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## ***Dnmt3a* Regulates Myeloproliferation and Liver-Specific Expansion of Hematopoietic Stem and Progenitor Cells**

Olga A. Guryanova<sup>1</sup>, Yen K. Lieu<sup>2</sup>, Francine E. Garrett-Bakelman<sup>3</sup>, Barbara Spitzer<sup>1</sup>, Jacob L. Glass<sup>3,4</sup>, Kaitlyn Shank<sup>1</sup>, Ana Belen Valencia Martinez<sup>5</sup>, Sharon A. Rivera<sup>2</sup>, Benjamin H. Durham<sup>1</sup>, Franck Rapaport<sup>1</sup>, Matthew D. Keller<sup>1</sup>, Suveg Pandey<sup>1</sup>, Lennart Bastian<sup>1</sup>, Daniel Tovbin<sup>1</sup>, Abby R. Weinstein<sup>1</sup>, Julie Teruya-Feldstein<sup>1</sup>, Omar Abdel-Wahab<sup>1,4</sup>, Valeria Santini<sup>5</sup>, Christopher E. Mason<sup>6</sup>, Ari M. Melnick<sup>3</sup>, Siddhartha Mukherjee<sup>2,\*</sup>, and Ross L. Levine<sup>1,4,7,\*</sup>

<sup>1</sup>Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY

<sup>2</sup>Department of Medicine and Irving Cancer Research Center, Columbia University, New York, NY

<sup>3</sup>Department of Medicine, Weill Cornell Medical College, New York, NY

<sup>4</sup>Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY

<sup>5</sup>Hematology Unit, University of Florence, Florence, Italy

<sup>6</sup>Department of Physiology and Biophysics, Weill Cornell Medical College, New York, NY

<sup>7</sup>Center for Epigenetics Research, Memorial Sloan Kettering Cancer Center, New York, NY

### **Abstract**

*DNMT3A* mutations are observed in myeloid malignancies, including myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), and acute myeloid leukemia (AML). Transplantation studies have elucidated an important role for *Dnmt3a* in stem cell self-renewal and in myeloid differentiation. Here we investigated the impact of conditional hematopoietic *Dnmt3a* loss on disease phenotype in primary mice. *Mx1-Cre*-mediated *Dnmt3a* ablation led to the development of a lethal, fully penetrant myeloproliferative neoplasm with myelodysplasia (MDS/MPN) characterized by peripheral cytopenias and by marked, progressive hepatomegaly. We detected expanded stem/progenitor populations in the liver of *Dnmt3a*-ablated mice. The MDS/MPN induced by *Dnmt3a* ablation was transplantable, including the marked hepatomegaly. Homing studies showed that *Dnmt3a*-deleted bone marrow cells preferentially migrated to the liver. Gene expression and DNA methylation analyses of progenitor cell populations identified

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\*Correspondence: Siddhartha Mukherjee, Department of Medicine and Irving Cancer Research Center, Columbia University, 4781 Broadway, New York, NY 10032. Phone: (212) 851 4617; ; Email: sm3252@columbia.edu. Ross L. Levine, Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, Box 20, New York, NY 10065. Phone: (646) 888 2767; fax: (646) 422 0890; ; Email: leviner@mskcc.org

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differential regulation of hematopoietic regulatory pathways, including fetal liver hematopoiesis transcriptional programs. These data demonstrate that *Dnmt3a* ablation in the hematopoietic system leads to myeloid transformation *in vivo*, with cell autonomous aberrant tissue tropism and marked extramedullary hematopoiesis (EMH) with liver involvement. Hence, in addition to the established role of *Dnmt3a* in regulating self-renewal, *Dnmt3a* regulates tissue tropism and limits myeloid progenitor expansion *in vivo*.

## Keywords

Dnmt3a; myelodysplasia; hematopoietic stem cells; extramedullary hematopoiesis; HSPC homing

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## Introduction

Aberrant epigenetic patterning is a cornerstone of the molecular pathogenesis of cancers, including myeloid malignancies<sup>1</sup>. *DNMT3A* (DNA methyltransferase 3A) mutations are detected in 8–10% of myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS)<sup>2–4</sup>, and in 20–25% of acute myeloid leukemias (AML)<sup>5–8</sup>. These mutations occur as monoallelic or biallelic nonsense/frameshift alterations, or a dominant-negative R882 substitution<sup>9, 10</sup>. *DNMT3A*-mutant AMLs exhibit global and site-specific alterations in DNA methylation<sup>10</sup>, also observed in chronic myelomonocytic leukemia (CMML) patients with *DNMT3A* mutations<sup>11</sup>.

Although these data underscore the importance of *DNMT3A* mutations to myeloid transformation, the specific mechanisms by which *DNMT3A* functions as a tumor suppressor have not been fully elucidated. It is possible that mutations in epigenetic modifier genes alter the epigenetic state of normal hematopoietic stem/progenitor cells (HSPC), which allows malignant cells to re-access earlier developmental transcriptional programs. Notably, such features as enhanced hematopoietic stem cell self-renewal, increased proliferative capacity, myeloid bias, and extramedullary hematopoiesis (EMH), are shared between fetal liver hematopoiesis and MDS/MPN<sup>12, 13</sup>.

Previous studies on *Dnmt3a* loss in adult hematopoiesis used transplantation assays to document expansion of the stem/progenitor compartment, most prominently long-term HSCs, a gain in self-renewal, and a decline in the output of differentiated progeny<sup>14</sup>. Moreover, a subset of recipients developed different hematologic malignancies<sup>15, 16</sup>. However, these studies did not assess the tumor suppressor function of *Dnmt3a* in the absence of the selective pressure of serial transplantation, or whether *Dnmt3a* loss is sufficient to induce transformation *in vivo*. We therefore characterized the impact of conditional *Dnmt3a* loss in the hematopoietic compartment to assess impact on disease phenotype *in vivo*, and on steady-state adult hematopoiesis, coupled with studies of the effect of *Dnmt3a* loss on DNA methylation and transcriptional state.

## Materials and Methods

### Animal studies

were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center. *Dnmt3a<sup>fl/fl</sup>* conditional knock-out (cKO) line<sup>17</sup> was reconstituted from frozen embryos (The Jackson Laboratory, Bar Harbor, ME), backcrossed to C57BL/6 background, and crossed to *Mx1-Cre*-deletor line. See supplemental methods.

### Colony-forming assay in semisolid media

was performed using previously published method<sup>18</sup> with modifications. See supplemental methods.

### Homing assay

was performed as described<sup>19</sup> with modifications. See also supplemental methods.

### Gene expression and DNA methylation analyses

were performed on FACS-sorted LSK and GMP cells. Enhanced Reduced Representation of Bisulfite Sequencing (ERRBS) was used to determine cytosine methylation patterns at base pair resolution<sup>20, 21</sup>. RNA was subjected to standard Illumina-based sequencing. For detailed description see supplemental methods.

### Statistical analysis

Except where indicated, data are presented as mean  $\pm$  standard deviation plotted using Prism version 6 (GraphPad Software). Statistical significance was determined using Student's *t*-test with Holm-Sidak correction method, with  $\alpha=5.0\%$ .

