Loss of Dnmt3a Immortalizes Hematopoietic Stem Cells In Vivo

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DATA AND SOFTWARE AVAILABILITY

The accession number for the raw and processed whole-genome bisulfite sequencing (WGBS), histone modification, and RNA sequencing data reported in this paper is GEO: GSE98191. UCSC Genome Browser tracks (mouse mm9) can be accessed from the hub http://lilab.research.bcm.edu/dldcc-web/lilab/hjpark/multipleTransplants/hub.txt. The accession number for the exome sequencing data reported in this paper is SRA: SRP133364.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.025.

DECLARATION OF INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

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SUMMARY
Somatic mutations in DNMT3A are recurrent events across a range of blood cancers. Dnmt3a loss of function in hematopoietic stem cells (HSCs) skews divisions toward self-renewal at the expense of differentiation. Moreover, DNMT3A mutations can be detected in the blood of aging individuals, indicating that mutant cells outcompete normal HSCs over time. It is important to understand how these mutations provide a competitive advantage to HSCs. Here we show that Dnmt3a-null HSCs can regenerate over at least 12 transplant generations in mice, far exceeding the lifespan of normal HSCs. Molecular characterization reveals that this in vivo immortalization is associated with gradual and focal losses of DNA methylation at key regulatory regions associated with self-renewal genes, producing a highly stereotypical HSC phenotype in which epigenetic features are further buttressed. These findings lend insight into the preponderance of DNMT3A mutations in clonal hematopoiesis and the persistence of mutant clones after chemotherapy.

In Brief
Jeong et al. show that a single genetic manipulation, conditional inactivation of the DNA methyltransferase enzyme Dnmt3a, removes all inherent hematopoietic stem cell (HSC) self-renewal limits and replicative lifespan. Deletion of Dnmt3a allows HSCs to be propagated indefinitely in vivo.

INTRODUCTION
Embryonic stem cells (ESCs) can be propagated indefinitely in vitro while maintaining their defining stem cell properties of self-renewal and differentiation. However, self-renewal of somatic stem cells such as hematopoietic stem cells (HSCs) appears to have a limit, as serial transplantation invariably results in loss of repopulation ability (Micklem et al., 1987; Siminovitch et al., 1964; Harrison and Astle, 1982). Understanding these limitations is
important for dissecting stem cell regulation and developing strategies to expand HSCs ex vivo for cell and gene therapy applications.

We previously showed that genetic inactivation of de novo DNA methyltransferase 3a (Dnmt3a) enhances self-renewal of murine HSCs (Challen et al., 2011, 2014). In humans, mutations in DNMT3A have been associated with clonal hematopoiesis of indeterminate potential (CHIP) in aging individuals (Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014). DNMT3A mutations in CHIP typically result in loss of activity through divergent mechanisms (Kim et al., 2013; Russler-Germain et al., 2014; Spencer et al., 2017), which probably confers enhanced self-renewal and enables them to slowly outcompete their normal counterparts over a long timescale.

Although loss of Dnmt3a promotes self-renewal, the degree of enhancement is undefined. Given that DNMT3A mutations are frequent in hematologic malignancies (Yang et al., 2015), are associated with a pre-malignant state (Shlush et al., 2014; Corces-Zimmerman et al., 2014), and can repopulate after chemotherapy (Pløen et al., 2014), it is critical to understand the mechanisms of resilience and longevity of DNMT3A mutant HSCs. Here we rigorously examine the replicative limits of HSCs lacking Dnmt3a.

