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Original Article

Dnmt3a haploinsufficiency cooperates with oncogenic *Kras* to promote an early-onset T-cell acute lymphoblastic leukemia

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Abstract: Mutations in *DNA methyltransferase 3A* (*DNMT3A*) are prevalent in various myeloid and lymphoid malignancies. The most common *DNMT3A* R882 mutations inhibit methyltransferase activity of the remaining wild-type DNMT3A proteins at a heterozygous state due to their dominant-negative activity. Reports and COSMIC database analysis reveal significantly different frequencies of R882 mutations in myeloid versus T-cell malignancies, inspiring us to investigate whether downregulation of *DNMT3A* regulates malignancies of different lineages in a dose-dependent manner. In a competitive transplant setting, the survival of recipients with $Kras^{G12D/+}$; $Dnmt3a^{+/-}$ bone marrow (BM) cells was significantly shortened than that of recipients with $Kras^{G12D/+}$ cells. Moreover, all of the recipients with $Kras^{G12D/+}$; $Dnmt3a^{+/-}$ cells developed a lethal T-cell acute lymphoblastic leukemia (T-ALL) without significant myeloproliferative neoplasm (MPN) phenotypes, while ~20% of recipients with $Kras^{G12D/+}$ cells developed MPN with or without T-ALL. This is in sharp contrast to the recipients with $Kras^{G12D/+}$; $Dnmt3a^{-/-}$ cells, in which ~60% developed a lethal myeloid malignancy (MPN or acute myeloid leukemia [AML]). Our data suggest that in the context of oncogenic *Kras*, loss of *Dnmt3a* promotes myeloid malignancies, while *Dnmt3a* haploinsufficiency induces T-ALL. This dose-dependent phenotype is highly consistent with the prevalence of *DNMT3A* R882 mutations in AML versus T-ALL in human.

Keywords: DNA methylation, DNMT3A, KRAS, T-ALL, MPN

Introduction

Unlike DNA sequence alterations causing inheritable impacts, epigenetics represents a labile and dynamic mechanism to regulate transcriptional potentials of a cell in response to diverse stimuli. DNA methylation, catalyzed by DNA methyltransferases (DNMTs) that add methyl groups to the 5-carbon of the cytosine ring, is an epigenetic mechanism that typically represses gene transcription [1-3]. Among DNMT family, DNMT1 primarily maintains pre-existing DNA methylation patterns, while DNMT3A and DNMT3B carry out *de novo* DNA methylation [1, 2]. Despite the long-recognized observation of aberrant DNA methylation levels and patterns in human cancers, their causal role in tumorigenesis and underlying genetic basis were first elucidated by the identification of *DNMT3A* mutations in AML [4-6]. Later, the concept that *DNMT3A* mutations are highly relevant to hematopoietic malignancies is supported by huge amount of sequencing results generated from multiple studies [7].

Recent sequencing efforts identify *DNMT3A* mutations in myeloid and lymphoid malignancies with distinct characteristics. The predominant R882 mutations, which locate in the catalytic domain of DNMT3A, are mostly at a heterozygous state [4, 6], and inhibit the methyltransferase activity of the remaining wild-type (WT) DNMT3A proteins due to their dominant-negative activity [8, 9]. Approximately 60% of

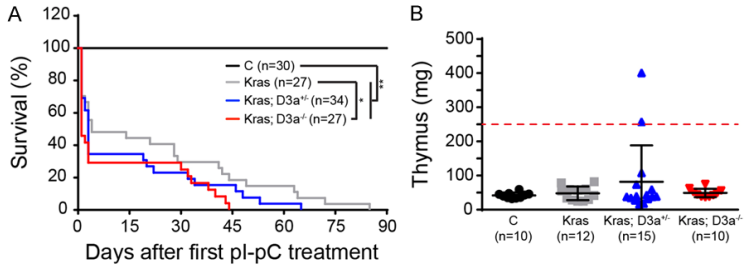

Figure 1. Loss of one *Dnmt3a* allele in primary *Kras*^{G12D} mice causes an early-onset T-ALL. A. Control (C), *Kras*$^{G12D/+}$ (Kras); *Kras*$^{G12D/+}$; *Dnmt3a*$^{+/-}$ (Kras; D3a$^{+/-}$), and *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ (Kras; D3a$^{-/-}$) mice were treated with pI-pC as described in Materials and Methods section. We define the day of first pI-pC injection as Day 1. 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. Quantification of thymus weight. Moribund mice with ≥250 mg of thymus weight (red dashed line) were considered as significant T-ALL.

