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Accessibility
DNA methyltransferase 1 functions through C/ebpa to maintain hematopoietic stem and progenitor cells in zebrafish

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Abstract

Background: DNA methyltransferase 1 (Dnmt1) regulates expression of many critical genes through maintaining parental DNA methylation patterns on daughter DNA strands during mitosis. It is essential for embryonic development and diverse biological processes, including maintenance of hematopoietic stem and progenitor cells (HSPCs). However, the precise molecular mechanism of how Dnmt1 is involved in HSPC maintenance remains unexplored.

Methods: An N-ethyl-N-nitrosourea (ENU)-based genetic screening was performed to identify putative mutants with defects in definitive HSPCs during hematopoiesis in zebrafish. The expression of hematopoietic markers was analyzed via whole mount in situ hybridization assay (WISH). Positional cloning approach was carried out to identify the gene responsible for the defective definitive hematopoiesis in the mutants. Analyses of the mechanism were conducted by morpholino-mediated gene knockdown, mRNA injection rescue assays, anti-phosphorylated histone H3 (pH3) immunostaining and TUNEL assay, quantitative real-time PCR, and bisulfite sequencing analysis.

Results: A heritable mutant line with impaired HSPCs of definitive hematopoiesis was identified. Positional cloning demonstrated that a stop codon mutation was introduced in $dnmt1$ which resulted in a predicted truncated Dnmt1 lacking the DNA methylation catalytic domain. Molecular analysis revealed that expression of CCAAT/enhancer-binding protein alpha (C/ebpa) was upregulated, which correlated with hypomethylation of CpG islands in the regulation regions of $cebpa$ gene in Dnmt1 deficient HSPCs. Overexpression of a transcriptional repressive SUMO-C/ebpa fusion protein could rescue hematological defects in the $dnmt1$ mutants. Finally, $dnmt1$ and $cebpa$ double null embryos exhibited no obvious abnormal hematopoiesis indicated that the HSPC defects triggered by $dnmt1$ mutation were C/ebpa dependent.

Conclusions: Dnmt1 is required for HSPC maintenance via C/ebpa regulation during definitive hematopoiesis in zebrafish.

Keywords: Dnmt1, C/ebpa, HSPCs, Zebrafish

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Background
In vertebrates, hematopoiesis takes place in two consecutive waves, primitive and definitive ones [1,2]. Primitive hematopoiesis, also known as embryonic hematopoiesis, predominantly produces erythroid and myeloid cells [3,4]; while definitive hematopoiesis, also called adult hematopoietic wave, generates hematopoietic stem cells (HSCs) that are capable of producing all lineages of blood [5]. The zebrafish (Danio rerio) is an excellent genetic system for the study of hematopoietic development [6,7], especially by characterization of thousands of mutants isolated from large-scale forward genetic screens [8,9]. In zebrafish, definitive HSCs arise from the ventral wall of the dorsal aorta (VDA), the zebrafish equivalent of the aorta/gonad/mesonephros (AGM) of mammals [10,11], then HSCs migrate through the caudal hematopoietic tissue (CHT) to the thymus and kidney marrow [12], where adult hematopoiesis occurs, similar to HSC migration through fetal liver and home to bone marrow in mammals.

The molecular regulation of hematopoiesis includes interactions of lineage-specific transcription factors and a series of epigenetic modifications, such as DNA methylation and covalent histone tail modifications [13]. DNA methylation is an important epigenetic regulation mechanism that regulates normal development through influencing gene transcription, genomic imprinting, and genome stability in mammal cells [14-16]. In hematologic malignancies, dysregulation of DNA methylation may result in global shifts in gene expression, which frequently leads to increased self-renewal in malignant blood cells at the expense of normal differentiation [17].

