M4/M5 AML are related malignancies in which oncogenic Ras signaling plays an essential role.

Although genetic mutations in a few genes are reported to be concurrent with oncogenic *RAS* mutations (9) in myeloid malignancies, their functional significances remain largely unknown. Acquisition of two copies of oncogenic *RAS* alleles, including $NRAS^{G12D/G12D}$ and $KRAS^{G13D/G13D}$, is associated with JMML/CMML progression in human and mouse (COSMIC database and (6, 10–12)). Consistently, $Nras^{G12D/G12D}$ mice develop JMML/MP-CMML phenotypes much more rapidly than $Nras^{G12D/+}$ mice (8), indicating that incremental activation of Ras signaling is a pathological mechanism contributing to JMML/CMML development. In contrast, knocking down Tet2 expression in $Nras^{G12D/+}$ bone marrow cells does not seem to promote JMML/CMML progression or its malignant transformation (13). These data suggest that the potential genetic interaction between oncogenic *RAS* and other concurrent mutations have to be validated *in vivo* on a case-by-case basis.

Recent work focusing on AML with a normal karyotype identified mutations in *DNA methyltransferase 3A* (*DNMT3A*) with biological, clinical, and potential therapeutic relevance in AML and other types of myeloid malignancies (9, 14). Approximately 90% of *DNMT3A* mutations occur as a single copy mutation over wild-type *DNMT3A* (15). Although the predominant mutation at the codon R882 has been shown to be a dominant-negative mutation (16, 17), loss of *Dnmt3a* in the mouse hematopoietic system does not induce leukemogenesis up to 6 months of age (18). In contrast, recipients transplanted with *Dnmt3a* deficient hematopoietic stem cells (HSCs) developed both myeloid and lymphoid malignancies (19). Furthermore, loss of *Dnmt3a* promotes lung tumor progression in oncogenic *Kras* mice (20). Consistent with this finding, a group of AML patients were identified who carried both oncogenic *RAS* and *DNMT3A* mutations (9, 21). However, it remains unclear whether these two mutations cooperate in myeloid leukemia development.

Here, we show that loss of *Dnmt3a* promotes multiple hematopoietic defects after a prolonged latency, which are distinct from recipients transplanted with $Dnmt3a^{-/-}$ HSCs.

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Downregulation of *Dnmt3a* (deleting a single copy or both copies) in oncogenic *Ras* models not only significantly promote JMML/MP-CMML progression but also leads to transformation to acute myeloid diseases in a cell autonomous manner. Our finding is consistent with COSMIC database mining results showing that oncogenic *RAS* and *DNMT3A* mutations were only concurrent in myeloid malignancies, including JMML, CMML, and AML. Further mechanistic studies demonstrate that *Dnmt3a* deficiency promotes myeloid diseases in oncogenic *Kras* model through rescuing $Kras^{G12D/+}$-mediated depletion of HSCs and increasing self-renewal of $Kras^{G12D/+}$ myeloid progenitor cells. These mutant myeloid progenitors could initiate myeloid malignancies in recipients and thus serve as leukemia initiating cells. Our results suggest that changes in epigenetic landscapes and signaling networks co-regulate hematopoietic stem and progenitor cells to promote myeloid leukemias.

Materials and Methods

Mice

All mouse lines were maintained in a pure C57BL/6 genetic background (>N10). *Dnmt3a* conditional knockout mice ($Dnmt3a^{fl/fl}$ (22); provided by Dr. Qiang Chang) were crossed to mice bearing a conditional oncogenic *Kras* ($Kras^{Lox\text{-}stop\text{-}Lox\ (LSL)\ G12D/+}$) or Mx1-Cre mice to generate mice carrying both alleles ($Kras^{LSL\ G12D/+}$; $Dnmt3a^{fl/+}$ and $Dnmt3a^{fl/+}$*; Mx1-Cre*, respectively). $Kras^{LSL\ G12D/+}$; $Dnmt3a^{fl/+}$ mice were further crossed to $Dnmt3a^{fl/+}$*; Mx1-Cre* mice to generate our experimental mice, including $Kras^{LSL\ G12D/+}$*;* $Dnmt3a^{fl/fl}$*; Mx1-Cre*, $Kras^{LSL\ G12D/+}$*; Mx1-Cre*, $Dnmt3a^{fl/fl}$*; Mx1-Cre*, and *Mx1-Cre* mice. CD45.1-positive congenic C57BL/6 recipient mice were purchased from NCI. Cre expression was induced through intraperitoneal injection of 2.5 μg/g body weight (GE Healthcare) of polyinosinic-polycytidylic acid (pI-pC) every other day for two times. All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by an Animal Care and Use Committee at UW-Madison. The program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Flow cytometric analysis of hematopoietic tissues

For lineage analysis of peripheral blood, bone marrow, and spleen, flow cytometric analyses were performed as previously described (4). Myeloid progenitors in bone marrow and spleen were analyzed as previously described (8). HSCs in bone marrow and spleen were analyzed as described in (23). Because hind limb bone marrow represents ~25% of total bone marrow, the number of HSCs in total bone marrow is calculated as 4 X the number of HSCs in hind limb bone marrow. The stained cells were analyzed on a FACS Calibur (BD Biosciences) or a MACSQuant Analyzer (Miltenyi Biotec Inc.). Antibodies specific for the following surface antigens were purchased from eBioscience: CD45.2 (104), B220 (RA3-6B2), CD19 (eBio1D3), Thy1.2 (53-2.1), Mac-1 (M1/70), Gr-1 (RB6-8C5), CD4 (GK1.5), CD8 (53-6.7), CD3 (145-2C11), IgM (II/41), IL7Rα (A7R34), Sca-1 (D7), TER119(TER-119), CD34 (RAM34), cKit (2B8). FcγRII/III (2.4G2) was purchased from BD Biosciences. CD150 (TC15-12F12.2) was purchased from Biolegend.

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Additional methods are described in Supplemental Methods.

Results

Somatic deletion of *Dnmt3a* promotes acute myeloid diseases in *Kras$^{G12D/+}$* mice

To determine whether *Dnmt3a* plays an important role in *Kras$^{G12D/+}$*-mediated hematopoietic malignancies, we generated *Dnmt3a$^{fl/fl}$; Mx1-Cre, Kras$^{LSL\ G12D/+}$; Mx1-Cre* and *Kras$^{LSL\ G12D/+}$; Dnmt3a$^{fl/fl}$; Mx1-Cre* mice. Administration of polyinosinic-polycytidylic acid (pI-pC) in these compound mice stimulates endogenous interferon (IFN) production and thus induces Cre expression from the IFN-α/β-inducible Mx1 promoter, which in turn leads to the expression of oncogenic Kras from its endogenous locus and/or somatic deletion of *Dnmt3a* (24). We refer to these pI-pC-treated compound mice as *Dnmt3a$^{-/-}$*, *Kras$^{G12D/+}$*, and *Kras$^{G12D/+}$; Dnmt3a$^{-/-}$* mice respectively, and pI-pC-treated Mx1-Cre or wild-type mice as control mice throughout this manuscript.