RESULTS

Loss of Dnmt3a Provides HSCs with Indefinite Longevity

We previously showed that Dnmt3a-null (Mx1-Cre:Dnmt3a<sup>fl/fl</sup>) HSCs treated with pIpC = Dnmt3a<sup>KO</sup> HSCs could self-renew for up to four rounds of serial transplantation (Challen et al., 2011). We terminated these experiments after four transplants because control HSCs failed to self-renew past this point. However, as Dnmt3a<sup>KO</sup> HSCs continued to show robust HSC repopulation, we hypothesized that Dnmt3a loss of function may remove inherent constraints on HSC self-renewal and longevity. Here, we tested these limits. Phenotypic HSCs (Lineage<sup>−</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>CD48<sup>−</sup>CD150<sup>+</sup>CD45.2<sup>+</sup>) were purified from prior recipients (CD45.1<sup>+</sup>) using flow cytometry. Two hundred HSCs were re-injected along with fresh whole bone marrow (WBM) competitor cells (CD45.1<sup>+</sup>) into new recipients (Figure 1A). Eighteen to 24 weeks later, recipients were sacrificed for analysis and continued HSC transplantation. After each transplant round, donor-derived (CD45.2<sup>+</sup>) HSCs were quantified (Figure 1B). After the third transplant, Dnmt3a<sup>KO</sup> HSCs failed to generate substantial peripheral blood progeny (Figures 1C and 1D). Nevertheless, robust repopulation of Dnmt3a<sup>KO</sup> HSCs was readily detectable in the bone marrow of recipient mice over 12 rounds of transplantation (Figure 1D). These HSCs displayed all the canonical markers of long-term HSCs (Figure S1A), and the expanded population was highly restricted to the HSC pool (Figure S1B). This degree of self-renewal far exceeds the potential of normal HSCs.

When Dnmt3a<sup>KO</sup> HSCs are forced to differentiate in the absence of WBM competitor cells, a variety of hematopoietic pathologies arise (Celik et al., 2015; Mayle et al., 2015). To determine whether the immortalized HSCs were transformed, we transplanted 1 × 10<sup>5</sup> ninth-generation transplant (Tx-9) Dnmt3a<sup>KO</sup> HSCs without competitor WBM. These animals succumbed to bone marrow failure (Figure 1E) with anemia and peripheral cytopenias.
Figure 1F), likely resulting from the inability of Dnmt3aKO HSCs to generate sufficient blood elements to sustain the recipients in the absence of wild-type (WT) support cells. Bone marrow histology (data not shown) showed no evidence of leukemic transformation. Expanded Dnmt3aKO HSCs were not mobilized in the blood (Figure S1C), and no extramedullary hematopoiesis was observed in the spleen (Figure S1D). Mice transplanted with control or Dnmt3aKO HSCs contained similar numbers of stromal cells (Figure S1E) and appropriate bone marrow localization (Figure S1F). Dnmt3aKO HSCs showed similar proximity to endothelial cells as control HSCs (Figure S1G) but were found to be closer to neighboring HSCs (Figure S1H), as would be expected from their clonal expansion.

**Dnmt3a Controls DNA Methylation at HSC Regulatory Elements**

We performed molecular comparisons of age-matched control and early-stage transplant Dnmt3aKO HSCs with late-stage transplant Dnmt3aKO HSCs. Global DNA methylation analysis (Figure S2A; Table S1) showed that Tx-11 Dnmt3aKO HSCs retained their overall methylation profile, with the majority of CpGs still methylated throughout the genome, but displayed DNA hypomethylation compared with control HSCs. This pattern was similar to, but more exaggerated than, the depletion of DNA methylation in early-stage transplant (Tx-3) Dnmt3aKO HSCs (Figure 2A).

Differentially methylated regions (DMRs) were defined as more than three CpGs within 300 bp that show >20% methylation change in the same direction. Of the genomic regions showing differential methylation both in Tx-3 Dnmt3aKO (to age-matched WT) and Tx-11 Dnmt3aKO (versus Tx-3) HSCs, 556 regions hypermethylated in Tx-3 were equally as likely to gain (297) or lose (259) DNA methylation in Tx-11 Dnmt3aKO HSCs (Figure 2B), suggesting that this hypermethylation was not stable. Conversely, 4,313 of 4,986 regions (86.5%) of the genome that lost DNA methylation in early-passage Dnmt3aKO HSCs (hypo-DMRs) showed a trend toward continued loss of methylation in later stage transplant Dnmt3aKO HSCs. There was significant enrichment for these “hypo_hypo” DMRs in stem cell enhancer elements (Figure 2C), but not CpG islands or gene promoters. “Hypo_hypo” DMRs were also enriched for transcription factor binding sites (TFBSs; Figure S2B), including hematopoietic regulators such as Gata2 (Figure S2C). This enrichment was not due to the difference in DMR numbers, as 100 random computational samplings of 600 “hypo_hypo” and “hypo_hyper” DMRs showed the same trend (Figure S2D).