DNMT3A mutations occur on R882 residues in AML [4, 6]. In contrast, the frequency of R882 mutations drops to 19% in adult T cell acute lymphoblastic leukemia (T-ALL), including early, cortical and mature subtypes [10]. The different frequency of R882 mutations in AML versus T-ALL is similar to what our observed in COSMIC database (v77), 60% and 24% for AML and T-ALL, respectively. Consistent with high frequency of dominant-negative R882 mutations in human AML, we and others recently demonstrated that complete loss of *Dnmt3a* accelerates oncogenic *Ras*-induced progression of MPN and promotes its transformation to AML in a fraction of oncogenic *Ras* mice [11, 12]. These findings inspire us to investigate whether downregulation of *DNMT3A* modulates the progression and/or transformation of myeloid versus lymphoid diseases in a dose-dependent manner.

To address this question, the conditional knock-in *Kras*$^{G12D/+}$ mouse model, which expresses oncogenic *Kras* from its endogenous promoter in hematopoietic cells, was used. *Kras*$^{G12D/+}$ mice and recipients transplanted with *Kras*$^{G12D/+}$ BM cells develop both MPN and T-ALL [13-17]. Here, we took this *Kras*$^{G12D/+}$ mouse model and a competitive bone marrow transplantation approach to study the cell-autonomous function of *Dnmt3a* haploinsufficiency on oncogenic *Kras*-induced leukemogenesis.

Materials and methods

Mice

All mouse lines were maintained in a pure C57BL/6 genetic background (>N10). *Dnmt3a* conditional knockout mice (*Dnmt3a*$^{fl/+}$), generated by Nguyen *et al.* [18], and kindly provided by Dr. Qiang Chang (UW-Madison), 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*$^{LSL\ G12D/+}$; *Dnmt3a*$^{fl/+}$; *Mx1-Cre*, *Kras*$^{LSL\ G12D/+}$; *Mx1-Cre*, and *Mx1-Cre* mice. CD45.1-positive congenic C57BL/6 recipient mice were purchased from National Cancer Institute (NCI, Bethesda, MD, USA). *Cre* expression was induced through intraperitoneal injection of 2.5 μg/g body weight of polyinosinic-polycytidylic acid (pI-pC) (GE Healthcare). 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.

Bone marrow transplantation

BM cells were isolated from various compound mice, which were injected pI-pC once. 2.5×10^5 BM cells (CD45.2$^+$) along with same number of competitor cells (CD45.1$^+$) were injected into lethally irradiated CD45.1$^+$ recipients as previously described [11]. Four weeks after transplantation, *Cre* expression was induced as described in Figure S1. Peripheral blood was collected regularly to monitor the development of hematopoietic malignancies.

Flow cytometric analysis

For lineage analysis of peripheral blood and thymus, flow cytometric analyses were performed as previously described [11, 19]. The stained cells were analyzed on a FACS Calibur (BD Biosciences). Antibodies specific for the following surface antigens were purchased from eBioscience: CD45.2 (104), Mac-1 (M1/70), Gr-1 (RB6-8C5), Thy1.2 (53-2.1), CD4 (GK 1.5), and CD8 (53-6.7).