## Results

### Hematopoietic-specific inactivation of *Dnmt3a* results in lethal hematologic disease

We first investigated the role of *Dnmt3a* in steady-state hematopoiesis. *Mx1-Cre*-driven recombination results in complete loss of *Dnmt3a* protein in the hematopoietic system (Figures 1A and S1A) and leads to a lethal, fully penetrant disease (median survival 48.6 weeks,  $p<0.0001$ , Figure 1B). None of the *Dnmt3a<sup>+/+</sup>:Mx1-Cre<sup>+</sup>* or *Dnmt3a<sup>fl/fl</sup>:Mx1-Cre<sup>-</sup>* animals developed hematologic abnormalities within a 90-week follow-up period. *Dnmt3a*-ablated mice developed progressive macrocytic anemia with anisocytosis, thrombocytopenia, and monocytosis (Figures 1C–H and S1B–G). Examination of the hematocrit and platelet counts over time revealed progressive anemia and thrombocytopenia (Figure 1I–J).

### *Dnmt3a* loss induces mature myeloid and myeloid progenitor expansion *in vivo*

Detailed analysis of the hematopoietic system in *Dnmt3a* KO mice found marked myeloid bias and myeloid and erythroid dysplasia in peripheral blood (Figures 2A–B) accompanied by hypercellular bone marrow (Figure S2A) with megakaryocyte dysplasia (Figure 2C). We found increased spleen size (Figures 2D) and effacement of splenic architecture by myeloid

infiltration, and scattered dysplastic megakaryocytes (Figure 2E), consistent with myeloproliferation, confirmed by flow cytometry (Figures 2F and S2B). We observed an increase in the stem-cell-enriched Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) and in Lineage<sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>+</sup> (LK) myeloid progenitor cells, with significant expansion of GMPs (Figure 2G–H). The findings of hypercellular bone marrow with dysplasia, myeloid bias in the peripheral blood, and extramedullary hematopoiesis is consistent with a myeloproliferative/myelodysplastic disorder (MDS/MPN).

### Impaired erythroid maturation after *Dnmt3a* loss

To gain insight into the mechanism of anemia in diseased *Dnmt3a*-null mice we examined erythroid maturation in bone marrow and spleens according to CD71 and Ter119 markers<sup>22, 23</sup> and found a decrease in the mature stage IV erythroblasts and an increase in stage II CD71<sup>+</sup>Ter119<sup>+</sup> cells, most pronounced in the spleens, without significant changes in apoptosis (Figure S2C–E), consistent with a previous report<sup>15</sup>. We also observed erythrophagocytosis in the spleens and livers of *Dnmt3a*-deleted animals (Figure S2F).

### Perturbation of the stem/progenitor compartment and increased self-renewal of *Dnmt3a* deficient hematopoietic cells

Previous studies found increased numbers of primitive HSCs, but not of immediate downstream progenitors, in recipient mice reconstituted with *Dnmt3a*-ablated HSCs<sup>14</sup>. In this setting the impact of stress from bone marrow transplantation could not be unequivocally separated from the cell-intrinsic HSC phenotype. Detailed analysis of HSPCs in diseased primary *Dnmt3a* KO animals showed a significant increase in the relative frequency of the immature LSK population. This expansion was due to elevated LSK CD48<sup>+</sup> cells while the LSK CD48<sup>-</sup>CD150<sup>+</sup> LT-HSC population remained unperturbed, and we observed an increase in committed myeloid progenitors (Figures 3A–B and S3A). Overall, *Dnmt3a*-deficient HSPCs were characterized by increased proliferation and decreased apoptosis (Figure 3C–D). The increased frequency of CD48<sup>+</sup> LSK cells accompanied by predominant myelopoiesis relative to lymphopoiesis, and increased self-renewal and multilineage reconstitution potential are characteristic of fetal hematopoiesis<sup>13</sup>. We observed a prominent cKit<sup>+</sup>CD41<sup>+</sup> population in the bone marrow of *Dnmt3a*-deleted mice, which is nearly absent in normal adult hematopoiesis (Figure S2G).

To assess the self-renewal potential of bone marrow cells we performed colony-forming assays. *Dnmt3a* loss resulted in continuous serial replating, while control cells rapidly exhausted their colony-forming ability (Figure 3E). In serial competitive transplantation assays *in vivo* *Dnmt3a* KO cells showed robust repopulation advantage compared to wild-type control (Figure S3B–C), which was more pronounced in the bone marrow compartment, and continued to increase with each round of transplantation (Figure 3F–G). These observations suggest that loss of *Dnmt3a* augments stem cell function *in vitro* and *in vivo*.

### ***Dnmt3a* loss results in hepatomegaly due to liver-specific myeloproliferation and extramedullary hematopoiesis**

All moribund *Dnmt3a*-deleted animals presented with distended abdomens and marked hepatomegaly (Figure 4A–B). Histopathologically *Dnmt3a* KO livers showed portal, lobular, sinusoidal infiltration by immature myeloid cells with open chromatin and prominent nucleoli, scattered megakaryocytes, and occasional blasts (Figure 4C), confirmed by FACS analysis that also showed prominent monocytosis (Figure 4D–E and S4A). Alkaline phosphatase (ALP) and bilirubin levels were elevated and albumin levels decreased (Figures 4F and S4B), consistent with impaired liver function.

Histological detection of megakaryocytes in infiltrated liver parenchyma after *Dnmt3a* inactivation suggested that liver was a site of hematopoiesis. Analysis of HSPCs residing in the liver detected myeloid progenitor LK and stem-cell-enriched LSK cells in *Dnmt3a*-null mice, but not in wild-type animals (Figure S4C). Plating of *Dnmt3a* KO liver cells yielded colonies with characteristic granulocyte-erythroid-macrophage-megakaryocyte (GEMM) and granulocyte-macrophage (GM) morphology that serially replated (Figure 4G) similar to continuously self-renewing *Dnmt3a*-ablated bone marrow cells. By contrast, cells derived from wild-type control livers were unable to give rise to hematopoietic colonies *in vitro*.

### ***Dnmt3a*-deleted bone marrow cells exhibit cell-intrinsic liver tropism**

As *Mx1-Cre* is able to mediate excision, in addition to HSCs, in hepatic and perivascular cells<sup>24, 25</sup>, we examined if extramedullary tropism of *Dnmt3a* KO hematopoietic cells was due to cell-intrinsic or niche-dependent mechanism by performing short-term homing studies (Figure 5A). While the ability of *Dnmt3a*-deficient cells to home to the bone marrow was unaltered, they showed preferential liver tropism (Figure 5B–C). Interestingly, CD45<sup>+</sup> *Dnmt3a* KO liver cells exhibited even stronger hepatotropism than bone marrow cells from the same animals (Figure S5A). These findings demonstrate that preferential liver homing of *Dnmt3a*-null hematopoietic cells is mediated by a cell-intrinsic mechanism.