Three active DNMTs, namely DNMT1 [18], DNMT3A, and DNMT3B [19,20], have been identified in mammals. DNMTs are highly evolutionarily conserved with a regulatory region attached to a catalytic domain [21]. DNMT1 is the most abundant DNA methyltransferase in mammalian cells and considered to be the key maintenance methyltransferase [22]. In mammals, DNMT1 null mutant embryonic stem cells are viable and contain a small percentage of methylated DNA and methyltransferase activity [23]. Mouse embryos homozygous for a deletion of Dnm1 die at 10 to 11 days gestation due to developmental defects [24]. Reduced Dnm1 activity in xenopus [25] and zebrafish [26,27] has similar consequences. Dnm1 also plays important roles in HSCs. The deletion of Dnm1 has no influence on the mature cells in the hematopoietic system but causes decreased niche retention and self-renewal and differentiation defects of HSPCs [28]. In acute myeloid leukemia (AML), the expression of DNMTs is upregulated [29]. Conditional knockout of Dnm1 blocks development of leukemia, and haploinsufficiency of Dnm1 is sufficient to delay progression of leukemogenesis and impair leukemia stem cell (LSC) self-renewal without altering normal hematopoiesis [30]. The precise mechanism of the Dnm1 regulation of HSC function requires further investigation.

In this study, a heritable zebrafish mutant line with hematopoietic defects identified through ENU-based forward genetic screening was found defective in dnm1 gene. Phenotype characterization of dnm1 mutant has uncovered severely impaired definitive hematopoiesis. Further molecular mechanistic studies revealed that cebpa was a Dnm1 downstream target gene and activated as a result from hypomethylation of its regulation regions in dnm1 mutants, which suggested cebpa was a key downstream target of dnm1 gene in HSPCs. We further demonstrated that the elevated C/ebpa activity was required and accounted for, at least in part, the defective definitive hematopoiesis.

Results
Zebrafish mutant line ldd794 displays impaired definitive hematopoiesis
To search for novel genes involved in regulating definitive hematopoiesis, we established an ENU-based genetic screening strategy to identify putative mutants with defects in definitive HSPCs in zebrafish. Whole-mount mRNA in situ hybridization (WISH) analysis of cmyb [31], a marker of HSPCs, was used to screen for mutants. In the mutant line ldd794, cmyb expression in homozygous embryos was reduced from 36 hours post-fertilization (hpf) in the AGM (Figure 1A, B) and was almost absent at 5 days post-fertilization (dpf) in the CHT (Figure 1I, J). Similarly, the expression of two other HSPC markers runx1 as well as scl were also decreased (Figure 1K-N), suggesting the definitive HSPCs were impaired. Ldd794 heterozygous fish was crossed with a Tg (cmyb:EGFP) homozygous individual (a stable zebrafish transgenic line expressing EGFP under the control of the cmyb promoter) [32]. The adult fishes carrying both dnm1 mutant allele and EGFP transgene were incrossed. As expected, the number of EGFP-positive cells was significantly decreased in the CHT of approximate 25% EGFP positive offspring at 4 dpf (Additional file 1: Figure S1A). This result further confirmed the HSPCs were specifically affected in ldd794 mutants.

The temporal and spatial expression patterns of a set of hematopoietic transcription factors and key genes involved in either lineage determination or differentiation were examined in ldd794 mutants. The expression of erythrocyte progenitor marker gata1 [33], mature erythrocyte marker hbae1 [34], myeloid-specific marker mpx [35], l-plastin [36] and lysozyme C [37], and lymphoid-specific marker rag1 [38] were all diminished in ldd794 homozygous mutants (Figure 1O-Z). The decrease of multiple hematopoietic lineages suggested that the deficiency of hematopoietic precursors and/or progenitors occurred in the dnm1 mutants.
Figure 1 (See legend on next page.)
To exclude the possibility that the defective definitive hematopoiesis was due to the preexisting primitive hematopoietic defects in ldd794 mutants, a series of markers involved in primitive hematopoiesis, such as cmyb, hbae1, pu.1, mpx, and lysozyme C were also examined at 22 hpf. No overt changes have been detected (Additional file 1: Figure S1B), suggesting that primitive hematopoiesis was not affected in the dnm1t1 mutants.

Since normal vasculogenesis is required for the birth of HSCs from the ventral wall of the dorsal aorta in the zebrafish embryo [10,11,39,40], we evaluated early vascular development by expression of vascular markers. No obvious differences in flk1 and ephrinB2 expressions were observed between ldd794 siblings and mutants (Additional file 1: Figure S1C), suggesting that the vascular system and artery-vein differentiation remained intact and the hematopoiesis defects were not due to impaired vasculature.