Somatic deletion of *Dnmt3a* significantly enhanced leukemia phenotypes and shortened the life-span of *Kras$^{G12D/+}$* mice (Fig. 1). Two days after the 2nd pI-pC injection, control, *Dnmt3a$^{-/-}$*, *Kras$^{G12D/+}$*, and *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* mice were sacrificed and assessed for hematopoietic phenotypes (Fig. 1B–1D). Consistent with previous reports (2–5), *Kras$^{G12D/+}$* mice developed an acute myeloproliferative neoplasm (MPN), closely resembling human JMML/MP-CMML. This disease was manifested by splenomegaly (Fig. 1B) with significant extramedullary hematopoiesis (Fig. 1D), expanded monocyte and neutrophil compartments in various hematopoietic tissues (Fig. 1C), elevated white blood cell counts in peripheral blood, and defective erythroid and megakaryocyte development (Table S1). Loss of *Dnmt3a* not only significantly enhanced the MPN phenotypes in *Kras$^{G12D/+}$* mice (Fig. 1B–1C and Table S1), but also promoted the development of acute myeloid leukemia (AML)-like phenotypes in about one third of the animals, as demonstrated by accumulation of immature myeloblast cells in the spleen (Fig. 1D).

Dnmt3a deficiency induces multiple hematopoietic defects

Although all *Dnmt3a$^{-/-}$* mice survived in an overtly healthy condition for 6 months, ~14% of *Dnmt3a$^{fl/fl}$; Mx1-Cre* mice without pI-pC treatment died with multiple hematopoietic defects within 13 months due to the leaky expression of Cre over time (Fig. 2A). In these moribund mice, the fraction of T cells in peripheral blood was significantly elevated but the thymus size and structure were essentially normal, indicating a chronic T cell expansion (Fig. 2B). Our result is consistent with a recent report that *Dnmt3a* prevents malignant mouse lymphopoiesis (25). In the enlarged spleen (~4–5 fold over control spleens), the normal architecture was completely effaced by extramedullary hematopoietic tissue that was dominated by erythroid and megakaryocytic elements with fewer numbers of maturing myeloid cells. Sheets of blasts were not evident. Consistent with the H&E sections, flow cytometric analysis of splenocytes also demonstrated the dominance of erythroid lineage cells, suggesting a stressed erythropoiesis (Fig. 2C). The markedly enlarged livers (~10–15 fold over control livers) were dramatically altered with only small islands of hepatocytes present separated by sheets of discohesive cells. Based on the histological evaluation, this is most consistent with a histiocytic or myeloid sarcoma (Fig. 2D).

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To validate our result, we set up another cohort of *Dnmt3a$^{fl/fl}$; Mx1-Cre* mice injected with pI-pC and monitored them closely. They consistently displayed a transient anemia two months after pI-pC injections with significantly lower red blood cell count, hemoglobin, and hematocrit (Fig. S1A). However, the other aspects of hematopoiesis were indistinguishable from those of control mice (Fig. S1B). Further analysis of erythropoiesis in bone marrow and spleen showed that the red pulp in *Dnmt3a$^{-/-}$* spleen was moderately enlarged (Fig. S2A). Consistent with this result, flow cytometric analysis using CD71 and TER119 demonstrated that the erythroid compartment in *Dnmt3a$^{-/-}$* spleen was moderately but significantly expanded with increased Region II cells and decreased Region IV cells (Fig. S2B–S2D). *In vitro* colony assay revealed that the number of CFU-E (colony forming unit-erythroid) progenitors in *Dnmt3a$^{-/-}$* spleen was significantly increased (Fig. S2E) without detectable change of their sensitivity to Erythropoietin (EPO) stimulation (Fig. S2F). These results suggest that loss of *Dnmt3a* affects erythroid differentiation, which can be compensated by stress erythropoiesis in spleen. Compared with *Dnmt3a$^{fl/fl}$; Mx1-Cre* mice without pI-pC treatment, *Dnmt3a$^{-/-}$* mice succumbed to similar hematopoietic defects (including T cell expansion in peripheral blood, stress erythropoiesis in spleen, and myeloid malignancies in liver) with a much higher penetrance (Fig. 2A).

To investigate cell autonomous role of *Dnmt3a* deficiency in leukemogenesis, we transplanted 2.5×10^5 *Dnmt3a$^{-/-}$* bone marrow cells along with same number of competitor cells into individual lethally irradiated mice (Fig. S3). To our surprise, only 2 out of 23 recipients died of hematopoietic maliganacies 300 days after transplantation (Fig. S3A). The first one was found dead with enlarged thymus and spleen, while the second one was sacrificed at the moribund stage and also showed enlarged spleen and thymus (Fig. S3B). Detailed flow cytometric analysis of the second mouse revealed a lethal acute T-cell lymphoblastic leukemia/lymphoma (T-ALL) throughout the entire hematopoietic system (Fig. S3C). Together, our results suggest that somatic downregulation of *Dnmt3a* does promote leukemogenesis as a first genetic hit.

Downregulation of *Dnmt3a* promotes myeloid diseases in *Nras$^{G12D/+}$* mice

Approximately 90% of *DNMT3A* mutations in human leukemia patients occur as a single copy mutation over wild-type *DNMT3A* (15). Although the predominant mutation at the codon R882 has been shown to be a dominant-negative mutation (16, 17), other mutations have not been characterized in detail. We investigated whether loss of a single copy of *Dnmt3a* would promote oncogenic Ras-induced leukemogenesis. As was seen in oncogenic *Kras*-induced non-small cell lung carcinoma (20), we did not observe significant acceleration or enhancement of MPN development in *Kras$^{G12D/+}$*; *Dnmt3a$^{+/-}$* mice compared with *Kras$^{G12D/+}$* mice (our unpublished data). This could be due to the strong MPN phenotypes in *Kras$^{G12D/+}$* mice. Therefore, we generated *Nras$^{G12D/+}$*; *Dnmt3a$^{fl/+}$*; *Mx1-Cre* mice to address this question (referred as *Nras$^{G12D/+}$*; *Dnmt3a$^{+/-}$* mice after pI-pC treatment). Compared with *Nras$^{G12D/+}$* mice, *Nras$^{G12D/+}$*; *Dnmt3a$^{+/-}$* mice indeed showed significantly shortened life-span and developed more severe MPN phenotypes (Fig. 3A and 3C). As with *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* mice, about one third of *Nras$^{G12D/+}$*; *Dnmt3a$^{+/-}$* mice developed AML-like phenotypes (Fig. 3B). Thus, our data indicate that downregulating Dnmt3a and oncogenic Nras cooperate to promote myeloid diseases *in vivo*.