### **Hematopoietic-specific *Dnmt3a* loss results in cell-autonomous myeloproliferation and liver-specific myeloid expansion in secondary recipients**

We tested the ability of *Dnmt3a*-deficient hematopoietic cells to re-initiate MDS/MPN by transplantation into sublethally-irradiated congenic hosts. No engraftment was observed 12 weeks post-transplant confirming that the hematologic disease represents MDS/MPN, and not AML. Conversely, transplantation into lethally-irradiated recipients resulted in a fully penetrant, rapidly fatal disease with a median latency of 13.4 weeks (Figure 5D,  $p < 0.0001$ ) that recapitulated the MDS/MPN seen in primary mice with hepatosplenomegaly (Figure 5E and S5B). Similar results were obtained when recipient mice were transplanted with CD45<sup>+</sup> cells isolated from *Dnmt3a*-deleted livers, suggesting that disease-initiating cells are present in the liver (data not shown). *Dnmt3a* KO-transplanted livers showed myeloid infiltration and histiocytic hyperplasia (Figure 5F). Recipient mice developed thrombocytopenia, decreased hematocrit and peripheral blood leukocytosis with monocytosis (Figures 5G–H and S5C–D). In contrast to primary *Dnmt3a* KO mice, transplant recipients showed both myeloid and T-lymphoid bias, and bi-lineage expansion (Figure S5E–F).

### Extramedullary hematopoiesis in MDS/MPN patients with *DNMT3A* mutations

To extend our studies to the human context we examined whether *DNMT3A* mutations are associated with EMH in a well-annotated cohort of 46 patients with the MDS/MPN overlap syndrome CMML<sup>11</sup>. *DNMT3A*-mutant patients had a higher rate of clinically evident EMH (Figure 5I): 2 of 4 *DNMT3A*-mutant patients (50%) presented with hepatosplenomegaly and lymphadenopathy due to extramedullary CMML, while only 7.14% (3/42) *DNMT3A*-WT patients showed similar extramedullary involvement ( $p=0.053$  and  $Phi=+0.39$ , 2-tailed Fisher's exact test). These data underscore the tumor suppressor role of *Dnmt3a* and association of *DNMT3A* loss with EMH in the clinical context.

### DNA methylation profiling reveals decreased methylation upon *Dnmt3a* loss at loci with regulatory potential

To assess whether the *Dnmt3a* KO phenotype was linked to aberrant epigenetic programming we analyzed DNA methylation patterns in LSK and GMP cells. Enhanced reduced-representation bisulfite sequencing (ERRBS) followed by unsupervised hierarchical clustering of genome-wide cytosine methylation profiles showed segregation of *Dnmt3a* wild-type and mutant samples (Figure S6A). Genome-wide CpG methylation values revealed regions of marked DNA demethylation upon *Dnmt3a* loss (Figure 6A;  $p<0.01$ , Wilcoxon two-tailed rank sum test). Of the 822 differentially methylated regions in GMPs (DMRs - methylKit:  $q<0.01$  and 20% methylation difference; methylSig: beta-binomial  $p$  0.25 and methylation difference 10%; Table S1) 797 (97%) were hypomethylated in *Dnmt3a*-ablated mice (Figure S6B) with particular enrichment for hypomethylated CpGs within introns, gene neighborhoods, and intergenic regions ( $p<0.01$ , 1-tailed Fisher's exact test; Figure 6B). We next focused our analyses on GMPs as we observed a marked increase in the proportion of GMPs *in vivo*. A trend toward hypomethylation was seen at gene promoters (Figures 6B and S6C). Overall, GMP-stage active enhancers showed a greater degree of methylation loss than promoters, exons, introns, gene neighborhoods, or intergenic regions ( $p<0.01$ , Wilcoxon one-tailed rank-sum test with continuity correction; Figure 6C). A subset of genes associated with hypomethylated DMRs had increased expression by the RNA-sequencing analysis (Figure S6E). Furthermore, in GMPs significantly hypomethylated regions were enriched in binding sites with homology to Ets family transcription factor Elk4, zinc finger and BTB domain containing transcription factor Zbtb3, and Forkhead class transcription factor FoxP1, implicated as tumor suppressors, while HoxA9 was among notable findings in LSKs (Figure S6D)<sup>26,28</sup>. Gene ontology analysis by biological process applied to DMCs within gene promoters revealed significant enrichment of genes involved in cell adhesion (*CD97*, *Icam2*, *Ppard*, *Cldn4*, *Eng*, *Ninj2*, protocadherins and cadherin-related protein genes), regulation of RNA polymerase II-driven transcription (*Srebfl*, *FoxH1*, *HoxB1*, *Cebpb*, *Hand1*, *Nr4a2*, *Tead2*, *Jak3*), and embryonic development (*HoxB1*, *Cebpb*, *Hand1*, *Hlx*, *HoxA6*, *HoxB6*; Figure 6E). These data suggest that the hypomethylation observed with *Dnmt3a* loss may have a functional role in regulating transcriptional gene states.



## Gene expression profiling identifies reprogramming towards a more primitive developmental state with *Dnmt3a* loss

RNA-sequencing identified 218 upregulated and 98 downregulated genes in *Dnmt3a*-null GMPs (Figure 6D; adjusted  $p < 0.1$ ), including genes implicated in embryonic and cancer stem cell phenotypes *cMyc*<sup>29</sup>, *Lifr*<sup>30</sup>, *Bmx*<sup>31</sup>, *Satb1*<sup>32</sup>, positive and negative regulators of tumor invasion *Adam3*, *Ptk7*<sup>33</sup>, serpin<sup>34</sup> and semaphorin<sup>35</sup> family proteins and ephrin receptor ligands, and networks associated with hematopoietic cell lineage, cell surface receptors, and regulation of immune systems (Figures 6F and S6F, Table S2). Among hypomethylated genes that showed corresponding expression changes, in addition to *Adam3* and *Ptk7*, were *Svep1*, *Hivep2*, *Lrp1*, *Cds1*, *Padi1*, *Zfp618* that regulate cell attachment and amplify cellular signaling responses from cytokines. Gene set enrichment analysis (GSEA) found enrichment of HSC signature genes<sup>36</sup> in *Dnmt3a*-null GMPs consistent with enhanced self-renewal. Comparison with the fetal hematopoiesis gene expression signature<sup>37</sup> (Figure 6G) along with positive enrichment for PRC2-regulated genes and negative enrichment for the ES cell differentiation genes confirmed transcriptional reactivation of early developmental programs after *Dnmt3a* loss. In line with previous reports<sup>6, 38</sup> we found increased expression of *Hox* genes and of their targets in *Dnmt3a*-null GMPs (Figure S6G–H). Increased expression of the cell adhesion molecules is consistent with hypomethylation within their promoters and altered cell tropism of the *Dnmt3a*-ablated bone marrow cells (Figures 6E and S6H). These data indicate that key regulator genes and gene networks are transcriptionally modified by *Dnmt3a* loss.