**Zebrafish ldd794 mutant encodes a truncated Dnmt1 lacking the enzymatic catalytic domain**

Positional cloning approach was applied to identify the gene responsible for the defective definitive hematopoiesis in ldd794 mutants. Bulk segregation analysis indicated that the potential mutation site was located on chromosome 3. The 528 putative mutant embryos (1,056 meioses) were further examined. Seven homologous recombinations of sslp1 (zC250L3) and eight homologous recombinations of sslp2 (zC74M13) from different embryos were identified, which enabled us to localize the mutation to a 0.45-MB region containing eight genes (Figure 2A). Complementary DNA (cDNA) sequencing of all eight candidate genes revealed that in dnm1t1, there was a T to A transversion, which introduced a stop codon at 743th amino acid, resulting in a predicted truncated Dnmt1 lacking the DNA methylation catalytic domain (Figure 2B-D). The gene synergy analysis revealed that Dnmt1 genomic context was highly conserved from zebrafish to human (Figure 2E).

**Loss of Dnmt1 catalytic activity leads to specific HSPC defects**

WISH analysis revealed that zebrafish dnm1t1 was expressed ubiquitously, including the hematopoietic regions (Additional file 1: Figure S2). The dnm1t1 specific antisense ATG-morpholino (MO) was injected into wild-type embryos at one-cell stage to reproduce the phenotypes observed in ldd794 mutant embryos. As expected, the expression of cmyb diminished from 36 hpf (Figure 3B, B') in the AGM and significantly reduced in the CHT at 5 dpf (Figure 3F, F'), which was exactly a phenocopy of ldd794 mutants. Unsurprisingly, erythrocytes, myeloid cells, and lymphocytes were decreased in dnm1t1 ATG morphants (Additional file 1: Figure S3). Furthermore, a dnm1t1 splicing MO, specifically affecting splicing of precursor dnm1t1 RNA transcripts (Additional file 1: Figure S4), was also able to mimic the Dnmt1 protein truncation identified in ldd794 mutants. A similar effect to that of ATG MO was observed (Figure 3C, C; G, G'), further confirming that the loss of Dnmt1 catalytic activity indeed specifically led to observed HSPC defects in the dnm1t1 mutants.

Finally, specific in vivo rescue experiments were carried out. Full-length zebrafish dnm1t1 mRNA was co-injected with dnm1t1 ATG MO into wild-type embryos. The results showed that dnm1t1 mRNA could efficiently rescue the hematopoietic defects in dnm1t1 morphants (Figure 3D, D; H, H'). In contrast, co-injection of the truncated dnm1t1 mutant mRNA with the dnm1t1 ATG MO was ineffective (data not shown). Consistently, dnm1t1 mRNA could also restore the defects of HSPCs in ldd794 mutant embryos (Figure 3I-N). These data demonstrated that the phenotype observed in dnm1t1 mutants was indeed Dnmt1 dependent.

To investigate whether the observed deficient Dnmt1-mediated phenotypes were due to abnormal cell proliferation or apoptosis of definitive HSPCs, the anti-phosphorylated histone H3 (pH3) immunostaining and TUNEL assay were performed, respectively. A decrease of pH3 and cmyb-EGFP double positive cells was detected while no obvious change of TUNEL and cmyb-EGFP double positive cells was found in dnm1t1 mutants (Figure 3O-U, Additional file 1: Figure S5). Similar results were observed in dnm1t1 morphants (data not shown), suggesting that the defects of HSPCs in dnm1t1-deficient embryos were caused by decreased proliferation.

**Increased cebpα expression was correlated with hypomethylation of CpG islands**

Given the fact that Dnmt1 predominantly methylates hemimethylated CpG dinucleotides in the mammalian
genome and facilitates repression in promoter regions, we speculate that the loss of Dnmt1 might activate some key negative regulators of definitive hematopoiesis.

C/ebpa, a member of the basic leucine zipper protein family of transcription factors [41,42], not only plays a pivotal role in granulopoiesis [43] but also regulates the self-renewal and proliferation of HSPCs at a much earlier stage during mouse hematopoiesis [44-47]. C/ebpa deficiency leads to hyperproliferation and increased self-renewal capacity in both fetal and adult HSCs [44,45,47], while activation of C/ebpa is sufficient to repress stem cell capacities and proliferation of HSCs [46]. More direct evidence is that C/EBPa has previously been reported as a cell-cycle inhibitor [48]. These evidences prompted us to test whether C/ebpa function was important for mediating the observed HSPC proliferation phenotypes in the *dnmt1* mutants.