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Loss of *Dnmt3a* rescues *Kras$^{G12D/+}$*-mediated HSC depletion and increases self-renewal of *Kras$^{G12D/+}$* myeloid progenitors

To study the mechanisms of how *Dnmt3a* deficiency promotes myeloid diseases in oncogenic *Kras* mice, we first examined the HSC compartment because mutant HSCs serve as JMML/MP-CMML initiating cells in these animals (4, 26). HSCs are defined as Lin^{-} CD41^{-} CD48^{-} c-Kit^{+} Sca-1^{+} CD150^{+} cells throughout the manuscript (Fig. 4A). Although the frequency of *Dnmt3a$^{-/-}$* HSCs was moderately increased in bone marrow compared to that of control HSCs, the absolute number was comparable in the whole body (total bone marrow + spleen) (Fig. 4B). Consistent with previous reports (5, 26), the HSC number in *Kras$^{G12D/+}$* mice was significantly less than that in control mice, indicating an oncogenic *Kras*-mediated HSC depletion. In contrast, the HSC compartment in *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* mice was comparable to that in control mice, suggesting that depletion of *Kras$^{G12D/+}$* HSCs was rescued by loss of *Dnmt3a*. The total number of multipotent progenitors (MPPs, defined as Lin^{-} CD41^{-} CD48^{-} c-Kit^{+} Sca-1^{+} CD150^{-} cells; Fig. 4A) in *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* mice was concomitantly increased compared to that in control and *Kras$^{G12D/+}$* mice (Fig. 4C), whereas the LSK (Lin^{-} Sca1^{+} cKit^{+}) compartment was comparable to controls (Fig. 4D).

We then analyzed the myeloid progenitors (MPs) in all groups of animals (Fig. 5). In *Dnmt3a$^{-/-}$* mice, the number of MPs, including common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs), was significantly decreased in bone marrow compared to that in control mice (Fig. 5A–5E). Consistently, *Dnmt3a$^{-/-}$* bone marrow cells formed significantly fewer colonies in semi-solid cultures (Fig. 5F). In contrast, *Dnmt3a* deficiency promoted further expansion of MP compartment in *Kras$^{G12D/+}$*-expressing spleens (Fig. 5A–5F). Moreover, although loss of *Dnmt3a* or expression of endogenous oncogenic *Kras* led to a moderate increase of transient self-renewal of MPs, *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* MPs demonstrated significantly higher self-renewal capability than *Kras$^{G12D/+}$* or *Dnmt3a$^{-/-}$* MPs in the replating assay (Fig. 5G). Together, our data suggest that loss of *Dnmt3a* promotes myeloid diseases in *Kras$^{G12D/+}$* mice through rescuing *Kras$^{G12D/+}$*-mediated HSC depletion and enhancing self-renewal of *Kras$^{G12D/+}$* MPs.

Deletion of *Dnmt3a* promotes myeloid malignancies in *Kras$^{G12D/+}$* mice in a cell autonomous manner

To determine whether *Dnmt3a* deficiency plays a cell autonomous role in promoting leukemogenesis in *Kras$^{G12D/+}$* model, we transplanted control, *Kras$^{LSL\ G12D/+}$; Mx1-Cre,* or *Kras$^{LSL\ G12D/+}$; Dnmt3a$^{fl/fl}$; Mx1-Cre* bone marrow cells into lethally irradiated mice (Fig. 6). Four weeks after transplantation, recipient mice were injected with pI-pC as before. We did not observe significant change of life span between recipients with *Kras$^{G12D/+}$* cells and those with *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* cells (Fig. 6A). This could be due to the leaky expression of oncogenic Kras but not efficient *Dnmt3a* deletion before the pI-pC induction.

Although different groups of donor cells engrafted recipients at a comparable level (Fig. 6B), recipients of *Kras$^{G12D/+}$*; *Dnmt3a$^{-/-}$* cells developed an acute myeloid disease with a much higher incidence than those with *Kras$^{G12D/+}$* cells (60% vs 20%; Fig. 6C).

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Consistently, the myeloid disease phenotypes characteristic in *Kras*$^{G12D/+}$ mice, including splenomegaly and expanded myeloid compartments in various hematopoietic tissues (Fig. 6D–6E) as well as defective phenotypes in erythoid and megakaryocytic lineages of cells (Table S2), were more prominent in recipients of *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ cells than those of *Kras*$^{G12D/+}$ cells. Similarly to primary non-transplanted mice, about one third of myeloid diseases that developed in recipients of *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ cells were AML-like, and the rest of them closely resembled a MPN (Fig. S4). In contrast, all the myeloid diseases that developed in recipients with *Kras*$^{G12D/+}$ cells were MPN. Genotyping of myeloid disease cells from recipient bone marrow revealed that WT *Kras* allele was significantly downregulated in 50–75% of samples (Fig. S5), which might be caused by clonal expansion of leukemia cells with uniparental disomy of the oncogenic *Kras* allele or deletion of WT *Kras* allele. In conclusion, our results indicate that loss of *Dnmt3a* and oncogenic *Ras* cooperate to promote myeloid malignancies, which is consistent with the identification of activating RTK/RAS pathway mutations in *Dnmt3a*$^{-/-}$ induced AML in mice (19, 27).

To test the human relevance of our finding, we conducted a COSMIC database search to quantify the incidence of myeloid versus lymphoid malignancies in leukemia patients with oncogenic *RAS* mutations (Fig. 6F). We found that ~50% of patients with an oncogenic *KRAS* mutation and ~70% of patients with an oncogenic *NRAS* mutation had a myeloid malignancy. Acquisition of additional copies of oncogenic *RAS* mutations did not significantly change the disease incidence. However, 100% of patients with both oncogenic *RAS* and *DNMT3A* mutations had developed myeloid malignancies, including JMML, CMML, and AML. This result suggests that oncogenic *RAS* and *DNMT3A* mutations intrinsically interact or cooperate to promote leukemogenesis in myeloid cells.

In addition to myeloid neoplasms, all the recipients of *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ cells developed T-ALL (Fig. S6). In comparison to the T-ALL that developed in recipients of *Kras*$^{G12D/+}$ cells, approximately 30% of *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ derived tumors contained a significant percentage of $CD4^{-}$ $CD8^{-}$ T-cells, suggesting a more immature phenotype. T-ALL developed in both groups of recipients carried Type 1 deletions in the *Notch1* locus, which render ligand-independent cleavage of Notch1 and elevated expression of intracellular C-terminus Notch1. Our results suggest that active Notch1 signaling promotes T-ALL development in both genetic contexts.

Kras$^{G12D/+}$; *Dnmt3a*$^{-/-}$ myeloid progenitors are capable of initiating myeloid malignancies in recipient mice

Because *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ bone marrow cells promoted highly penetrant myeloid malignancies in a cell-autonomous manner (Fig. 6) and *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ MPs displayed enhanced self-renewal *in vitro* (Fig. 5F), we investigated whether these mutant progenitors could initiate myeloid diseases in recipient mice and thus serve as leukemia initiating cells. Mutant LSK cells and LK $Sca1^{-}$ MPs were sorted and transplanted with competitor cells into lethally irradiated recipients (Table 1). All the recipients with mutant LSK cells succumbed to hematopoietic malignancies, one with T-ALL and MPN, one with MPN, and one with AML. Six out of seven recipients with mutant MPs died and careful analysis of four mice revealed that three died with MPN and one with AML. Our results

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indicate that some of *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ MPs are transformed to leukemia initiating cells.