Because loss of DNA methylation in Dnmt3aKO HSCs is particularly concentrated in DNA methylation canyons (Jeong et al., 2014), we examined canyons in late-passage HSCs. There are 1,093 canyons in WT HSCs (Jeong et al., 2014), which can be subdivided on the basis of histone marks into 565 active (H3K4me3+), 205 bivalent (H3K4me3+ H3K27me3+), and 323 inactive (H3K27me3+) canyons. Targeted loss of DNA methylation at active canyon walls was previously noted in early-passage Dnmt3aKO HSCs (Jeong et al., 2014). In Tx-11 Dnmt3aKO HSCs, there was further erosion of these walls (Figure 2D), and hypomethylation extended from the canyon edges (Figure S2E). In contrast, bivalent and inactive canyons displayed increased DNA methylation in Tx11 Dnmt3aKO HSCs (Figure 2E) and no changes in the canyon border region (Figure S2E). Hypermethylation of inactive canyons did not lead to altered gene expression, as these genes are typically expressed at negligible levels (Figure
However, genes in bivalent canyons, exemplified by Cxcl12 (Figure 2E), showed repression following hypermethylation with extended passage. As many genes contained in such canyons are important for HSC lineage commitment, this hypermethylation may be a mechanism that inhibits differentiation of the mutant HSCs.

RNA sequencing (RNA-seq) was performed to determine the impact of DNA methylation changes on gene expression. In general, genes that were differentially expressed between control and Tx-3 Dnmt3aKO HSCs showed similar expression patterns in Tx-9 Dnmt3aKO HSCs (Figure S2F). We noted previously that early-passage Dnmt3aKO HSCs exhibited increased expression of genes associated with HSC identity (Challen et al., 2011), defined as “HSC fingerprint” genes (Chambers et al., 2007). This trend continued in Tx-9 Dnmt3aKO HSCs. Although the majority of HSC fingerprint genes are upregulated in mutant HSCs, exceptions include several imprinted genes (Figure S2G) such as Ndn, Gtl2, and Peg3 implicated in stem cell function (Kubota et al., 2009; Qian et al., 2016; Berg et al., 2011). In summary, lack of Dnmt3a over serial passage stabilizes the self-renewing epigenome and leads to an inability to silence genes associated with maintenance of HSC identity.

**Differentiation Capacity Is Lost but Transformation Potential Is Retained in Immortalized Dnmt3aKO HSCs**

We rescued late-passage Dnmt3aKO HSCs with enforced expression of Dnmt3a to determine if differentiation capacity could be restored. Tx-11 Dnmt3aKO HSCs were transduced with a lentivirus expressing full-length Dnmt3a (with bicistronic GFP) and transplanted. Re-expression of Dnmt3a led to the emergence of GFP+ cells in the peripheral blood at 4 weeks post-transplant (Figures S3A and S3B), which was not observed from transduction of the same HSCs with the control lentivirus. But ultimately, this initial output was not sustained (Figure S3C). However, ectopic expression of Dnmt3a did abrogate clonal expansion of the mutant HSCs in the bone marrow (Figure 3A). Although the total HSC frequency was identical in recipients of Dnmt3aKO HSCs transduced with control or Dnmt3a-expressing lentivirus, GFP+ HSCs were severely depleted when expression of Dnmt3a was restored (Figure 3B). Re-expression of Dnmt3a induced proliferation (Figures 3C and S3D) and increased apoptosis (Figures 3D and S3E), which was associated with upregulation of the pro-apoptotic genes Bbc3 (puma) and Bax (Figure 3E).

To evaluate differentiation capacity without cellular competition, myeloid potential of GFP+ Dnmt3aKO HSCs was quantified by colony-forming assay. Myeloid potential of Tx-12 Dnmt3aKO HSCs was severely compromised, and their differentiation was not rescued by complementation with Dnmt3a-expressing lentivirus (Figure 3F). Moreover, the colonies that were produced by late-passage Dnmt3aKO HSCs were predominantly uni-lineage (Figure 3F). Similarly, re-expression of Dnmt3a was not able to restore T cell potential on OP9-DL1 co-culture (Schmitt and Zúñiga-Pflücker, 2002). Although Dnmt3a expression increased CD4+ T cell production, this output was marginal compared with control and early-passage Dnmt3aKO HSCs (Figure S3F). But analysis of the double-negative (DN; CD4− CD8a−) population did show development through the early stages of T cell maturation. In fact, re-expression of Dnmt3a generated an abnormal CD25bright DN2 cell...
population (Figure S3G), consistent with our observation that Dnmt3a is necessary for developmental progression of T cell progenitors (Kramer et al., 2017).