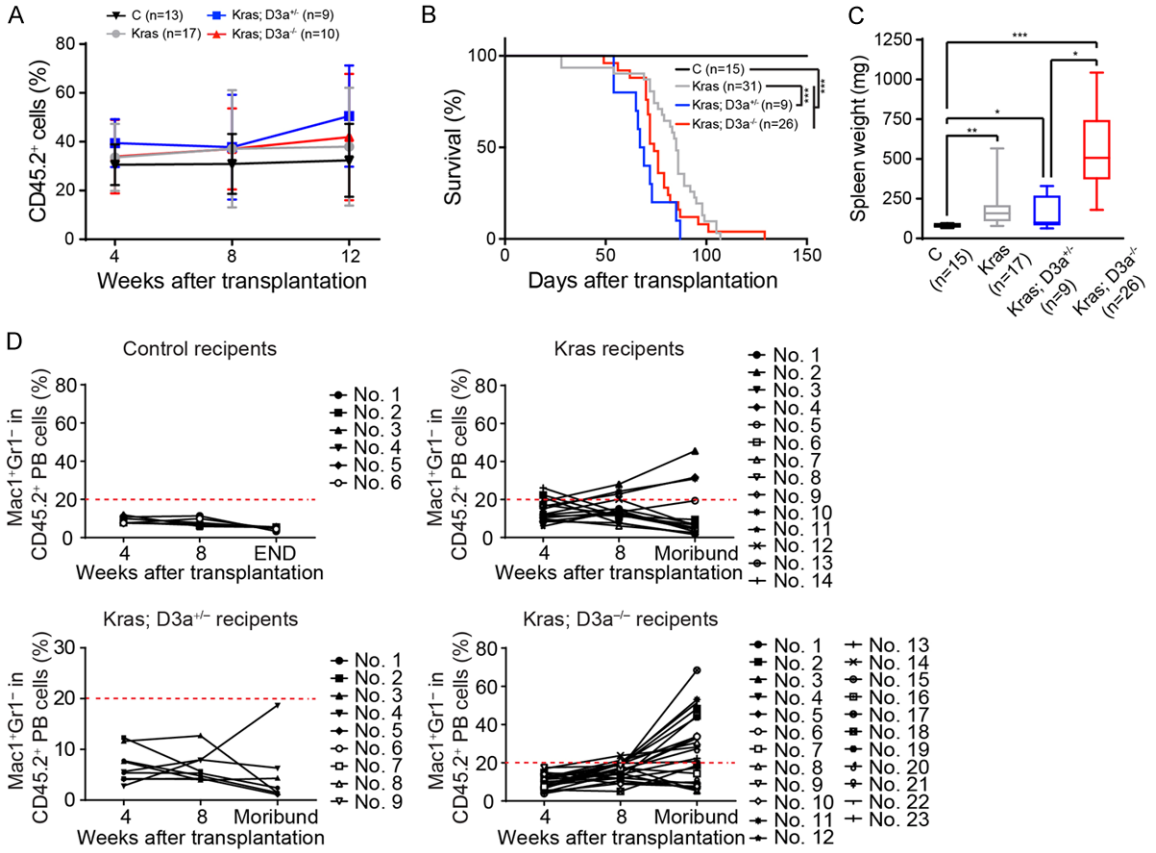

Figure 2. Recipients transplanted with *Kras; D3a*$^{+/-}$ cells have a shortened survival than *Kras* recipients. Lethally irradiated mice were transplanted with 2.5×10^5 BM cells from control (C), *Kras*$^{G12D/+}$ (Kras), *Kras*$^{G12D/+}$; *Dnmt3a*$^{+/-}$ (Kras; D3a$^{+/-}$) or *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ (Kras; D3a$^{-/-}$) mice along with same number of competitor cells as described in Materials and Methods. A. Quantification of donor-derived cells in different groups of recipients. The results were presented as mean +/- s.d. B. 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. C. Quantification of spleen weight. The results were presented as box-and-whisker diagram. D. Quantification of donor-derived monocytic cells in the peripheral blood of individual recipients. Monocytosis is defined as ≥20% monocytes in donor-derived peripheral blood cells (red dashed line). **P*<0.05; ***P*<0.01; ****P*<0.001.

Complete blood count (CBC) and histopathology

CBC analysis was performed using a Hemavet 950FS (Drew Scientific). Mouse tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich) and further processed at the Histology Lab of the University of Wisconsin Carbone Cancer Center (UWCCC).

Statistics

Kaplan-Meier survival analysis was performed and survival differences between groups were assessed with the Log-rank test, assuming significance at *P*<0.05. Unpaired 2-tailed Student's *t*-test was used to determine the significance between two data sets, assuming significance at *P*<0.05.

Results and discussions

Dnmt3a haploinsufficiency in oncogenic Kras hematopoietic cells causes a more rapid lethality without significant MPN phenotypes in a bone marrow transplantation setting

In order to study the dosage effect of *Dnmt3a* in myeloid versus lymphoid diseases, the *Kras*$^{G12D/+}$; *Mx1-Cre* (*Kras*), *Kras*$^{G12D/+}$; *Dnmt3a*$^{fl/+}$; *Mx1-Cre* (*Kras*; *D3a*$^{+/-}$) and *Kras*$^{G12D/+}$; *Dnmt3a*$^{fl/fl}$; *Mx1-Cre* (*Kras*; *D3a*$^{-/-}$) mice were generated. The *Mx1-Cre* mice were used as control throughout this paper. Treatment of

Table 1. Complete blood count (CBC) of moribund recipients

Parameters	Control (n=14)	*Kras* (n=16)	*Kras*; *D3a*$^{+/-}$ (n=7)	*Kras*; *D3a*$^{-/-}$ (n=10)
WBC (10^3 cells/μl)	8.9±2.7	8.5±11	14.7±21.8	14.5±21.7
RBC (10^6 cells/μl)	9.4±0.9	8.6±1.2	10.4±1.1*,##	4.7±2***,###,¶¶¶
Hemoglobin (g/dL)	13.6±1.5	12.2±1.9*	15.8±1.6**,###	8.9±2.7***,###,¶¶¶
Hematocrit (%)	43.1±3.9	40.5±5.4	47±4.8#	29.4±9.1***,##,¶¶¶
MCV (fL)	46.1±2	47.1±2.3	45.1±1.3	55.1±8.9**,##,¶
MCH (pg)	14.5±0.9	14.1±0.7	15.2±0.6##	17.1±1.7***,###,¶
MCHC (g/dL)	31.4±2.6	30±2.3	33.6±0.8###	30.6±2.1¶¶
RDW (%)	16.3±2.3	19.2±2.9**	15.4±2##	21.7±2.8***,#,¶¶¶
Platelet (10^3 cells/μl)	910±187	622±214***	483±307**	260±137***,###