Discussion

In this manuscript, we demonstrate that somatic downregulation of *Dnmt3a* induces leukemogenesis as a first genetic hit. Downregulating *Dnmt3a* cooperates with oncogenic *Ras* to promote myeloid malignancies in a cell autonomous manner in mice, consistent with the genetic findings in human leukemia patients. Furthermore, our data suggest that *Dnmt3a* deficiency promotes myeloid diseases through regulating the functions of *Kras*$^{G12D/+}$ HSCs and MPs (Fig. 7).

Although the predominant mutation at the codon R882 of *DNMT3A* is a dominant-negative mutation (16, 17), loss of *Dnmt3a* in the mouse hematopoietic system does not induce leukemogenesis up to 6 months of age (18). However, we found that *Dnmt3a*$^{-/-}$ mice developed multiple hematopoietic defects after a prolonged latency (Fig. 2). Our results are consistent with human studies reporting that *DNMT3A* mutations in HSCs lead to clonal hematopoietic expansion and thus act as the first/early genetic event during AML development (28–31). Interestingly, the hematopoietic phenotypes developed in *Dnmt3a*$^{-/-}$ mice (T cell expansion in peripheral blood, stress erythropoiesis in spleen, and myeloid malignancies in liver) are distinct from those in recipients transplanted with *Dnmt3a*$^{-/-}$ HSCs (19, 27) or total bone marrow cells (Fig. S3), which primarily developed MDS/AML and T-ALL. This could be due to both cell-autonomous and cell-nonautonomous effects of *Dnmt3a* deficiency in non-transplanted *Dnmt3a*$^{-/-}$ mice. For example, we previously showed that pI-pC injection could sufficiently induce Cre expression in liver (6). Noticeably, the survival of our BMT cohort mice is significantly prolonged than those reported (19, 27). This difference might be due to the lower number of *Dnmt3a*$^{-/-}$ HSCs we transplanted and the presence of competitor cells.

We found that *Dnmt3a* deficiency and *Kras*$^{G12D/+}$ cooperate to induce myeloid malignancies in a cell autonomous manner *in vivo. Dnmt3a*$^{-/-}$ significantly promoted JMML/CMML progression and shortened the survival of *Kras*$^{G12D/+}$ mice (Fig. 1). Recipients transplanted with *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ bone marrow cells developed JMML/CMML at a much higher incidence than those transplanted with *Kras*$^{G12D/+}$ cells (Fig. 6). Moreover, ~33% of animals developed an AML-like disease. Similarly, loss of one copy of *Dnmt3a* also promoted myeloid diseases in *Nras*$^{G12D/+}$ mice, consistent with the previous study using a retroviral construct to overexpress oncogenic Nras in *Dnmt3a*$^{-/-}$ bone marrow cells (19). However, the survival data from our genetic model is very different from that of RasG12D overexpression study, which could be mainly explained by the expression levels of oncogenic Nras (endogenous level versus overexpression). Indeed, although recipients transplanted with *Kras*$^{G12D/+}$ or *Nras*$^{G12D/+}$ cells do not develop AML spontaneously (4, 6, 26, 32), recipients with bone marrow cells overexpressing NrasG12D develop a highly penetrant CMML/AML rapidly (33). The *in vivo* genetic interaction between oncogenic Ras and *Dnmt3a*$^{-/-}$ in myeloid diseases is also supported by the identification of activating RTK/RAS pathway mutations (*c-Kit*^{V750M} and *Kras*^{G12D}) in *Dnmt3a*$^{-/-}$ induced AML in mice (19, 27). We believe that our results are highly relevant to human physiology because

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the combination of oncogenic *RAS* (including both *NRAS* and *KRAS*) and *DNMT3A* mutations exclusively occurred in patients with myeloid leukemias, including JMML, CMML, and AML (Fig. 6F).

Our results demonstrate that genetic interaction between *Dnmt3a* deficiency and $Kras^{G12D/+}$ regulates the functions of HSCs and MPs to drive myeloid leukemogenesis. We recently showed that stronger oncogenic Ras signaling (e.g. $Nras^{G12D/G12D}$) shifts HSC self-renewal to differentiation and leads to HSC exhaustion (23). Similar mechanisms might be also applicable to $Kras^{G12D/+}$ HSCs. However, $Kras^{G12D/+}$ -mediated HSC depletion is antagonized by loss of *Dnmt3a*, which is likely to contribute to the significantly increased incidence of myeloid malignancies in recipients with $Kras^{G12D/+}$; $Dnmt3a^{-/-}$ cells. Our result is consistent with a previous study reporting that $Dnmt3a^{-/-}$ maintains HSC self-renewal in the presence of oncogenic Ras (19).

We found that expression of endogenous oncogenic Ras (e.g. $Kras^{G12D/+}$ or $Nras^{G12D/+}$)promotes progenitor cell growth *in vivo* and *in vitro* (2, 3, 5–7), in sharp contrast to the negative effect of RasG12D overexpression on progenitor growth *in vitro* (19). This is likely due to different expression levels of oncogenic Ras used in the studies. Consistent with a previous report (27), we observed moderately increased self-renewal of $Dnmt3a^{-/-}$ MPs in a re-plating assay. Moreover, $Kras^{G12D/+}$ and $Dnmt3a^{-/-}$ act synergistically to promote self-renewal in MPs, which are transformed to leukemia initiating cells and drive AML-like diseases *in vivo*. Our data provide a strong rationale to target both epigenetic regulators and aberrant cytokine signaling in treating myeloid leukemias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Mouse genotype abbreviations

Control	pI-pC treated Mx1-Cre or wild-type
$Kras^{G12D/+}$	recombined $Kras^{G12D/+}$ heterozygous (pI-pC treated LSL $Kras^{G12D/+}$; Mx1-Cre)
$Dnmt3a^{-/-}$	recombined Dnmt3a knockout (pI-pC treated $Dnmt3a^{fl/fl}$; Mx1-Cre)
$Kras^{G12D/+}$; $Dnmt3a^{-/-}$	recombined $Kras^{G12D/+}$ heterozygous deficient for Dnmt3a (pI-pC treated LSL $Kras^{G12D/+}$; $Dnmt3a^{fl/fl}$; Mx1-Cre)

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Nras$^{G12D/+}$; Dnmt3a$^{+/-}$	recombined Nras$^{G12D/+}$ and Dnmt3a$^{+/-}$ double heterozygous (pI-pC treated LSL Nras$^{G12D/+}$; Dnmt3a$^{fl/+}$; Mx1-Cre).