## Discussion

Our findings emphasize the crucial role of *Dnmt3a* in the pathogenesis of hematologic malignancies, by showing *Dnmt3a* in primary mice gives rise to a fully penetrant, lethal myeloid disease. Although studies of *Dnmt3a*-deficient hematopoiesis in adoptive transfer experiments has produced invaluable insights<sup>14, 16</sup>, the variable impact on disease phenotype may be due to the selective pressure of transplantation, which has been suggested to be necessary to unmask the malignant transformation induced in *Dnmt3a*-deficient HSCs<sup>15</sup>. Our data provide an unequivocal role for *Dnmt3a* in inducing myeloid transformation in primary mice in the absence of transplantation. Our studies and previous studies relied on interferon-activated *Mx1*-Cre excision<sup>14, 16</sup> which may force HSCs to enter cell cycle<sup>39, 40</sup>. Hence an inflammatory component may be an important contributing factor into the pathogenesis of hematopoietic failure syndromes, consistent with clinical observations<sup>41</sup>. *Dnmt3a* loss resulted in expansion of the immature compartment in the bone marrow, including LSK and GMP cells, concomitant with pronounced monocytosis. This pattern of quantitative changes reveals compromised differentiation at several stages, paralleling high-risk subtypes of MDS and a disproportionate association of *DNMT3A*-mutations with monocytic disease observed in the clinic<sup>42, 43</sup>. These findings reinforce the clinical relevance of *Dnmt3a*-deficient hematopoiesis as a faithful animal model of human disease, including MDS/MPN.

*Dnmt3a*-deficient hematopoiesis shares similarities with HSC expansion within the fetal liver, including expansion of CD48<sup>+</sup> LSK ST-HSCs and MPPs and lower relative

frequencies of CD150<sup>+</sup>CD48<sup>-</sup> LSK LT-HSCs, preferential myelopoiesis with lower Gr-1 expression, and changes in erythroid maturation, including a prominent CD41<sup>+</sup> cKit<sup>+</sup> population not found in adult hematopoiesis. Most importantly, deletion of functional *Dnmt3a* leads to robust gain in self-renewal and long-term multilineage reconstituting potential, which is a hallmark of fetal hematopoiesis<sup>13, 37, 44, 45</sup>. We observed enrichment of the HSC and fetal liver gene expression signatures in *Dnmt3a*-null GMPs, along with upregulation of embryonic and cancer stem cell-associated genes. It is tempting to speculate that these phenotypic changes arise due to partial reprogramming towards developmentally more primitive state by impaired epigenetic control following *Dnmt3a* loss. Further studies examining expression of fetal stem/progenitor markers and transcriptomic switches will be necessary to address the potential role of *Dnmt3a* function in hematopoietic development.

The most striking finding in *Dnmt3a*-deleted myeloproliferation/myelodysplasia is the hepatomegaly due to EMH and liver-specific myeloproliferation. The ability of adult hematopoietic cells to home, colonize, and mature in the liver reflects primitive developmental patterns of hematopoiesis and re-establishment of a stem cell niche<sup>46</sup>. Reminiscent of fetal hematopoiesis, hepatic EMH in *Dnmt3a*-null animals was predominantly localized within sinusoids. The mechanism of homing to the liver is distinct from other organs, as the lower blood flow and fenestrated endothelium in the sinusoids do not require selectin-mediated rolling for leukocyte recruitment, suggesting other adhesion mechanisms play a prominent role<sup>47</sup>. Gene expression analysis of *Dnmt3a*-deleted GMPs identified enrichment of a cell adhesion signature that may account for the enhanced liver tropism. At the same time, elevated expression of the extracellular matrix degradation and tissue invasion genes may contribute to cell mobilization from the bone marrow to the liver<sup>48, 49</sup>, facilitated through epigenetic changes after *Dnmt3a* loss targeting genomic loci with regulatory potential, including Ets binding sites<sup>50</sup>. These observations may have important implications for the treatment of *DNMT3A*-mutated MDS in humans as EMH in the liver may serve as a safe haven environment and a reservoir for the disease-initiating clone. Future interrogation of the specific cell adhesion determinants will help clarify molecular mechanisms of hepatotropism of *Dnmt3a*-ablated hematopoietic cells.

Taken together, *Dnmt3a* inactivation in primary animals leads to the development of a myeloid malignancy with features of human MDS/MPN, highlighting the relevance of this model to chronic myeloid neoplasms. We speculate that increased epigenetic plasticity allows reactivation of developmental gene expression programs. Subsequent studies are needed to explore the role for concomitant disease alleles, and to determine if therapies can be developed to prevent and/or reverse the myeloid transformation induced by *Dnmt3a* loss.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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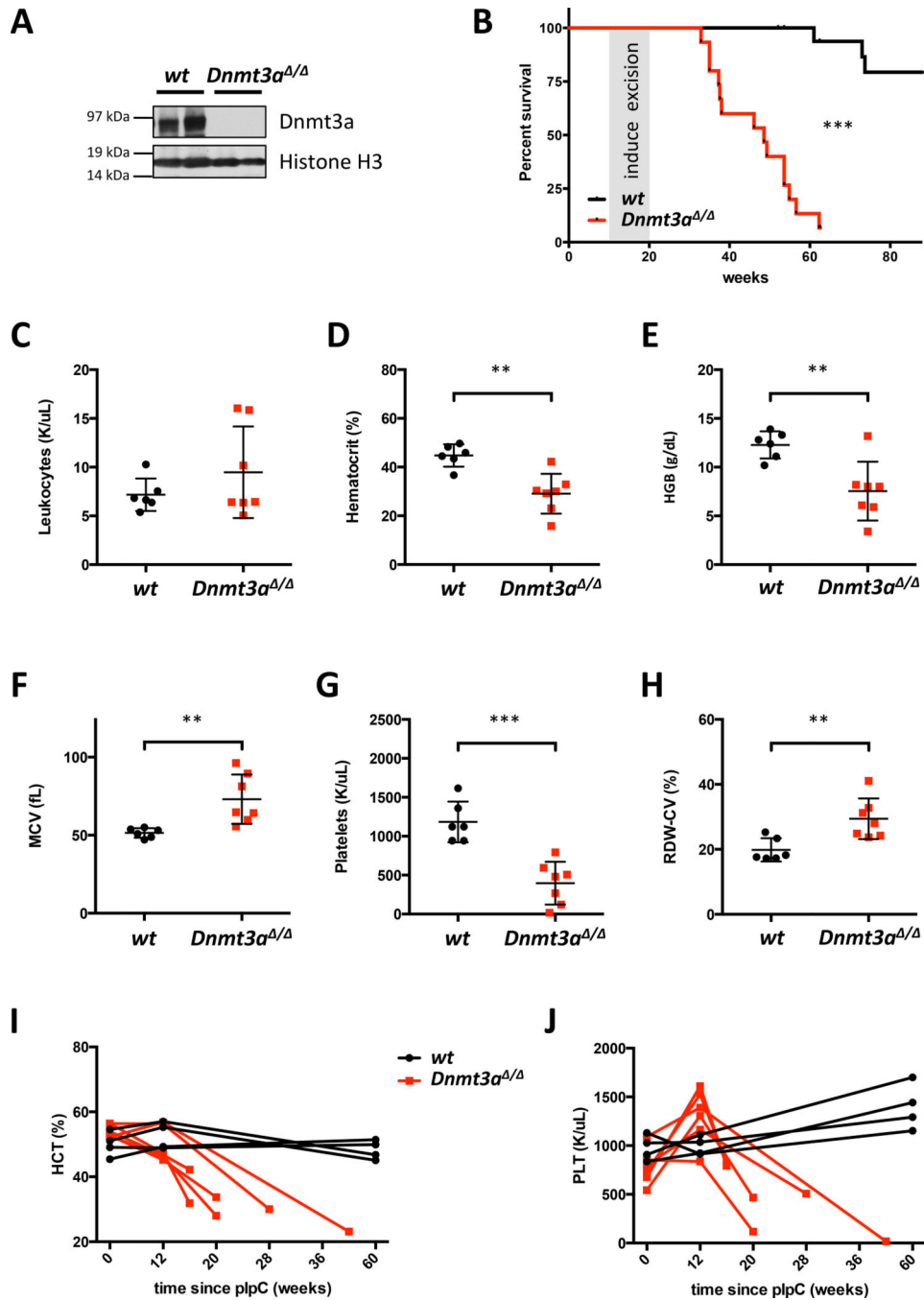
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**Figure 1. Primary *Dnmt3a* conditional knock-out mice have decreased survival and develop peripheral blood cytopenias**

A. *Dnmt3a* protein levels after excision in the spleens of *Dnmt3a<sup>fl/f</sup>:Mx1-Cre<sup>+</sup>* and control mice.