All results are present as means ± s.d. Student *t*-test is performed. Comparing to control: *P<0.05, **P<0.01, ***P<0.001. Comparing to *Kras*: #P<0.05, ##P<0.01, ###P<0.001. Comparing to *Kras*; *D3a*$^{+/-}$: ¶P<0.05, ¶¶P<0.01, ¶¶¶P<0.001.

pI-pC twice induced a rapid lethality in *Kras*, *Kras*; *D3a*$^{+/-}$, and *Kras*; *D3a*$^{-/-}$ mice (**Figure 1A**). Although *Kras*; *D3a*$^{+/-}$ mice tended to survive shorter than *Kras* mice, their difference did not reach a statistical significance. In contrast to *Kras*; *D3a*$^{-/-}$ mice that developed more severe MPN phenotypes than *Kras* mice [11], detailed analysis of bone marrow, spleen, and peripheral blood did not demonstrate any significant differences of MPN-like phenotypes between *Kras* and *Kras*; *D3a*$^{+/-}$ mice (our unpublished observations). Notably, none of *Kras* or *Kras*; *D3a*$^{-/-}$ mice died with a significant T-ALL, while two primary *Kras*; *D3a*$^{+/-}$ mice developed a prominent T-ALL, which was characterized by enlarged thymus (≥250 mg), within a very short period of time (**Figure 1B**). This result suggested that *Dnmt3a* haploinsufficiency cooperated with oncogenic *Kras* to promote an early-onset T-ALL.

To validate this result, we treated the compound mice with pI-pC once and then euthanized them for the competitive BM transplantation. Four weeks after transplantation, we injected pI-pC twice to achieve a better recombination efficiency (Figure S1). All recipients showed similar contribution of donor-derived CD45.2$^+$ cells in the peripheral blood (**Figure 2A**). The survival of recipients transplanted with *Kras*; *D3a*$^{+/-}$ cells was significantly shortened than that of *Kras* recipients, but was indistinguishable from that of *Kras*$^{G12D/+}$; *D3a*$^{-/-}$ recipient mice (**Figure 2B**). As we previously reported [11], deletion of two *Dnmt3a* alleles further enhanced oncogenic *Kras*-induced MPN-like phenotypes, including splenomegaly (**Figure 2C**), expanded donor-derived monocytic compartment (**Figure 2D**), anemia, and thrombocytopenia (**Table 1**). Moreover, three *Kras*$^{G12D/+}$; *D3a*$^{-/-}$ recipients developed AML-like phenotypes (Figure S2). In contrast, deletion of one *Dnmt3a* allele antagonized oncogenic *Kras*-induced anemia phenotypes and did not significantly promote other MPN-like phenotypes (**Figure 2C**, **2D** and **Table 1**). Of note, none of the recipients of *Kras*; *D3a*$^{+/-}$ cells developed significant monocytosis (≥20% monocytes in donor-derived peripheral blood cells) (**Figure 2D**), characteristic of oncogenic *Kras*-induced MPN. These results suggest that unlike *Dnmt3a* deficiency, *Dnmt3a* haploinsufficiency does not significantly promote oncogenic *Kras*-induced myeloid diseases.

Deletion of one Dnmt3a allele in oncogenic Kras hematopoietic cells promotes an early-onset T-ALL in a cell-autonomous manner

We turned to investigate the effect of *Dnmt3a* dosage in lymphoid diseases. Notably, all recipients transplanted with *Kras*; *D3a*$^{+/-}$ BM cells died with an enlarged thymus, which was predominantly populated with donor-derived T cells (**Figure 3A** and **3B**). Additionally, 5 out of 9 (56%) moribund *Kras*; *D3a*$^{+/-}$ recipients developed T cell lymphocytosis (≥40% T cells in donor-derived peripheral blood cells) (**Figure 3C**). In contrast, complete loss of *Dnmt3a* significantly attenuated *Kras*$^{G12D/+}$-induced thymic enlargement and only 6 out of 23 (26%) moribund *Kras*; *D3a*$^{-/-}$ recipients developed T cell lymphocytosis (**Figure 3A** and **3C**). In 5 *Kras*; *D3a*$^{-/-}$ recipients, the frequency of donor-derived T cells in malignant thymus was reduced due to myeloid cell infiltration (**Figure 3B**). Detailed analysis of moribund recipients