We then considered whether differentiation could bypass Dnmt3a. Ebf1 is a master regulator of B cell potential (Lin and Grosschedl, 1995) located in a canyon that becomes hypermethylated with extended passage of Dnmt3a KO HSCs. Tx-12 Dnmt3a KO HSCs were transduced with control, Dnmt3a-expressing, or Ebf1-expressing lentivirus and co-cultured on OP9 stromal cells for 14 days. The B cell (B220+ CD19+) differentiation deficit in early-passage Dnmt3a KO HSCs was restored by expression of Dnmt3a, and like control HSCs, B cell output of early-passage Dnmt3a KO HSCs was enhanced by overexpression of Ebf1 (Figure 3G). In contrast, overexpression of neither Dnmt3a nor Ebf1 was able to restore any B cell potential to late-passage Dnmt3a KO HSCs (Figure 3G).

If differentiation potential was completely silenced, perhaps immortalized Dnmt3a KO HSCs would be incapable of malignant transformation, given that some differentiation is required for generation of acute myeloid leukemia (AML) (Ye et al., 2015). Tx-12 Dnmt3a KO HSCs were transduced with a lentivirus expressing KrasG12D, a common mutation co-occurring with DNMT3A mutations in AML (Ley et al., 2013), and transplanted. Once GFP+ cells began emerging in the blood, mice rapidly succumbed (Figure 3H) to a fully penetrant AML with a c-Kit+ CD11b+ phenotype (Figure 3I). Thus, although differentiation was irreversibly blocked in immortalized Dnmt3a KO HSCs, they retained the potential for malignant transformation when presented with an appropriate co-operating mutation.

Molecular Analysis of Dnmt3a Rescue, Dominant-Negative Mutant, and Clonal Hematopoiesis

DNA methylation was compared between Tx-12 Dnmt3a KO HSCs transduced with either control or Dnmt3a-expressing lentivirus. Of 361 genomic regions hypomethylated in Tx-11 Dnmt3a KO HSCs versus Tx-3 Dnmt3a KO HSCs, 280 (77.6%) became hypermethylated in Tx-12 Dnmt3a KO HSCs following re-expression of Dnmt3a, indicating that DNA methylation was re-established at the correct regions, including canyon boundaries (Figure 4A) and enhancers (Figure 4B). The re-establishment of DNA methylation patterns did not necessarily correlate with corresponding gene expression differences (Figure S4A), but perhaps this would normalize with further re-expression of Dnmt3a (Figure S4B).

We also compared the DNA methylation changes in Dnmt3a KO HSCs with those in the context of mutations in patients. The most prevalent DNMT3A mutation is a missense at amino acid 882 (Ley et al., 2010). This mutation creates a dominant-negative protein with reduced DNA methyltransferase capacity (Russler-Germain et al., 2014; Kim et al., 2013). Global DNA methylation was performed on Tx-3 Dnmt3aR878H/+ HSCs (Dnmt3aR878, mouse homolog of human DNMT3A R882H) (Guryanova et al., 2016) and compared with Tx-3 Dnmt3a KO HSCs. Tx-3 Dnmt3aR878 HSCs displayed a self-renewal advantage over control HSCs, but not to the same degree as Dnmt3a KO HSCs (Figure S4C). On a global scale, Dnmt3aR878 showed hypomethylation throughout the genome, although not to the same degree as Dnmt3a KO HSCs (Figure 4C). There was high correlation between the regions affected by changes in DNA methylation in Dnmt3aR878 and Dnmt3a KO HSCs. Of the DMRs shared between Dnmt3aR878 and Dnmt3a KO HSCs compared with WT, 95.0%
showed hypermethylation in both mutant genotypes, while 95.6% (6,742 of 7,032) underwent hypomethylation in both mutant HSC genotypes. Of the 12,644 hypo-DMRs in Dnmt3a<sup>R878</sup> HSCs compared with WT, 6,724 (53.2%) are hypo-DMRs in Dnmt3a<sup>KO</sup> HSCs at the exact same genomic co-ordinates, with this fraction increasing if the genome windows are extended. Although overall methylation patterns were highly overlapping between the Dnmt3a mutant HSCs, there were distinct focal changes. For example, Dnmt3a<sup>R878</sup> HSCs did not exhibit the hypermethylation of enhancer elements of differentiation factors such as Pax5 and Bcl11b (Figure S4D). Similarly, the HoxB gene cluster, which becomes significantly hypomethylated in Dnmt3a<sup>KO</sup> HSCs, did not show the same loss of DNA methylation in Dnmt3a<sup>R878</sup> HSCs (Figure 4E) and corresponding transcriptional upregulation (Figure 4F). A CpG island in the Gata3 promoter is particularly sensitive to Dnmt3a loss of function (Challen et al., 2011), but DNA methylation remained intact for this region in Dnmt3a<sup>R878</sup> HSCs (Figure 4G), and gene expression was unchanged (Figure 4H). These findings suggest that Dnmt3a<sup>KO</sup> and Dnmt3a<sup>R878H</sup> mutations in HSCs result in similar overall DNA methylation changes, but methylation differences at specific stem cell enhancer elements may be important for the differentiation defects in the loss-of-function model.