Firstly, to test whether cebpa was upregulated in *dnmt1*-deficient HSPCs, *dnmt1* MO was injected into Tg (*cmyb*:EGFP) embryos at one-cell stage, then *cmyb*-EGFP positive cells were sorted and collected at 3 dpf (Figure 4A). RT-PCR results showed that the expression of *cebpa* indeed increased in *dnmt1*-deficient *cmyb*-EGFP positive cells (Figure 4B).

Secondly, we checked the DNA methylation status of the CpG islands located near the zebrafish *cebpa* gene. The phenotype of hematopoietic defects is caused by mutation in *dnmt1*. (A) Genetic mapping of ldd794 on chromosome 3. The numbers above the SSLP markers indicate the number of recombinants in 1,056 meioses analyzed. Fine mapping using SSLPs narrowed down the region between sslp1 and sslp2 markers. The red asterisk indicates the mutation position within *dnmt1* gene. (B) Sequence traces of pooled sibling and pooled *dnmt1* mutant cDNAs. A T to A transversion at 2,229th nucleotide resulted in a stop codon in *dnmt1* (marked by red asterisk). (C) Sequence result of grandparents. The sequence result indicated that this pair of ldd794 mutants (screened out in ENU project) was heterozygote carrying one mutated allele of *dnmt1* (marked by red asterisk). (D) Structure of wild-type and mutant Dnmt1 protein. (E) Synteny analysis of *dnmt1*. 
Figure 3. Dnmt1 mRNA rescue and anti-phosphorylated histone H3 (pH3) immunostaining assays in dnmt1-deficient embryos.

(A-H) Dnmt1 mRNA rescue assay at 36 hpf (A-D) and 5 dpf (E-H) Dnmt1 ATG morpholino (4 ng) and splicing-specific morpholino (8 ng) effectively reproduced decreased cmyb expression phenotype in wild-type embryos (B, C, F, G). Dnmt1 mRNA rescued the defects of dnmt1 morphants. (I-N) Rescue assay of wild-type dnmt1 mRNA in ldd794 dnmt1 mutants at 36 hpf (K) and 3 dpf (N). Wild-type dnmt1 mRNA effectively restored cmyb expression in ldd794 dnmt1 mutants at 36 hpf (K) and 3 dpf (N). (A-N') Magnified images of the boxed regions in A to N, respectively. Red arrows indicate cmyb-positive HSPCs in the AGM or CHT.

(O-T) The proliferation of HSPCs was reduced in dnmt1−/− mutants. Double immunostaining of cmyb-EGFP (O-P) and anti-PH3 (Q-R) in the CHT of Tg(cmyb-EGFP) line at 72 hpf. The bottom panel shows merged images (S, T).

(U) Statistics of PH3 and cmyb-EGFP double positive cells.
Up to 40 kb long of upstream, the coding region, and the downstream genomic sequence of the zebrafish cebpa gene were searched for potential CpG islands. A total of 14 candidate CpG islands were found, and their DNA methylation statuses were evaluated by bisulfite sequencing analysis. The hypermethylation was found in four CpG island regions (BSP1 to BSP4) near the cebpa gene in wild-type cmyb-EGFP positive cells. As expected, a much lower methylation status was found in these four CpG islands in MO-injected cmyb-EGFP positive cells (Figure 4C, Additional file 1: Figure S6). Taken together, cebpa expression is likely regulated by Dnmt1 activity.

Cebpa upregulation was involved in the defective HSPC phenotypes in dnmt1-deficient embryos

A series of rescue assays were carried out in order to verify whether elevated expression of cebpa is responsible for dnmt1 mutant phenotype. In our previous work, two repressive forms of C/ebpa have been constructed [49], SUMO2-C/ebpa, mimicking the constitutively sumoylated form of C/ebpa and POZ-C/ebpa, mimicking the SUMO-mediated repressive form. The mRNAs of these two constructs were individually injected into the wild-type embryos with dnmt1 ATG MO, respectively. Both were shown to be effective in rescuing cmyb expression (Figure 5A-H) in the morphants. The similar rescuing results were also observed in ldd794 mutant embryos overexpressing SUMO2-C/ebpa repressive protein (Figure 5I-N).