B. Survival of *Dnmt3a* KO ( $n=15$ ) and control ( $n=18$ ) mice. *Cre*-mediated excision was induced by poly(I:C) injections (please see Supplementary Methods). Animals sacrificed as controls for diseased *Dnmt3a* KO mice were censored. Reasons for euthanasia in 2 control animals were rectal prolapse and severe bite wounds due to fighting; cause of death in 1 mouse was undetermined. None of the control mice exhibited signs of hematologic disease.

C. White blood cell counts at disease onset in *Dnmt3a*-ablated mice ( $n=6-7$ ).

D–H. *Dnmt3a*-deleted mice develop macrocytic anemia ( $n=6-7$ ) with decreased hematocrit (D) and hemoglobin levels (E), increased mean corpuscular volume (F), thrombocytopenia (G), and anisocytosis (H) (\*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ ).

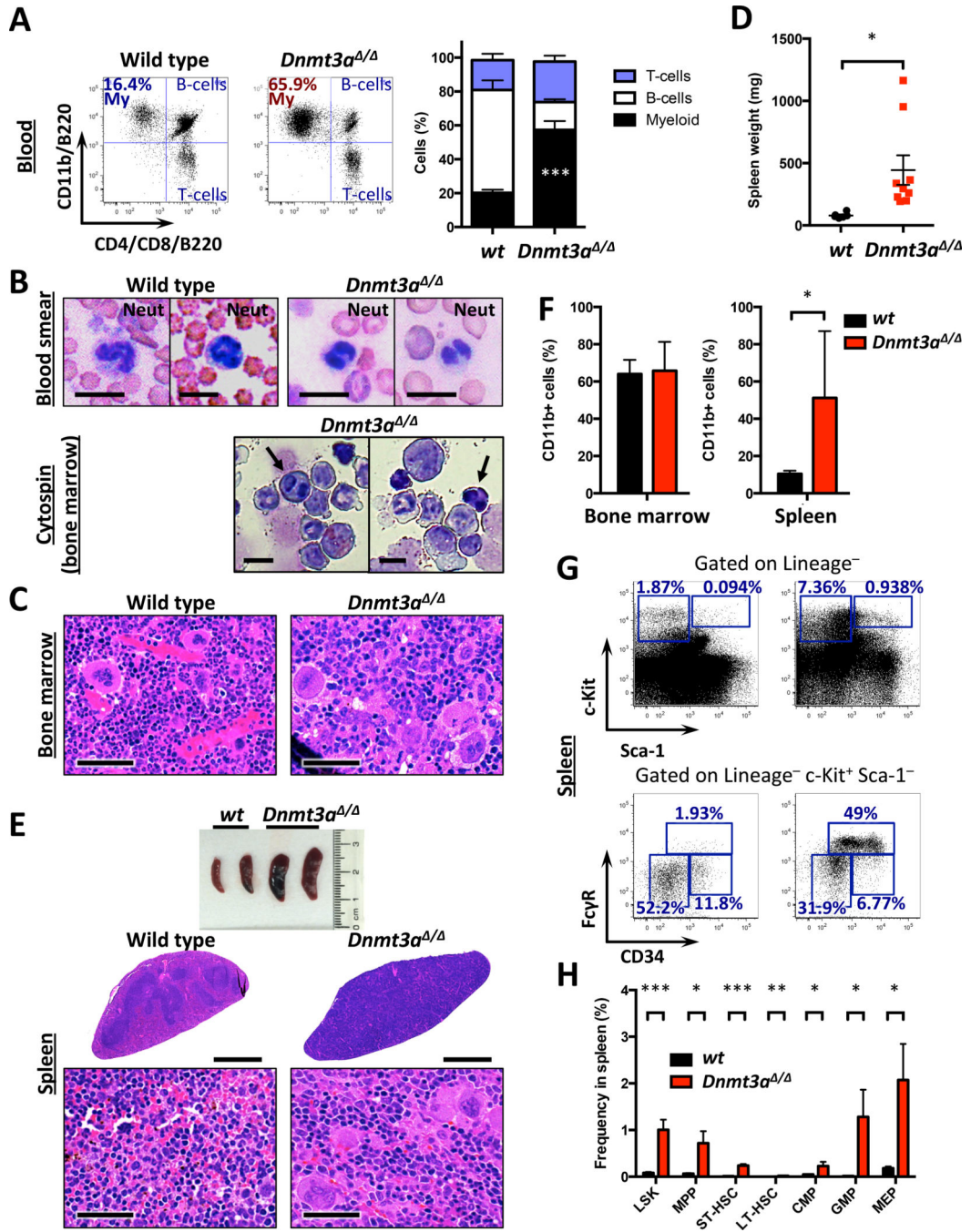
I–J. Hematocrit (I) and platelet counts (J) over time in 6 *Dnmt3a* KO and 2 representative control mice.