The expansion of Dnmt3a<sup>KO</sup> HSCs over extended passage was reminiscent of human CHIP, which can persist in a benign state for decades. To determine if this HSC expansion was associated with acquisition of co-operating mutations, exome sequencing was performed. The overall number of somatic variants was reduced in Tx-12 Dnmt3a<sup>KO</sup> HSCs compared with early-passage HSCs (Figure 4I), but the variant allele fraction (VAF) of these variants was higher (Figure 4J), indicating the transplanted HSC pool was becoming more homogeneous over time. A reduced number of variants is consistent with reduced clonal complexity, while an increase in the VAF of individual variants (Tx-3 Dnmt3a<sup>KO</sup> HSCs = 0.2400, Tx-12 Dnmt3a<sup>KO</sup> HSCs = 0.4381) is consistent with heterozygous polymorphisms originating from dominant clones. However, analysis of the high-confidence somatic variants did not identify any acquired mutations which have been associated with human CHIP (Table S4).

As Dnmt3a has been suggested to regulate telomeres (Gonzalo et al., 2006) and telomere shortening limits HSC transplantability (Allsopp et al., 2003), we computationally predicted telomere length (Ding et al., 2014). Tx-12 Dnmt3a<sup>KO</sup> HSCs showed no erosion of telomere length (Figure S4E; Table S4). Cumulatively, these data suggest Dnmt3a loss of function is sufficient to bias HSC fate decisions and initiate the pre-malignant condition of CHIP but is not sufficient to drive malignant transformation.

**DISCUSSION**

Here we show that loss of Dnmt3a endows HSCs with immortality in vivo. The self-renewal potential of Dnmt3a<sup>KO</sup> HSCs far exceeds that of normal HSCs and the lifespan of the mice from which they were derived. Our data establish that HSCs do not have an inherently finite lifespan but that loss of Dnmt3a augments epigenetic features that enforce self-renewal and enable HSCs to be propagated indefinitely. Further examination of the mechanisms perpetuating immortality in Dnmt3a<sup>KO</sup> HSCs may provide a window for artificially
extending the lifespan of HSCs, an important biomedical application in the context of the aging human population.

The differentiation block of immortal $Dnmt3a^{KO}$ HSCs cannot be rescued by re-expression of $Dnmt3a$ or transcription factors such as Ebf1. But re-expression of $Dnmt3a$ abrogated the self-renewal phenotype, suggesting the roles of $Dnmt3a$ in self-renewal and differentiation can be uncoupled. Our data also demonstrate that malignant transformation is agnostic to DNA methylation state: both early-passage (Mayle et al., 2015; Celik et al., 2015) and late-passage (this study) $Dnmt3a^{KO}$ HSCs will generate malignancies when an appropriate cooperating mutation is acquired. How oncogenic signals can overcome the differentiation block of $Dnmt3a^{KO}$ HSCs to generate disease warrants further investigation.