It is worth noting that two dnmt1 mutant lines with liver and pancreas development defects were reported [50]. As expected, the similar phenotypes were also detected in our ldd794 mutants by assessing the expression of hepatocytes marker lfabp and pancreas marker trypsin (Additional file 1: Figure S7). Intriguingly, the rescue effects of SUMO2-C/ebpa on liver and pancreas development defects were not observed (Additional file 1: Figure S7), suggesting the role of Dnmt1 in liver and pancreas development was unlikely cebpa dependent.

Taken together, these data strongly suggest that elevated cebpa function is involved in HSPC defects of dnmt1 mutants.

Cebpa function was pivotal to Dnmt1 regulated maintenance of definitive HSPCs

We have generated a cebpa null mutant zebrafish line by TALEN approach (submitted elsewhere). The phenotype was similar to that observed in mice. While no mature
myelocytes were detected by Sudan Black staining, the expression of cmyb remained unchanged, if not a marginal increase. This cebpa mutant line was then crossed with ldd794 heterozygotes. The adult fishes carrying both cebpa and dnmt1 mutant alleles were incrossed. We found only in the presence of C/ebpa that cmyb expression was severely decreased (Figure 6B, B'). By contrast, in the cebpa and dnmt1 double null mutants, the expression level of cmyb remained normal (Figure 6D, D'). In parallel, similar results were observed in cebpa null embryos knocked down with dnmt1 MO (Additional file 1: Figure S8). These results indicate that the defective HSPCs triggered by dnmt1 mutation require intact C/ebpa function. Therefore, cebpa is a key target gene of dnmt1 and its regulated function is involved in the definitive HSPCs maintenance.

**Discussion**

In this study, a zebrafish mutant line with definitive hematopoiesis defects was identified. The specific phenotype was caused by a premature termination codon in the dnmt1 gene, which resulted in a truncated Dnmt1 protein lacking its catalytic domain. DNMT1 protein is an important DNA methyltransferase to silence and regulate genes by methylation of DNA regions without changing the genomic DNA sequence [23]. Lack of Dnmt1 function resulted HSPC proliferation block and shortage of differentiated blood lineages. We demonstrated that normal Dnmt1 function was critical in regulating cebpa gene expression, and intact C/ebpa function was required for HSPC proliferation block triggered by the absence of Dnmt1 function. Our
studies provided new evidence for that cella is a downstream target of Dnmt1 in regulating HSPC proliferation during normal hematopoiesis.

Many lines of evidences demonstrate that DNA methylation influences gene expression during embryogenesis [51-53]. In mice, a Dnmt1 mutation led to a recessive lethal phenotype with stunted and delayed development [24]. Similarly, two other zebrafish mutants related to dnmtn were shown to have defects in pancreas development at a late embryonic stage and resulted in embryonic lethality [50]. In line with these mutant phenotypes, our ldd794 homozygous mutants usually die at 8 dpf with abnormal hematopoiesis and other organ formation such as the liver, further suggesting that reduced DNA methylation causes developmental abnormality and embryonic lethality. It is worth noting that ldd794 heterozygotes do not display any observable phenotype, implying that the mutated dnmtn allele does not play a dominant negative role.

Our developmental and molecular analyses showed that the lack of Dnmt1 enzymatic activity in ldd794 mutants led to severe reduction in HSPC numbers as well as impaired production of all three major lineages but accompanied by normal vascular development during early development. These observations are in an agreement with the ones from mouse studies [28]. Bone marrow transplant assays revealed that Dnmt1 affected HSCs in a cell-autonomous manner [28]. Our findings, together with those in mice, demonstrate that Dnmt1 has a conserved role in definitive hematopoiesis.

The phenotype with lower number of HSPCs in CHT might be due to either reduced proliferation or increased apoptosis of HSPCs. The pH3 and TUNEL assays suggested that the definitive HSPC defects are not due to increased apoptosis but likely caused by decreased proliferation of HSPCs. These results are also consistent with the fact that the frequencies of apoptotic cells in total BM, CMPs, GMPs, or MEPs remained unchanged in mouse mutants [28].