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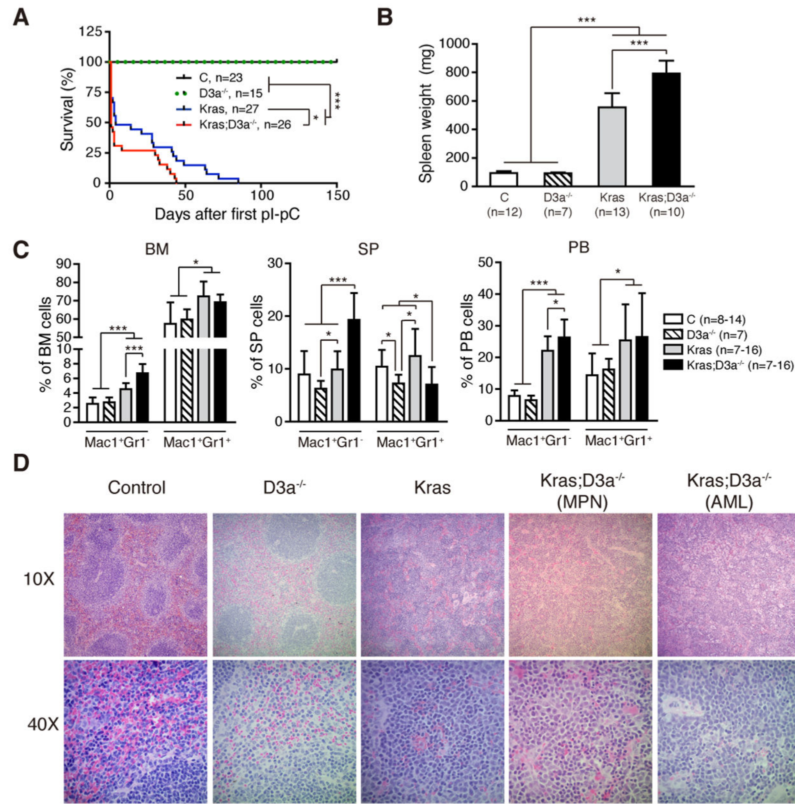

Figure 1. Loss of *Dnmt3a* promotes myeloid diseases in *Kras*$^{G12D/+}$ mice

Control (C), *Dnmt3a*$^{-/-}$ (D3a$^{-/-}$), *Kras*$^{G12D/+}$ (Kras), and *Kras*$^{G12D/+}$*; Dnmt3a*$^{-/-}$ (Kras; D3a$^{-/-}$) mice were treated with pI-pC as described in Materials and Methods. We define the day of 1st pI-pC injection as Day 1. Treated mice were sacrificed on Day 5 for analysis of different hematopoietic tissues. (A) Kaplan-Meier survival curves of different groups of mice were plotted against days after first pI-pC injection. P values were determined by the Log-rank test. (B,C) Quantification of spleen weight (B) and myeloid cell compartment in bone marrow (BM), spleen (SP) and peripheral blood (PB) (C). (D) Representative spleen histologic H&E sections from different groups of animals are shown. The results are presented as means ± SD. * $P<0.05$ and *** $P<0.001$. MPN, myeloproliferative neoplasm; AML, acute myeloid leukemia.

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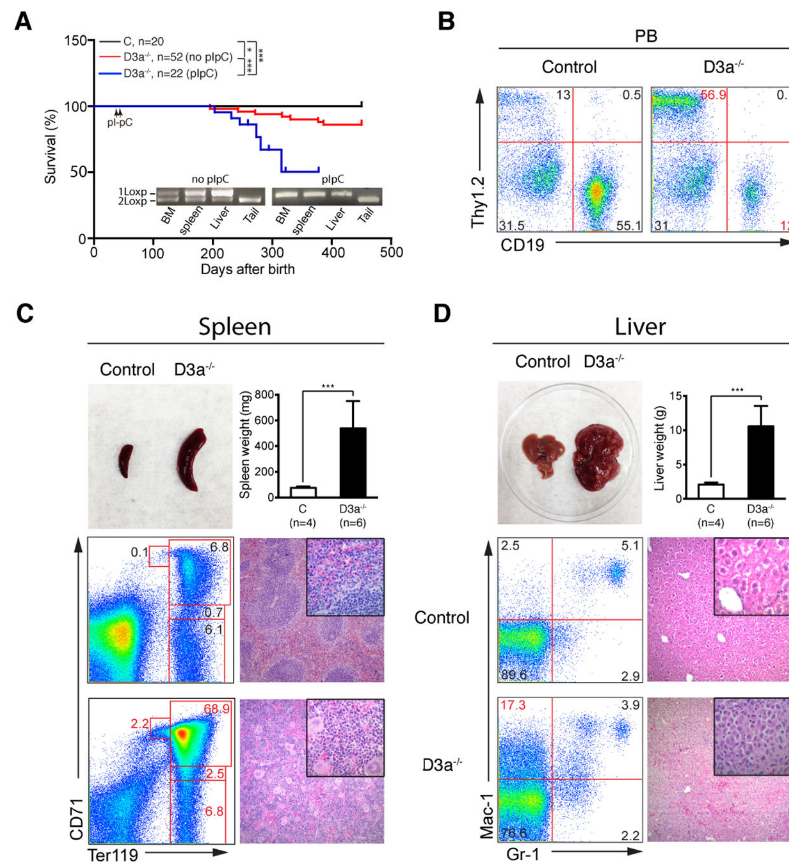

Figure 2. Loss of *Dnmt3a* induces multiple hematopoietic defects *in vivo*

The untreated and pI-pC treated *Dnmt3a*$^{fl/fl}$; *Mx1-Cre* mice were monitored for an extended period of time until a moribund stage. (A) Kaplan-Meier survival curves of different groups of mice were plotted against days after birth. Bone marrow (BM), spleen (SP), and liver cells from moribund mice were genotyped to evaluate recombination efficiency of *Dnmt3a*. P value was determined by the Log-rank test: *P<0.05. (B) Representative analyses of peripheral blood T cells (Thy1.2) and B cells (CD19) from moribund *Dnmt3a*$^{-/-}$ mice and age-matched control mice are shown. (C) Splenomegaly and representative histologic H&E sections and erythroblast analysis of spleen from moribund *Dnmt3a*$^{-/-}$ mice and age-matched control mice are shown. (D) Hepatomegaly and representative histologic H&E sections and myeloid cell analysis of liver from moribund *Dnmt3a*$^{-/-}$ mice and age-matched control mice are shown. The results are presented as means ± SD. *** P<0.001.