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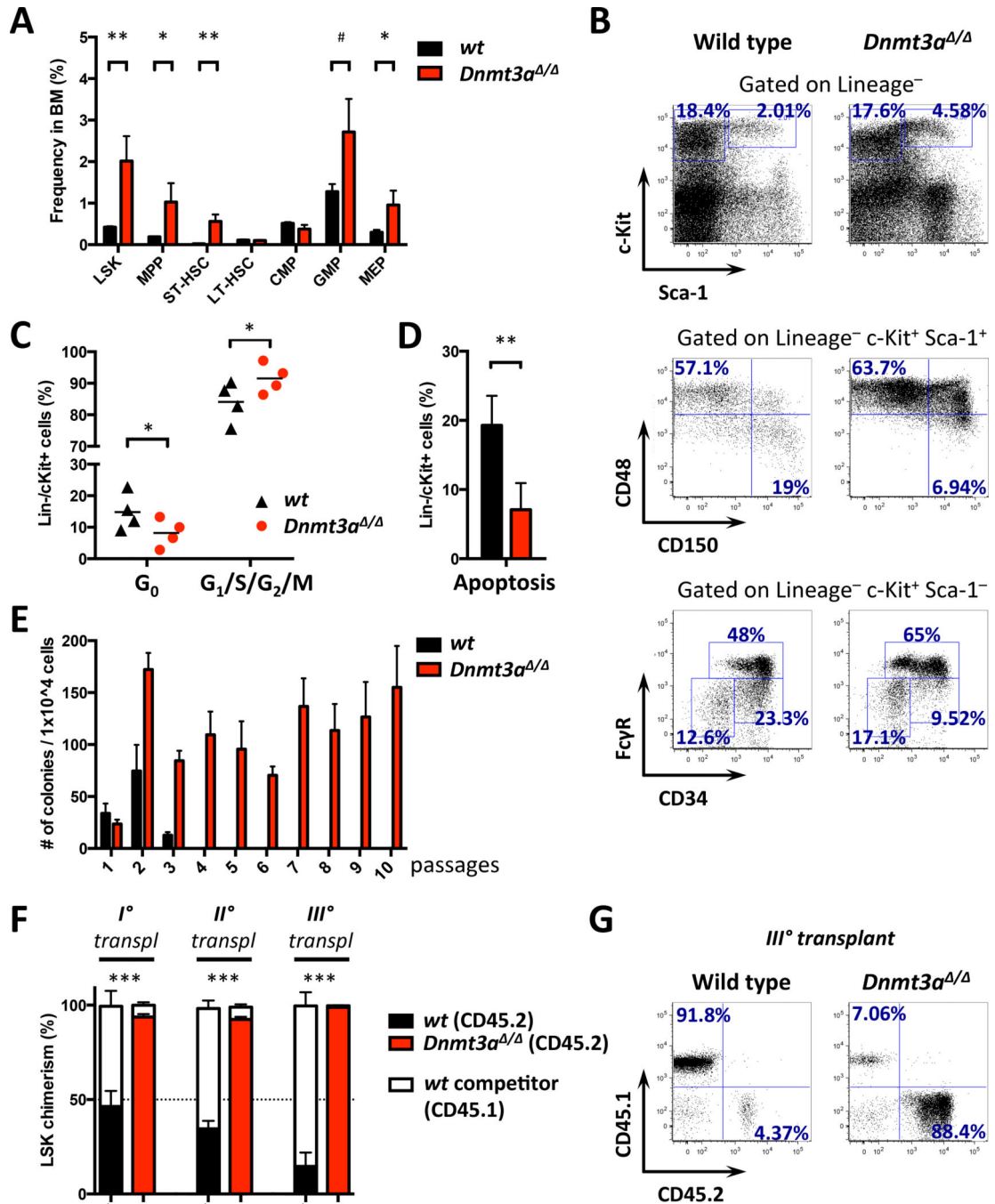
**Figure 2. *Dnmt3a*-deleted mice develop myelodysplasia/myeloproliferation with extramedullary hematopoiesis**

A. Myeloid bias in peripheral blood of *Dnmt3a*-null mice ( $n=7-8$ ; \*\*\*,  $p<0.001$ ).

B. Peripheral blood smears and bone marrow cytopsin preparations showing dysplasia of myeloid and erythroid lineages in *Dnmt3a*-null but not control mice, manifesting as nuclear hyposegmentation reminiscent of pseudo-Pelger-Huet neutrophils classically noted in human MDS and nucleated red blood cells (arrows) with dyserythropoiesis characterized by irregular nuclear contours. Bar – 10  $\mu$ m.



- C. Representative H/E-stained sections of control and *Dnmt3a* KO sterna. Arrows – megakaryocyte dysplasia. Bar – 50  $\mu$ m.
- D. Spleens weights in *Dnmt3a* KO and control mice at disease onset ( $n=6-9$ ; \*,  $p<0.05$ ).
- E. Gross pathology and H/E-stained sections of representative control and *Dnmt3a*-null spleens. Arrows – megakaryocytic infiltration. Bar – 1000  $\mu$ m (low magnification), and 50  $\mu$ m (high magnification).
- F. Quantification of the CD11b<sup>+</sup> cells shows elevated levels of myeloid cells in spleens of *Dnmt3a*-deleted mice at time of disease onset ( $n=5-6$ ; \*,  $p<0.05$ ).
- G. Representative FACS plots of immature stem and progenitor cells in the spleens of *Dnmt3a*-ablated and control mice.
- H. Quantification of data presented in G ( $n=4-6$ ; \*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ ).



**Figure 3. Conditional deletion of *Dnmt3a* results in perturbation of the hematopoietic stem and progenitor compartment and gain of self-renewal potential**

A. Relative stem and progenitor cell frequencies in *Dnmt3a* KO and control bone marrow at disease onset ( $n=4-6$ ; #,  $p=0.065$ ; \*,  $p<0.05$ ; \*\*,  $p<0.01$ ).

B. Representative FACS plots for data in A.

C–D. Increased proliferation and decreased quiescence (C) and decreased apoptosis (D) in *Dnmt3a*-deleted compared to wild-type HSPCs in animals competitively transplanted with bone marrow cells derived from *Dnmt3a*-null (CD45.2) and wild-type (CD45.1) mice.

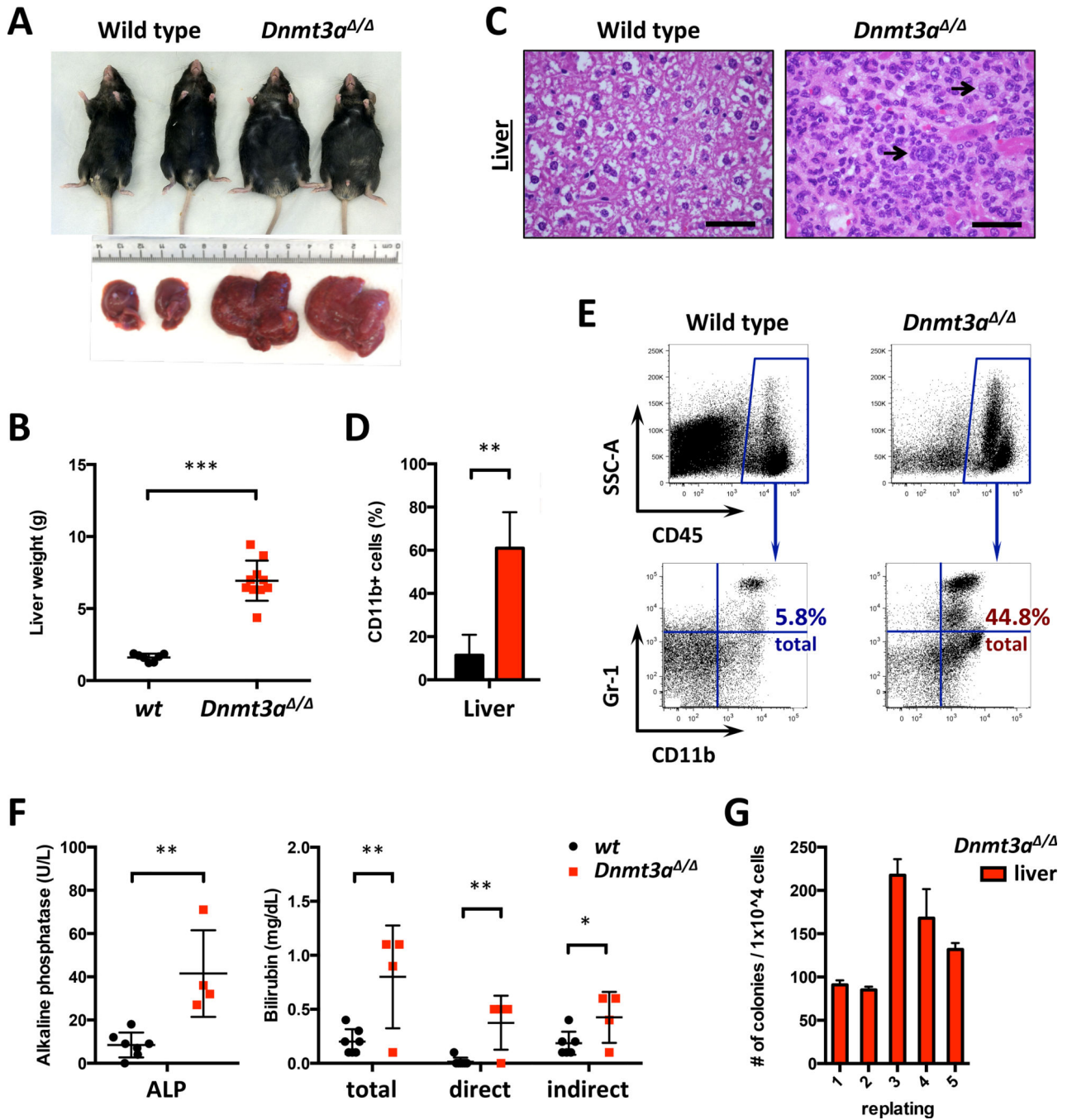
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Analysis was performed in 4 transplant recipients 9 months after transplantation (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