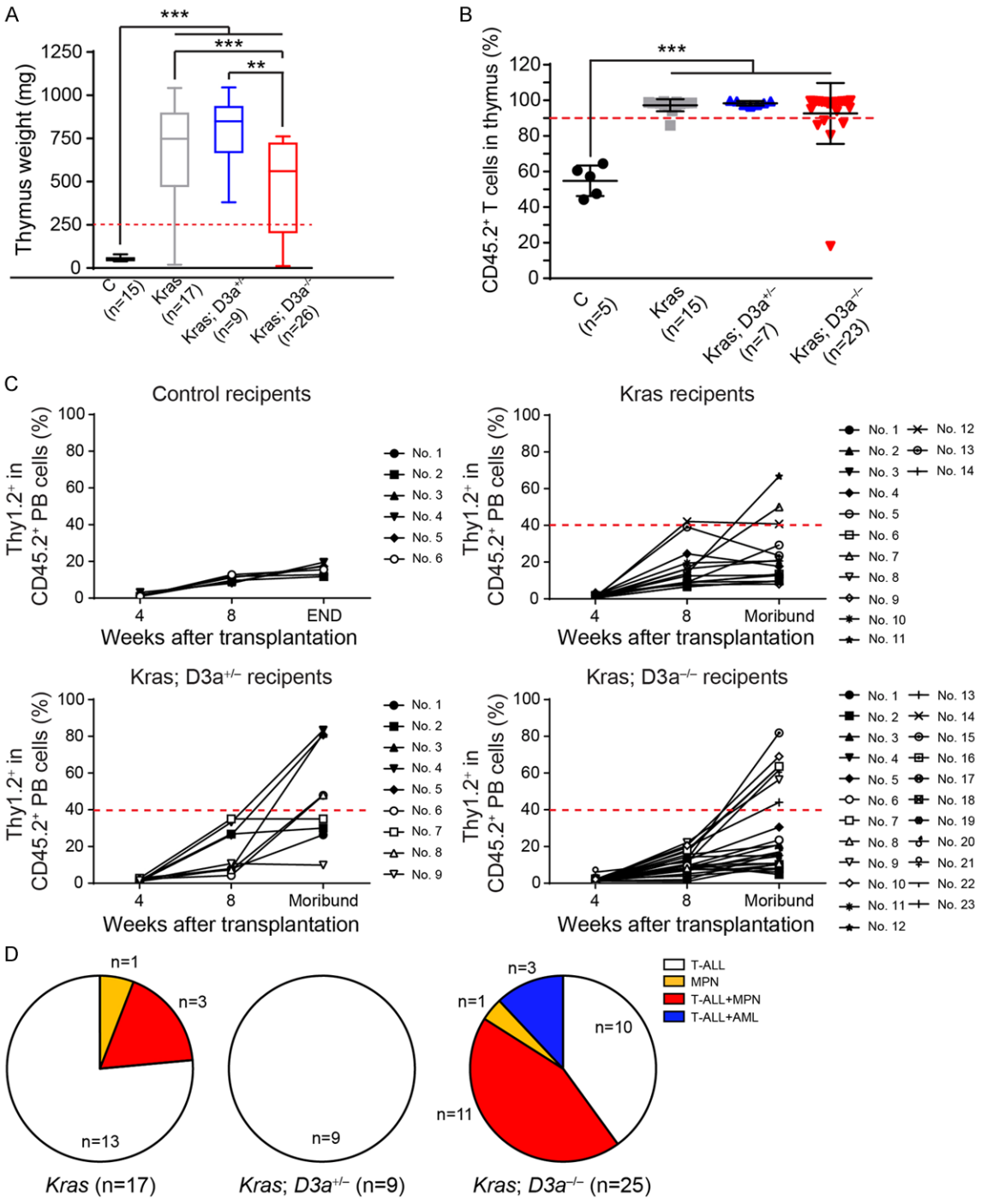

Figure 3. *Dnmt3a* haploinsufficiency cooperates with oncogenic *Kras* to promote the progress of T-ALL. Lethally irradiated mice were transplanted with 2.5×10^5 BM cells from different groups of compound mice along with same number of competitor cells as described in Materials and Methods. A. Quantification of thymus weight. Moribund mice with ≥250 mg of thymus (red dashed line) were considered to develop T-ALL. The results were presented as box-and-whisker diagram. B. Quantification of donor-derived thymocytes. The results are presented as mean ± s.d. ***P*<0.01; ****P*<0.001. C. Quantification of donor-derived T cells in the peripheral blood of individual recipients. T cell lymphocytosis is defined as ≥40% T cells in donor-derived peripheral blood cells (red dashed line). D. Disease incidences in recipient mice transplanted with *Kras*$^{G12D/+}$, *Kras*; *D3a*$^{+/-}$ or *Kras*; *D3a*$^{-/-}$ BM cells.

indicated that *Dnmt3a* haploinsufficiency cooperated with oncogenic *Kras* to promote development of T-ALL in a cell-autonomous manner (**Figure 3D**).