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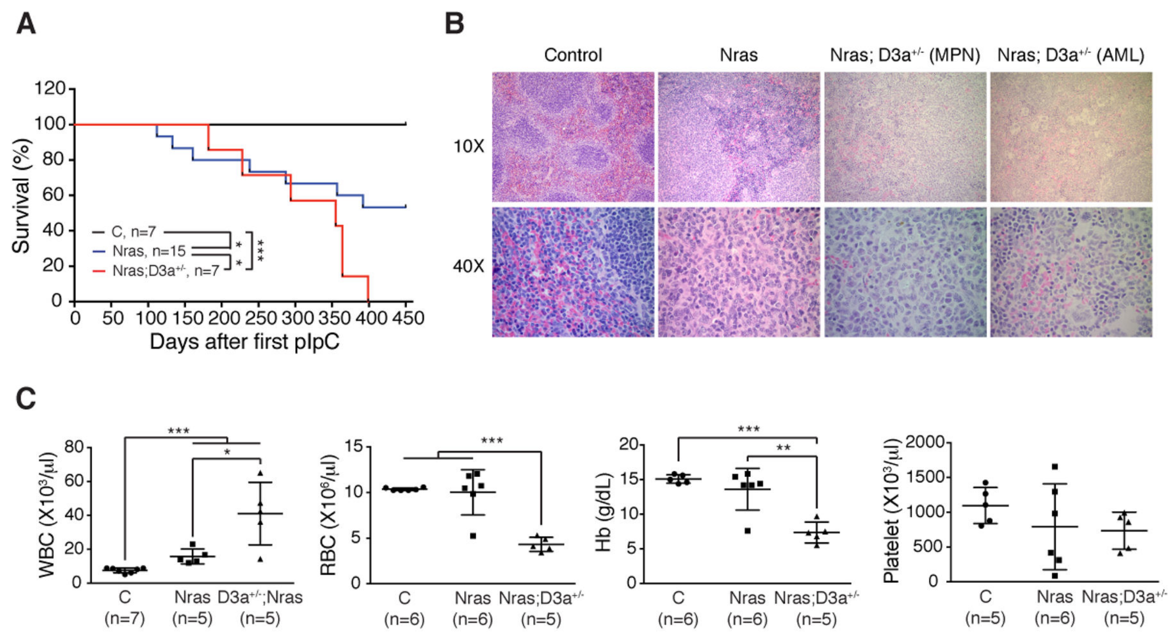

Figure 3. Downregulation of Dnmt3a expression promotes $Nras^{G12D/+}$-induced myeloid diseases

Control (C), $Nras^{G12D/+}$ (Nras), and $Nras^{G12D/+}; Dnmt3a^{+/-}$ (Nras; D3a$^{+/-}$) mice were injected with pI-pC as described in Methods. (A) Kaplan-Meier survival curves of different groups of mice were plotted against days after first pI-pC injection. P values were determined by the Log-rank test. (B) Representative spleen histologic H&E sections from moribund $Nras^{G12D/+}$ and $Nras^{G12D/+}; D3a^{+/-}$ mice and from age-matched control mice. (C) Complete blood count (CBC) from moribund $Nras^{G12D/+}; D3a^{+/-}$ mice and from 60-week old control and $Nras^{G12D/+}$ mice. The results are presented as means ± SD. * P<0.05, ** P<0.01 and *** P<0.001.

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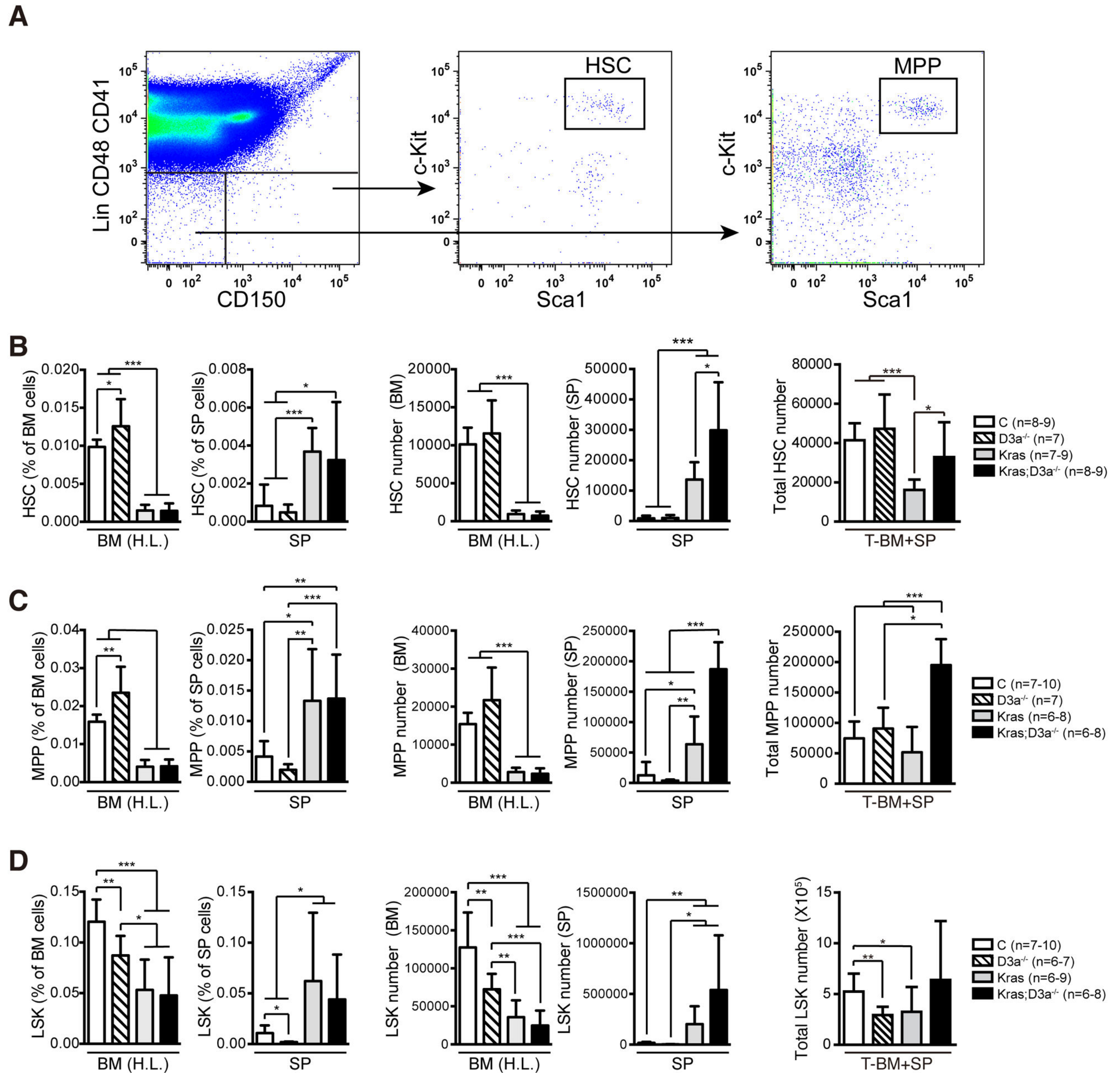