Given the fact that C/ebpa is a critical transcription factor for granulopoiesis [43], we have expected that its activation might induce accelerated myeloid differentiation. However, downregulated mpx expression in dnmtn mutant does not support an increased myeloid differentiation process. Meanwhile, an increased number of reports have demonstrated that C/ebp is an important modulator of HSPC function [44-47]. Supporting this idea, we have found that hypomethylation of the cella regulatory region as a result of Dnmt1 deficiency is directly associated with HSPC impairment. Although the possibility of other negative regulators being activated cannot be excluded completely, HSPC proliferation defect caused by dnmtn mutation is indeed C/ebp dependent; as in the cella dnmtn double null mutants, the cellb expression appeared to be normal. It was reported that C/EBPa negatively regulated n-myc, and the loss of C/EBPa resulted in de-repression of n-myc in mice HSCs [47]. Indeed, our Q-PCR analysis revealed that in cmyb-EGFP positive cells sorted from dnmtn MO knockdown embryos, n-myc had a much lower expression level (data not shown), which
might account for the pronounced decreased proliferation of HSPCs. Finally, one recent report revealed that a non-coding RNA arising from the CEBPA gene locus could influence the methylation level of CEBPA promoter by inhibiting DNMT1 protein binding to the regulatory region of CEBPA gene [54], which also supported our findings that Dnmt1 acts directly on cebpa promoter.

Taken together, our findings and others point out that the regulation of C/ebpa function during hematopoiesis takes place at multi-levels, including epigenetic modification, transcriptional regulation, and post-translational modification, which allow C/ebpa to exert its distinguished role in a fine-tuned manner.

Conclusions

Our studies for the first time clarify the possible molecular mechanism of Dnmt1 involved in HSPC maintenance during definitive hematopoiesis in zebrafish. The fact that C/ebpa functions as a critical downstream effector of Dnmt1 provides new insights of Dnmt1-regulated hematopoiesis.

Methods

Zebrafish maintenance and ENU mutagenesis

Zebrafish were maintained and staged under standard conditions as described previously [55]. Zebrafish embryos were cultured in “egg water” consisting of 0.03% sea salt and 0.002% methylene blue. A 0.0045% 1-phenyl-2-thiourea (Sigma-Aldrich, St. Louis, MO, USA) was used to prevent melanization and facilitate in situ hybridization analysis of gene expression [55]. ENU mutagenesis on Tubingen (Tu) strain was carried out as described [56]. The WIK line was used as the mapping strain. The zebrafish maintenance and study protocols were approved by the Institutional Review Board of the Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Whole-mount in situ hybridization (WISH)

Digoxigenin (DIG)-labeled RNA probes were synthesized with T3 or T7 polymerase (Ambion, Life Technologies, Carlsbad, CA, USA), using linearized cDNA plasmid constructs. Whole-mount mRNA in situ hybridization was performed as described previously [57]. The DIG-labeled probes were detected using alkaline phosphatase-coupled anti-digoxigenin Fab fragment antibody (Roche, Basel, Switzerland) with BCIP/NBT staining (Vector Laboratories, Burlingame, CA, USA).

Mapping and positional cloning of ldd794

Ldd794 (Tu background) carrying the mutant allele were outcrossed to the polymorphic wild-type strain WIK for positional cloning. The genome for linked SSLP (simple sequence length polymorphism) markers were scanned by bulk segregation analysis using standard methods [58]. For fine mapping, ldd794 mutant embryos were genotyped with SSLP markers to narrow down the genetic interval. The cDNAs of candidate genes were sequenced from pooled mutant RNA, and candidate mutation was confirmed by sequencing the genomic DNA of individual mutant embryo. All primers used for positional cloning and dnmt1 sequencing are provided in Additional file 2.

Morpholinos and mRNA microinjection

Morpholinos (MOs) and mRNA were injected into embryos at one-cell stage. Morpholino oligonucleotides were designed by and ordered from Gene Tools. The morpholino sequences are as follows: for dnmt1 MO, 5′-ACAAT GAGGTCTTGGTAGGCATTTC-3′ (4 ng/embryo) [27]; and for dnmt1 splicing MO, 5′-CCACCCCTCAAAA CAATAACAGTGT-3′ (8 ng/embryo). Capped mRNA samples were transcribed from linearized plasmids (mMessage Machine; Ambion), purified, and diluted to 100 ng/ul (dnmt1 and dnmt1 mutant mRNA) or 50 ng/ul (SLIM02-C/ebpa and POZ-C/ebpa mRNA) for injection of embryos at one-cell stage.