E. CFU assay on bone marrow cells from *Dnmt3a*-null or control littermates. Representative results of 3 independent experiments, each performed in triplicate.

F. CD45.1/CD45.2 chimerism in bone marrow LSK cells over three rounds of serial competitive transplantation. Test cells (CD45.2, red bars – *Dnmt3a* KO, black bars – WT) were competed against wild-type (CD45.1, white bars) and analyzed 16 weeks after transplantation ( $n=5$ ; \*\*\*,  $p < 0.001$ ). Dotted line represents CD45.1/CD45.2 cell ratio at time of injection (50%).

G. CD45.1/CD45.2 peripheral blood chimerism in tertiary recipients 16 weeks after transplantation in D. Representative FACS plots.



**Figure 4. Hepatomegaly due to myeloproliferation and monocytic infiltration in conditional *Dnmt3a* knock-out mice**

A. Gross pathology of livers and overall appearance of control and diseased *Dnmt3a*-deleted mice, representative of all animals studied.

B. Liver weights in *Dnmt3a*-KO and WT mice at disease onset ( $n=7-10$ ; \*\*\*  $p<0.001$ ).

C. H/E-stained liver sections; arrows – scattered megakaryocytes in *Dnmt3a*-ablated but not control mice. Bar – 50  $\mu$ m.

D. Quantification of CD11b<sup>+</sup> cells in livers of control and diseased *Dnmt3a* KO mice ( $n=3-6$ ; \*\*,  $p<0.01$ ).

E. Representative FACS plots for data presented in (D).

F. Serum alkaline phosphatase (ALP) and bilirubin levels in control and diseased *Dnmt3a*-deleted mice ( $n=4-7$ ; \*,  $p<0.05$ ; \*\*,  $p<0.01$ ).

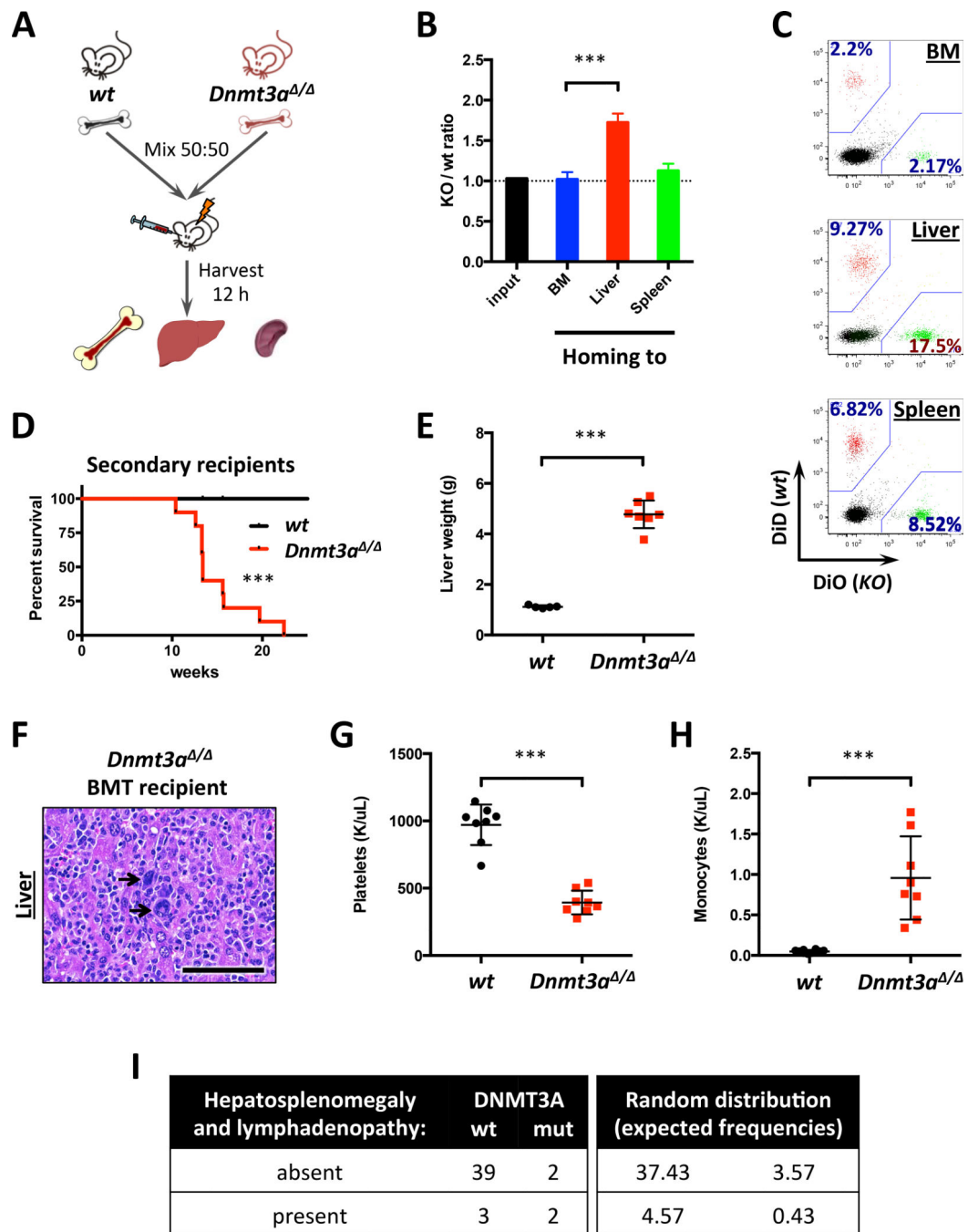
G. Colony-forming assay using liver cells from diseased *Dnmt3a* knock-out mouse, single experiment performed in triplicate.