Figure 4. Loss of *Dnmt3a* rescues *Kras*$^{G12D/+}$-mediated HSC depletion

Control (C), *Dnmt3a*$^{-/-}$ (D3a$^{-/-}$), *Kras*$^{G12D/+}$ (Kras), and *Kras*$^{G12D/+}$*; Dnmt3a*$^{-/-}$ (Kras; D3a$^{-/-}$) mice were injected with pI-pC as described in Methods. Treated mice were sacrificed on Day 5 for analysis of hematopoietic stem cells (HSCs) (B), multipotential progenitor cells (MPPs) (C) and Lin^{-} Sca-1^{+} c-Kit^{+} cells (LSKs) (D) in hind limb bone marrow (BM (H.L.)) and spleen (SP). (A) Representative example of staining and gating for HSCs and MPPs. (B–D) The absolute HSC, MPP and LSK numbers in BM (H.L.) and SP were calculated based on bone marrow or spleen cell numbers and frequencies. Because BM (H.L.) represents 25% of whole body bone marrow, the total number of stem/progenitor cells per animal was calculated as the sum of stem/progenitor cell number in spleen and four

fold of stem/progenitor cell number in BM (H.L.). The results are presented as means ± SD. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

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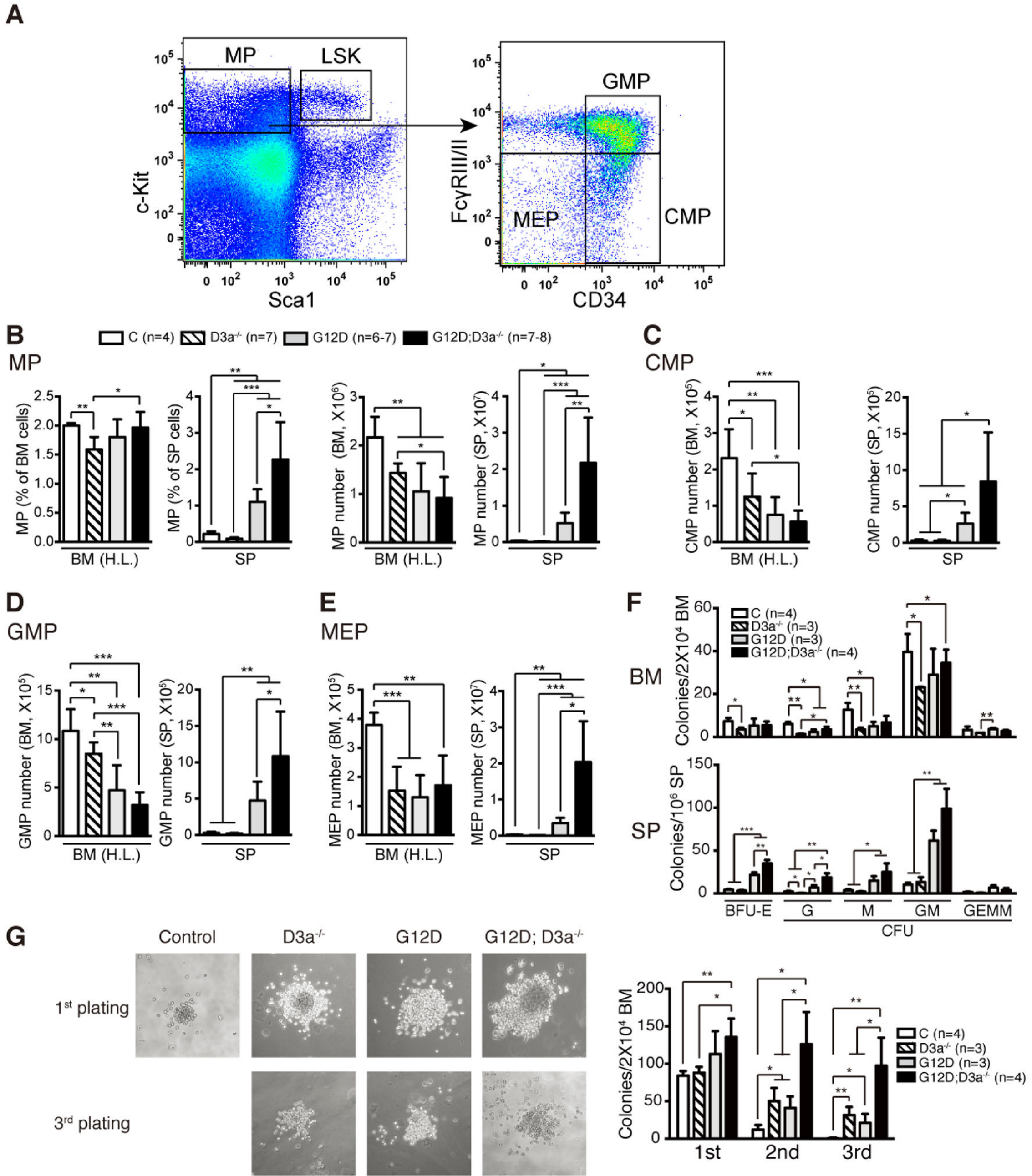

Figure 5. Loss of *Dnmt3a* increases transient self-renewal capability of *Kras*$^{G12D/+}$ myeloid progenitor cells

Control (C), *Dnmt3a*$^{-/-}$ (D3a$^{-/-}$), *Kras*$^{G12D/+}$ (Kras), and *Kras*$^{G12D/+}$*; Dnmt3a*$^{-/-}$ (Kras; D3a$^{-/-}$) mice were injected with pI-pC as described in Methods. Treated mice were sacrificed on Day 5 for analysis of myeloid progenitor cells. (A) Representative example of staining and gating for myeloid progenitors (MPs), common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs). (B–E) Quantitative analysis of MPs (B), CMPs (C), GMPs (D), and MEPs (E) in hind limb bone marrow (BM (H.L.)) and spleen (SP). (F) Quantification of various types of

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hematopoietic colonies cultured for 7 days in methylcellulose-based medium M3434. (G) Assessment of transient self-renewal capability of myeloid progenitors. The results are presented as means ± SD. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