Although we use an artificial system of serial transplantation, we consider the extent to which these insights may be extrapolated to humans. $DNMT3A$ mutations are the most common mutation in CHIP (Xie et al., 2014; Jaiswal et al., 2014; Genovese et al., 2014), with increasing frequency with age (McKerrell et al., 2015; Young et al., 2016). But as CHIP is identified by assaying peripheral blood, if $DNMT3A$ mutant human HSCs also show compromised differentiation, we predict the proportion of mutant HSCs in the bone marrow is underestimated by current studies. Our experiments are also distinct from the human scenario in that each transplant of $Dnmt3a^{KO}$ HSCs contains fresh WT bone marrow replete with normal HSCs. In the aging human, WT HSCs diminish in function (Pang et al., 2011), offering less competition to emerging $DNMT3A$ mutant clones. Even partial loss of $DNMT3A$ function through weak heterozygous mutations, likely enables mutant HSCs to outcompete WT counterparts. We also show that a dominant-negative $Dnmt3a$ mutation (R878) equivalent to the frequent human R882 mutation results in very similar DNA methylation changes to the null allele, suggesting most $DNMT3A$ mutations likely produce similar molecular and cellular consequences at least at the level of clonal expansion.

The phenotype of $Dnmt3a^{KO}$ HSCs is unique. Mutation of some other genes can enhance HSCs capacity (Rossi et al., 2012), but none can remove the inherent limits on self-renewal and replicative lifespan like $Dnmt3a$. Our data add to the emerging view that HSCs are in constant competition with their siblings. Perturbations that confer an advantage to one HSC over another will be selected depending on the specific context. With the enormous self-renewal capacity of $Dnmt3a$-null HSCs demonstrated here, a single mutant HSC in human bone marrow can outcompete WT counterparts over a period of many years, even if initially vastly outnumbered.

**EXPERIMENTAL PROCEDURES**

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures.

**Mice and Transplantation**

All animal procedures were approved by Institutional Animal Care and Use Committees and performed in strict adherence to Washington University institutional guidelines. All mice were C57BL/6 background. Recipient CD45.1 mice (8–10 weeks of age; strain #002014;
The Jackson Laboratory) were given a split dose of 10.5 Gy irradiation. Mx1-Cre(+):Dnmt3a\textsuperscript{fl/fl} mice have been previously described by our group (Challen et al., 2011). Control mice were Mx1-Cre(+):Dnmt3a\textsuperscript{+/+} mice. Deletion of floxed alleles was induced by intraperitoneal injection of 300 μg plpC (#p1530; Sigma-Aldrich)six times every other day inaduit mice (10–12 weeks old). Equal numbers of male and female mice were used.

For competitive transplants, 200 HSCs (Lineage\textsuperscript{−}[Gr-1, Mac-1, B220, CD3e, Ter119], c-Kit\textsuperscript{+}, Sca-1\textsuperscript{+}, CD48\textsuperscript{−}, CD150\textsuperscript{+}) were transplanted with 2.5 × 10\textsuperscript{5} WT CD45.1 competitor bone marrow. For serial transplantation, 200 donor-derived HSCs were isolated from the previous recipients 18–24 weeks post-transplant and transplanted into new lethally irradiated recipients along with fresh WT competitor bone marrow. Recipients were bled through the retro-orbital route and analyzed for donor-derived lineage contribution by flow cytometry.

**Quantification and Statistical Analysis**

Statistical comparisons between groups were evaluated using Student’s t test or ANOVA as appropriate using Prism 6 (GraphPad). All data are presented as mean ± SE. Time to morbidity is presented in Kaplan-Meier survival curves and analyzed using the log-rank test. Statistical significance is denoted as *p < 0.05, **p < 0.01, and ***p < 0.001; n indicates the number of biological replicates within each group.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Serial propagation of HSCs for 12 transplant generations, over 60 months in vivo
- Augmentation of modifications at enhancers and canyons to buttress self-renewal
- Differentiation becomes irreversibly compromised, but transformation potential remains
Figure 1. Loss of Dnmt3a Provides HSCs with Indefinite Longevity

(A) Schematic representation of serial HSC transplantation process. Tx, transplant stage; HSCs, hematopoietic stem cells; WBM, whole bone marrow.

(B and C) Representative flow cytometry plots showing donor-derived cell (CD45.2+) contribution to bone marrow HSC compartment (B) and peripheral blood (C) at the end of indicated stage of transplantation. N.D., not determined.

(D) Quantification of donor HSC-derived peripheral blood chimerism (dashed gray line) compared with absolute number of donor-derived HSCs per mouse generated from Dnmt3aKO HSCs at the end of each transplant (n = 5–29 recipients per transplant generation).

(E) Time to morbidity in mice transplanted with 1 × 10⁵ Dnmt3aKO HSCs in the absence of WBM competitor cells.