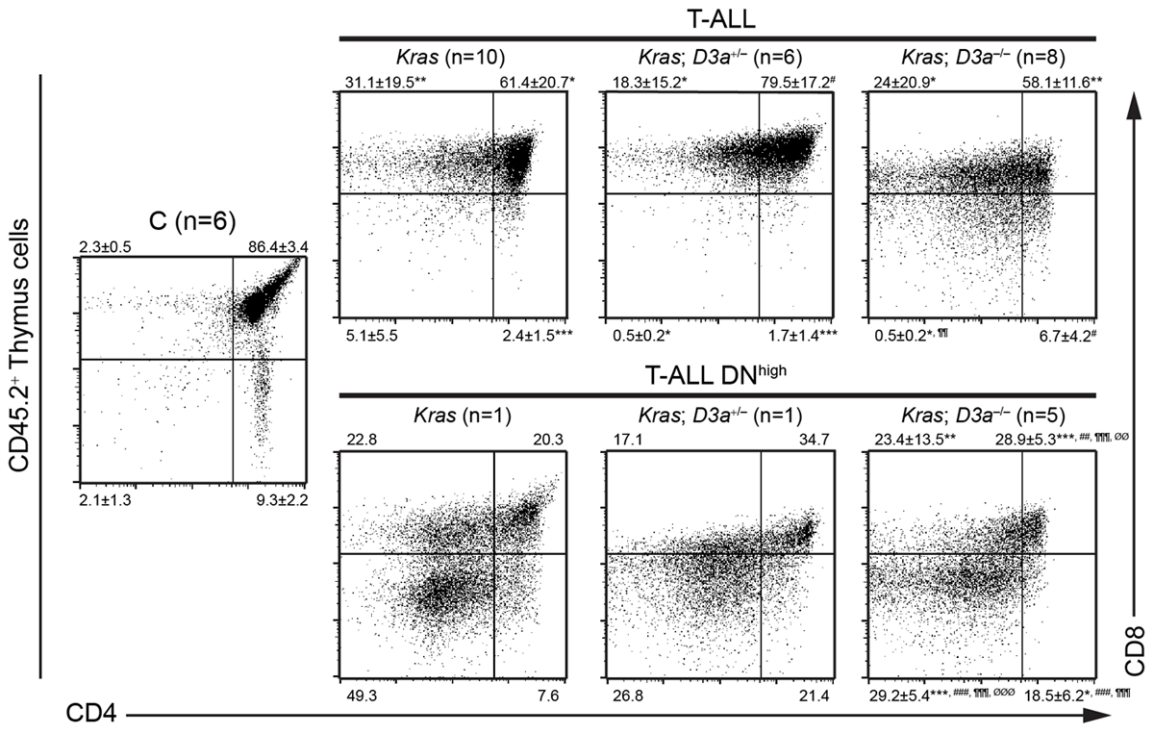

Figure 4. Loss of *Dnmt3a* in *Kras*^{G12D} hematopoietic cells appears to expand the compartment of CD4 and CD8 double negative thymocytes. Lethally irradiated mice were transplanted with 2.5×10^5 BM cells from different groups of compound mice along with same number of competitor cells as described in Materials and Methods. Thymocytes at different stages of T cell differentiation were quantified. The results are presented as mean ± s.d. Comparing to control: **P*<0.05; ***P*<0.01; ****P*<0.001. Comparing to *Kras* (T-ALL): #*P*<0.05; ##*P*<0.01; ###*P*<0.001. Comparing to *Kras*; *D3a*$^{+/-}$ (T-ALL): ¶¶*P*<0.01; ¶¶¶*P*<0.001. Comparing to *Kras*; *D3a*$^{-/-}$ (T-ALL): ØØ*P*<0.01; ØØØ*P*<0.001.