Anti-phosphorylated histone H3 (pH3) immunostaining and TUNEL assay

Three days post-fertilization (dpf), Tg (cmyb:EGFP) embryos were fixed in 4% paraformaldehyde (PFA). After dehydration and rehydration, the embryos were treated with Proteinase K (10 mg/ml) for 30 min at RT and re-fixed in 4% PFA for 20 min. After blocking with blocking buffer (2 mg/ml BSA + 10% FBS + 0.3% Triton-X100 + 1% DMSO in PBST), the embryos were stained with mouse anti-GFP (Invitrogen, Carlsbad, CA, USA) and rabbit anti-phosphohistone H3 antibody (Santa Cruz) primary antibody at 4°C overnight. Alexa Fluor 488-conjugated anti-mouse (Invitrogen) and Alexa Fluor 594-conjugated anti-rabbit (Invitrogen) were used as secondary antibodies. Images were taken using Olympus FV 1000 confocal microscopy equipped with the FV10-ASW version 3 software.

Terminal transferase UTP nick end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit, TMR red (Roche), according to the manufacturer’s recommendations.

Genomic DNA and RNA isolation

Tg (cmyb:EGFP) embryos at one-cell stage were injected with dnmt1 ATG morpholino. Cells positive for cmyb:EGFP were sorted and collected from homogenized embryos at 3 dpf. Genomic DNA (gDNA) and total mRNA were extracted using the TRIzol reagent (Life Technologies) according to the manufacturer’s instructions.
Quantitative real-time PCR
Reverse transcription was carried out using the super script first-strand synthesis system (Life Technologies) according to the manufacturer's instructions. Real-time quantitative PCR (Applied Biosystems, Foster City, CA, USA) was used for relative quantification of cebpa gene expression. The expression level of cebpa was normalized to the expression of housekeeping gene GAPDH. The primers used for real-time quantitative PCR were listed in Additional file 2.

Bisulfite sequencing PCR (BSP) assay
The DNA methylation assay was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendations. The treatment of DNA with bisulfite results in the selective conversion of unmethylated cytosine to uracil, whereas methylated cytosine remains unchanged. Methprimer (http://www.urogenes.org/methprimer/) was used to predict the CpG islands. The following primers were used for bisulfite-specific polymerase chain reaction regulation of the cebpa gene: BSP1 Fw: 5′-GTTTATA GAAGTTGTAGAGGGG-3′ and Rv: 5′-ACAAACC CAACCCCTCCTTATTTAT-3′; BSP2 Fw: 5′-TTTTTATTGATGTTTTGTTTTAGG-3′ and Rv: 5′-ATAAAAATTACACCCAAAATTCAAACACCAACCA-3′; BSP3 Fw: 5′-TATTGTA TAATTTAGTATGAAATTGTTTT-3′ and Rv: 5′-AACATTTAACATATATTTACCAAATACCAT-3′; BSP4 Fw: 5′-ATAATTTTTGTGTAGATTATAATGTTTT-3′ and Rv: 5′-TACTCCATATAACACATTTATCCAAATACCA-3′. PCR products were subcloned into pMD18-T Vector (Takara, Kyoto, Japan), and transformed bacteria were cultured overnight. Clones (eight to ten) of each BSP were sequenced for confirmation.

Additional file 1: Supplementary figures and figure legends.
Additional file 2: Table S1. The sequence information of SSLP markers, dnmt1 sequencing primers, and Q-PCR primers.

Abbreviations
Dnmt1: DNA methyltransferase 1; HSPCs: Hematopoietic stem and progenitor cells; C/EBPa: CCAAT/enhancer-binding protein alpha (zebrafish gene: cebpa); C/EBPa: Mouse gene; C/EBPa: Mouse protein; C/EBPa: Human gene; C/EBPα: Human protein; HSCs: Hematopoietic stem cells; VDA: Ventral wall of the dorsal aorta; AGM: Aorta/gonad/mesonephros; CHT: Caudal hematopoietic tissue; AML: Acute myeloid leukemia; LSC: Leukemia stem cell; ENU: N-ethyl-N-nitrosourea; WISH: Whole-mount in situ hybridization; hpf: Hours post-fertilization; dpf: Days post-fertilization; pSH: Phosphorylated histone H3; TUNEL: Terminal transferase UTP nick end labeling; BSP: Bisulfite sequencing PCR.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
XL performed the experiments and analyzed the data. XJ, YX, HY, KM, YC, and VJ assisted with the experiments. MD, WP, SC, ZC, Hdt, LZ, YZ, JZ, and JZ designed the research plan. JZ and JZ wrote the paper. All authors read and approved the final manuscript.

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