(F) Blood counts from moribund mice showing leukopenia, anemia, and thrombocytopenia. Gray shaded areas indicate normal range for WT mice. See also Figure S1.
Figure 2. Dnmt3a Controls DNA Methylation at HSC Regulatory Elements

(A) Hierarchical clustering on the basis of CpG methylation ratio of control, Tx-3, and Tx-11 Dnmt3aKO HSCs.

(B) Fraction of hyper- or hypo-methylated DMRs in Tx-3 Dnmt3aKO HSCs (versus control HSCs) that became further hyper- or hypo-methylated in Tx-11 Dnmt3aKO HSCs (versus Tx-3 Dnmt3aKO HSCs).

(C) Enrichment analysis of 4,313 “hypo_hypo” regions compared with 673 control regions (“hypo_hyper”). Size of data points represents the overlap percentage with the size of the corresponding regulatory regions in the denominator.

(D) DNA methylation levels of active, bivalent, and inactive canyons in control (WT), Tx-3 Dnmt3aKO, and Tx-11 Dnmt3aKO HSCs. Flanking regions are extended by the same length as the corresponding canyon (±1x).

(E) DNA methylation profile of active, inactive, and bivalent canyon loci by WGBS. The height of each bar represents the DNA methylation level of an individual CpG. Also shown for Cxcl12 are histone marks defining bivalent canyons and RNA-seq expression.

(F) Expression level changes of genes within active and bivalent canyon regions. See also Figure S2.
Figure 3. Differentiation Capacity Is Lost but Transformation Potential Is Retained in Immortalized Dnmt3aKO HSCs

(A) Representative flow cytometry plots showing bone marrow analysis of mice transplanted with Tx-11 Dnmt3aKO HSCs transduced with control (GFP) or Dnmt3a-expressing (Dnmt3a) lentivirus 18 weeks post-transplant.

(B) Frequency of transduced Tx-11 Dnmt3aKO HSCs in bone marrow of recipient mice 18 weeks post-transplant.

(C) Cell cycle analysis of the indicated genotype/transplant stage showing proportion of quiescent (G0) HSCs.

(D) Quantification of apoptotic HSCs of the indicated genotype/transplant stage.

(E) Expression of pro-apoptotic genes in Tx-11 Dnmt3aKO HSCs transduced with control (GFP) or Dnmt3a-expressing (Dnmt3a) lentivirus.

(F) Clonogenic myeloid potential of HSCs from the indicated genotype/transplant stage.

(G) B cell potential of HSCs from the indicated genotype/transplant stage transduced with control (GFP), Dnmt3a-expressing, or Ebf1-expressing lentivirus.

(H) Time to morbidity in mice transplanted with Tx-12 Dnmt3aKO HSCs transduced with lentivirus expressing KrasG12D.

(I) Phenotype of KrasG12D-driven AML.
Mean ± SEM values are shown. *p < 0.05, **p < 0.01. See also Figure S3.
Figure 4. Molecular Analysis of Dnmt3a Rescue, Dominant-Negative Mutant, and Clonal Hematopoiesis

(A and B) WGBS profiles showing increased DNA methylation (green boxes) in Gata2 canyon (A) and Gpr56 enhancer (B) in Tx-11 Dnmt3a KO HSCs transduced with Dnmt3a-expressing lentivirus.

(C) DNA methylation ratio of CpGs throughout the genome in HSCs of the indicated genotypes.

(D) Methylation levels of CpGs within gene bodies, promoters, and enhancers.

(E) DNA methylation profiles of the HoxB locus. Green box shows hypomethylation in Dnmt3aKO, which is not observed in Dnmt3aR878 HSCs.

(F) Expression levels of HoxB genes in Tx-3 Dnmt3aKO and Tx-3 Dnmt3aR878 HSCs. Data are expressed as relative fold change to control comparators in each sequencing experiment (n = 2–4 biological replicates per genotype).

(G and H) DNA methylation profile of Gata3 locus showing hypomethylation of Dnmt3aKO (3aKO) HSCs (green box) (G), corresponding with increased gene expression (H), not conserved in Dnmt3aR878 (R878) HSCs. Expression data are expressed as relative fold change to control comparators in each sequencing experiment (n = 2–4 biological replicates per genotype).
(I and J) Total number of somatic variants (I) and variant allele fraction (J) in HSCs from the indicated genotype/transplant stage.
See also Figure S4.