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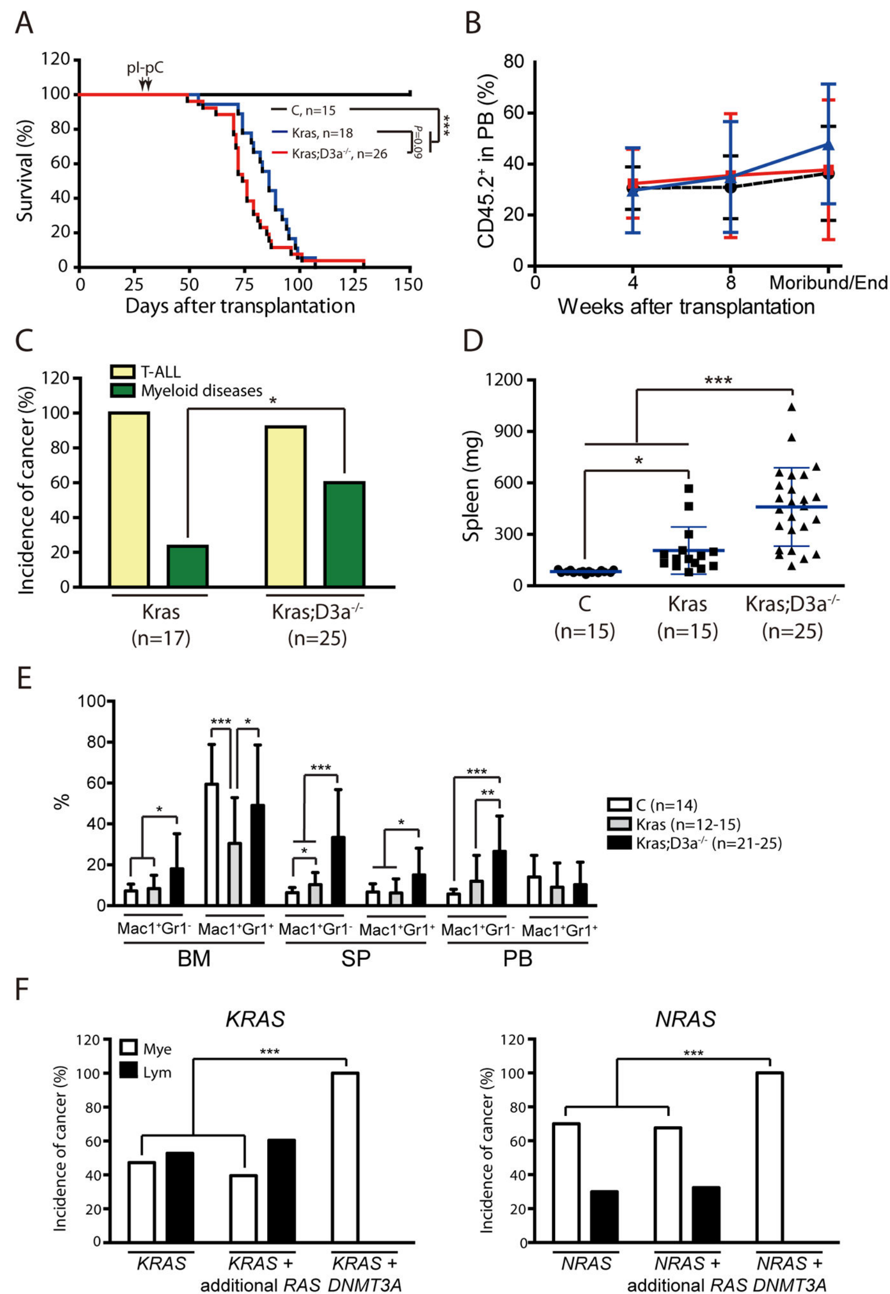

Figure 6. Recipients transplanted with *Kras*$^{G12D/+}$*; Dnmt3a*$^{-/-}$ cells develop myeloid diseases with a high penetrance

Lethally irradiated mice were transplanted with 2.5×10^5 bone marrow cells from different groups of mice along with same number of competitor cells. Four weeks after transplantation, Cre expression was induced by pI-pC injections. (A) Kaplan-Meier survival curves of different groups of recipient mice were plotted against days after transplantation. P values were determined by the Log-rank test. (B) The percentages of donor-derived cells in different groups of recipients. (C) Disease distribution patterns in recipient mice transplanted with *Kras*$^{G12D/+}$ or *Kras*$^{G12D/+}$*; Dnmt3a*$^{-/-}$ cells. We define the mice with a myeloid disease when donor-derived monocytes consist >20% of white blood cells in

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peripheral blood. Chi-square analysis was performed. (D–E) Quantitative analysis of spleen weight (D), and donor-derived monocytes and neutrophils in bone marrow (BM), spleen (SP), and peripheral blood (PB) (E) from moribund recipient mice. The results are presented as means ± SD. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. (F) Human patients with either oncogenic *KRAS* or *NRAS* mutations from COSMIC database v67 were divided into 3 groups: (1) samples with only one copy of *KRAS* or *NRAS* mutation, (2) samples with additional *RAS* mutations, including more than one copy of *KRAS* or *NRAS* mutations, or containing mutations in both *NRAS* and *KRAS* genes, (3) samples with concurrent *DNMT3A* and one copy of *KRAS* or *NRAS* mutation. The patients were further stratified based on their disease types, lymphoid or myeloid malignancies. P values were determined by the Chi-square analysis. *** $P<0.001$.

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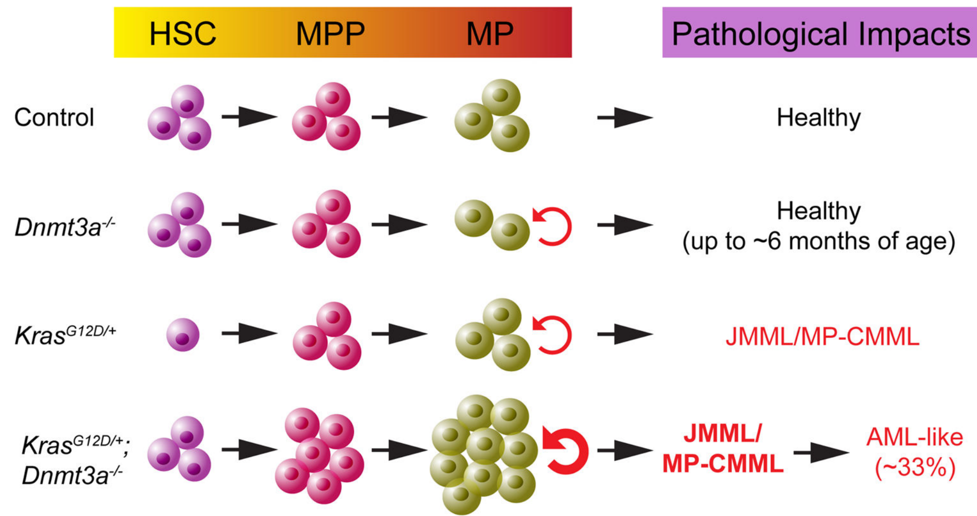

Figure 7.

Schematic illustration summarizes the phenotypes in different genetic groups of animals.

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Table 1

Summary of diseased recipient mice transplanted with Kras; D3a$^{-/-}$ LSK or MP cells

Donor cells (#/recipient)	Competitors (#/recipient)	Recipients (#)	Diseased animals (%)	Disease diagnosis(#)
LSK (1,000)	BM (200,000)	3	100	T-ALL and MPN (1) MPN(1) AML (1)
MP (15,000)	BM (200,000)	7	86	MPN (3) AML (1)

LSK (Lin^{-} Sca1^{+} cKit^{+}) and MP (myeloid progenitor) cells were flow sorted from *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ bone marrow and transplanted with congenic competitor cells (BM: bone marrow) into lethally irradiated recipients. Mice were monitored closely until a moribund stage. Note: some animals died before we could catch and analyze them. T-ALL, acute T-cell lymphoblastic leukemia/lymphoma; MPN, myeloproliferative neoplasm; AML, acute myeloid leukemia.