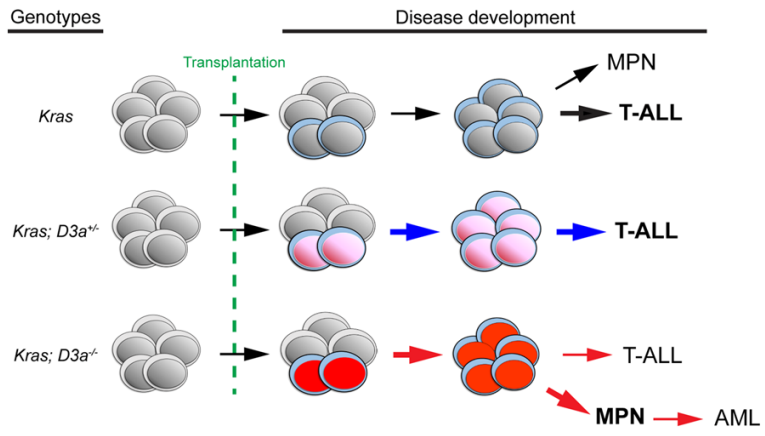

Figure 5. Schematic illustration summarizes that the downregulation of *Dnmt3a* expression promoted oncogenic *Kras*-induced MPN versus T-ALL in a dose-dependent manner. Upon competitive transplantation of BM cells (1:1 ratio of competitor: donor), *Dnmt3a* deficiency cooperates with oncogenic *Kras* to accelerate MPN progression and induce its transformation to AML in a fraction of mice, while *Dnmt3a* haploinsufficiency collaborates with oncogenic *Kras* to promote an early-onset T-ALL. Of note, all recipients showed similar contribution (~40%) of donor-derived CD45.2$^+$ cells in the peripheral blood. The thickness of the arrow represents the relative penetrance of a disease.

Immunophenotypic and gene expression analyses of primary human T-ALL cells reveal that the heterogeneity of T-ALL is partially related to arrest at distinct stages of development [20]. For producing mature CD4$^+$ or CD8$^+$ T cells, the committed lymphoid progenitor cells in the BM migrate to the thymus, and differentiate into CD4 and CD8 double negative (DN) committed T-cell precursor cells. The DN cells are further committed into CD4 and CD8 double positive (DP) cells, and finally differentiate into CD4$^+$ or CD8$^+$ T cells [21]. To study the impact of *Dnmt3a* downregulation in T-cell development, the enlarged thymus without significant myeloid cell infiltra-

tion were selected for further analysis. Consistent with previous results [13, 14], ~90% of *Kras* recipients expanded donor-derived CD8$^+$ T cells in thymus. This was also seen in ~86% and ~62% of *Kras*$^{G12D/+}$; *D3a*$^{+/-}$ and *Kras*$^{G12D/+}$; *D3a*$^{-/-}$ recipients, respectively (**Figure 4**). Interestingly, ~38% of recipients transplanted with *Kras*$^{G12D/+}$; *D3a*$^{-/-}$ cells displayed an increasing amount of DN thymocytes (T-ALL DN^{high}), whereas only 1 out of 11 *Kras*$^{G12D/+}$ and 1 out of 7 *Kras*$^{G12D/+}$; *D3a*$^{+/-}$ mice expanded the compartment of DN thymocytes (**Figure 4**). As all oncogenic *Ras*-induced T-ALL models, T-ALL cells from *Kras*; *D3a*$^{+/-}$ recipients carried Type 1 deletion of *Notch1* (Figure S3), which renders ligand-independent activation of Notch signaling. Our results are consistent with a previous report that transplantation of *Dnmt3a*$^{-/-}$ hematopoietic stem cells (HSCs) induces high incidence of myeloid malignancies and low frequency of lymphoid malignancies, including both T-ALL and its early immature subtype, in recipient mice. These malignant T cells also acquire *Notch1* mutations [12]. Furthermore, our results are consistent with previous studies identifying *KRAS* [22] and *DNMT3A* mutations [10, 23, 24] from patients with early immature subtypes of T-ALL.

Taken together, our data suggest that downregulation of *Dnmt3a* expression promotes oncogenic *Kras*-induced MPN versus T-ALL in a dose-dependent manner (**Figures 2** and **3**). Complete loss of *Dnmt3a* cooperates with oncogenic *Kras* to accelerate MPN progression and induce its transformation to AML, while *Dnmt3a* haploinsufficiency collaborates with oncogenic *Kras* to promote an early-onset T-ALL in the bone marrow transplantation setting (**Figure 5**). Our observation is consistent with a previous report that global methylation pattern in *Dnmt3a*-null driven T-ALL cells is distinct from that in MPN cells [12]. Our result also provides an explanation why the frequency of the dominant-negative *DNMT3A* R882 mutations in human T-ALL is much lower than that in myeloid malignancies.

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Disclosure of conflict of interest

None.

Authors' contribution

The contributions of individual authors are listed below: YIC for experimental design & execution as well as writing manuscript; GK, PST, and YSY for experimental execution; EAR for histopathology analysis and writing manuscript; JZ for experimental design and writing manuscript.