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**Figure 5. Conditional loss of *Dnmt3a* results in cell-autonomous liver tropism**

A. Schematic depiction of the homing assay workflow.

B. Relative homing tropism calculated as *Dnmt3a* KO/wt ratio in the bone marrow, livers and spleens of recipient mice ( $n=4$ ; \*\*\*,  $p<0.001$ ). Dotted line represents ratio at time of injection (1.0).

C. Representative FACS plots showing distribution of fluorescently labeled WT and *Dnmt3a* KO cells homed to the indicated sites, gated on  $CD45^+$ .

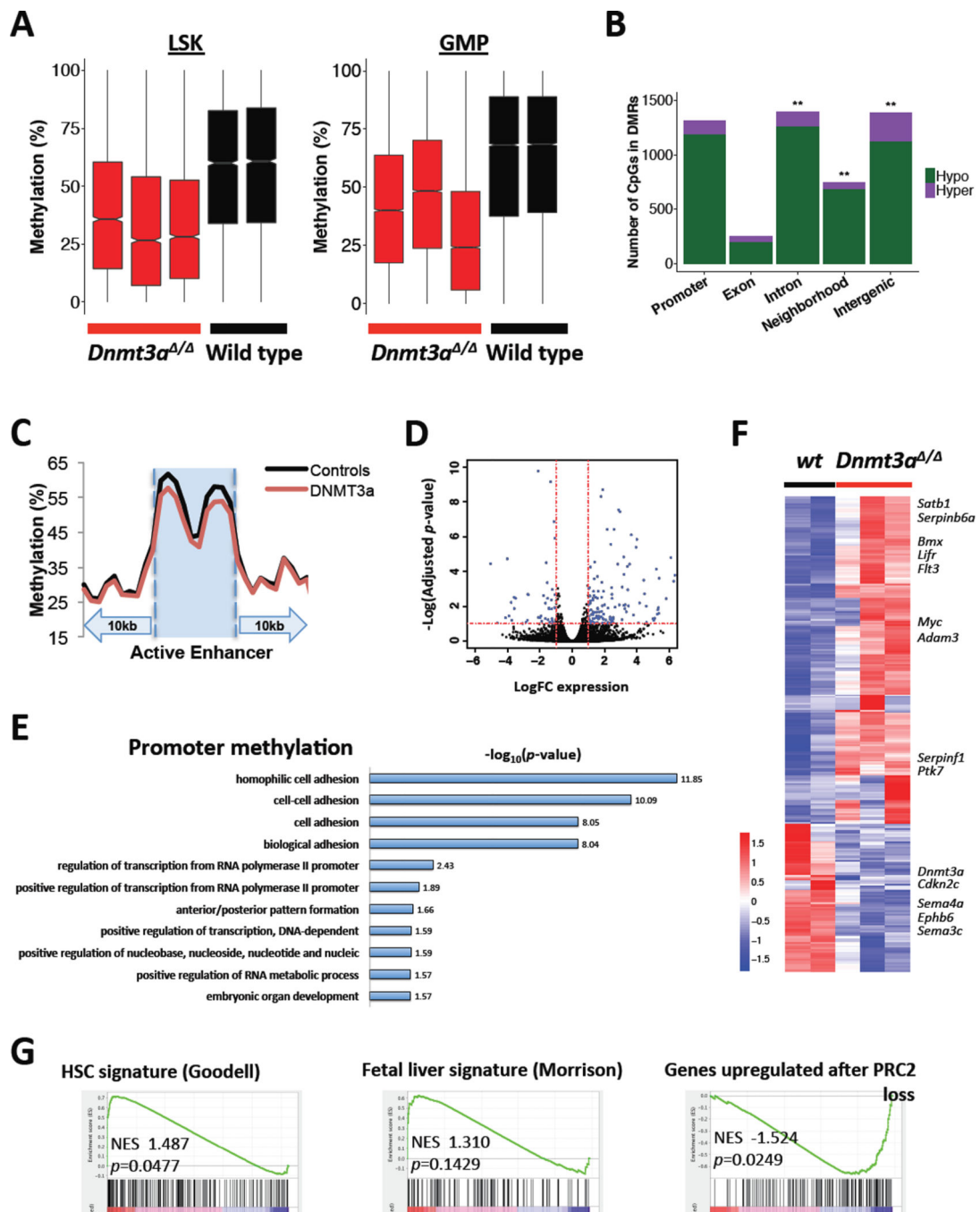


D. Survival of lethally-irradiated recipient mice transplanted with total bone marrow from diseased *Dnmt3a*-null or control animals ( $n=10$ ; \*\*\*,  $p=0.0003$  by Gehan-Breslow-Wilcoxon test).

E–F. Liver weights (E;  $n=5–7$ ; \*\*\*,  $p<0.001$ ) and H/E-stained sections (F) in primary recipients transplanted with *Dnmt3a*-ablated bone marrow cells at time of disease onset and in control wild-type mice. Arrows – megakaryocytes indicative of EMH.

G–H. Platelet (G) and monocyte (H) counts in the peripheral blood of mice transplanted with *Dnmt3a*-null bone marrow ( $n=8$ ; \*\*\*,  $p<0.001$ ).

I. Increased frequency of extramedullary hematopoiesis in CMML patients with *DNMT3A* mutations. Left panel represents observed frequencies of hepatosplenomegaly and lymphadenopathy in patients with different *DNMT3A* (wt or mutant) status ( $n=46$ ); right panel shows expected frequencies for random distribution ( $p=0.053$ ,  $\Phi=+0.39$ , 2-tailed Fisher's exact test).



**Figure 6. ERRBS DNA methylation and gene expression profiling identifies genomic features dysregulated by *Dnmt3a* loss**

A. *Dnmt3a*-null LSK and GMP cells show overall methylation loss relative to wild-type in differentially methylated regions (DMRs).

B. CpGs contained in DMRs are enriched within introns, gene neighborhoods, and intergenic regions. CpGs with lower %methylation in *Dnmt3a*-null GMP cells compared to wild-type are defined as hypomethylated, and those with greater methylation in *Dnmt3a*-null GMP cells as hypermethylated (\*\*, genomic compartments enriched for DMR-contained CpGs relative to other regions of the genome, Fisher's exact test, one-tailed,  $p<0.01$ ).

C. *Dnmt3a*-null GMPs show methylation loss at GMP active enhancers relative to wild-type. GMP active enhancers  $\pm 10$ kb were divided into 30 bins and the average methylation value in each was plotted.

D. Differential expression of genes covered by RNA-sequencing shows preferential upregulation of a subset of genes in *Dnmt3a*-null GMPs compared to wild-type controls. Dotted lines represent significance cutoffs set at  $\pm 1$  Log(FC expression) (vertical) or  $-1$  Log(adjusted *p*-value) (horizontal).

E. List of gene ontology terms by DAVID functional annotation tool derived from DMCs mapping to gene promoters in GMP cells from *Dnmt3a*-null and control animals.

F. Heatmap of differentially expressed genes (adjusted  $p < 0.1$ ) based on RNA-sequencing of GMPs derived from *Dnmt3a*-null and wild-type control animals, z-score scale.

G. GSEA plots showing enrichment of the HSC gene signature<sup>36</sup>, modified fetal liver HSC signature<sup>37</sup>, and PRC2\_EED\_UP.V1\_UP<sup>51</sup>, in *Dnmt3a*-null GMPs compared to wild-type controls.