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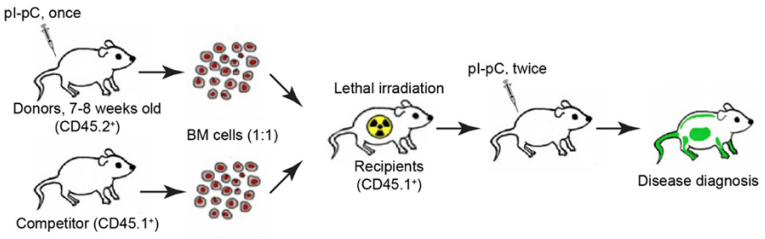

Figure S1. The procedure of bone marrow transplantation. Compound mice were injected pI-pC (2.5 μg/g body weight) once. Bone marrow (BM) cells were isolated from various compound mice. 2.5×10^5 BM cells ($CD45.2^+$) along with same number of competitor cells ($CD45.1^+$) were injected into lethally irradiated $CD45.1^+$ recipients. Four weeks after transplantation, we injected pI-pC twice to achieve a better recombination efficiency. Peripheral blood was collected regularly to monitor the development of hematopoietic malignancies.

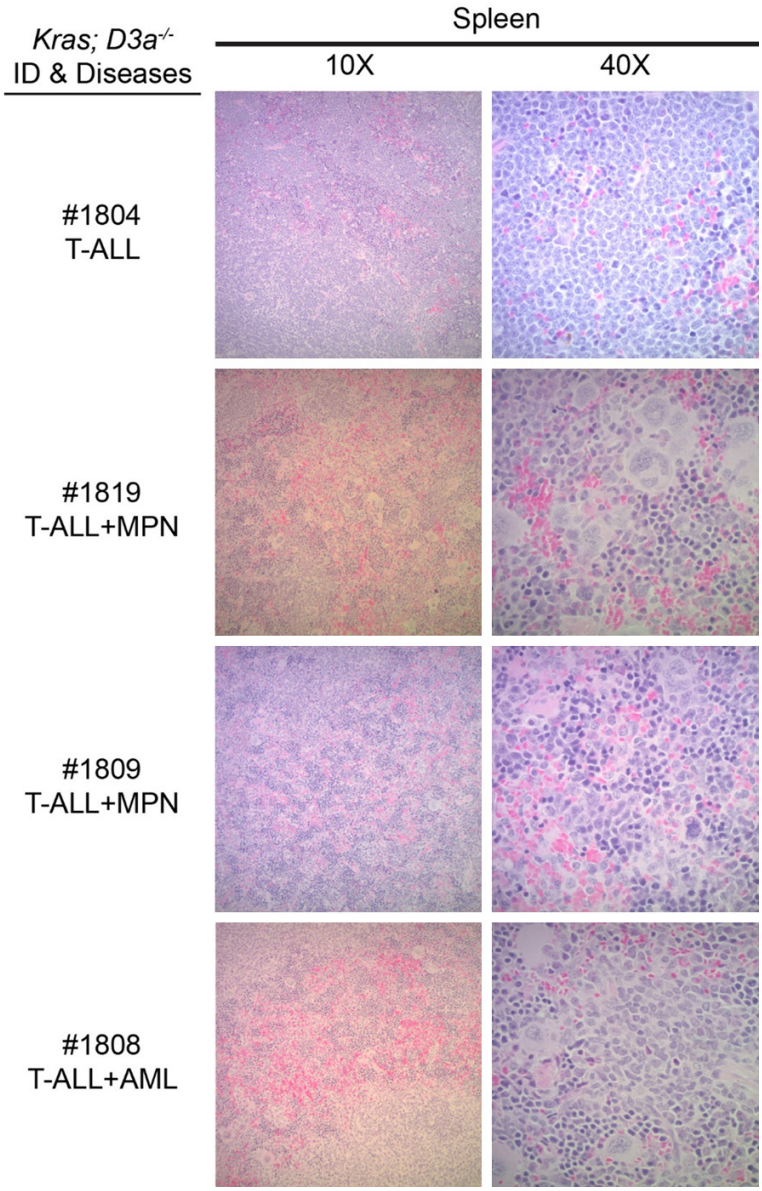

Figure S2. Representative spleen histologic H&E sections from moribund recipient mice that were transplanted with *Kras*$^{G12D/+}$; *Dnmt3a*$^{-/-}$ bone marrow cells.

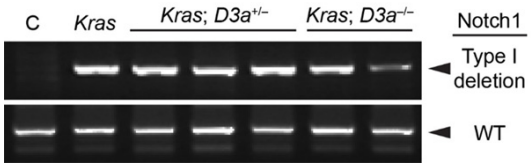

Figure S3. Analysis of *Notch1* type I deletion in different groups of recipient mice with T-ALL.