Figures and figure supplements

Epigenetic regulation of hematopoiesis by DNA methylation

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Figure 1. The DNA methyltransferase3bb.1 gene is expressed in developing hematopoietic stem and progenitor cells and is regulated by hematopoietic stem and progenitor cell (HSPC)-specific pathways. (a) Camera lucida drawing of a 24 hpf zebrafish embryo with a red box noting the approximate region of the trunk shown in in situ hybridization images. (b,c) Whole-mount in situ hybridization of a 36 hpf zebrafish trunk probed for dnmt3bb.1, showing expression in the ventral floor of the dorsal aorta (arrows in panel c). Yellow inset box in panel b indicates the magnified area shown in panel c. (d) Diagram corresponding to panel c showing dorsal aorta (red), cardinal vein (blue), and dnmt-positive cells in the floor of the dorsal aorta (yellow). (e,f) Double in situ hybridization of a 36 hpf zebrafish trunk probed for dnmt3bb.1 (e, blue) and c-myb (f, red). The c-myb positive HSPC progenitors also stain for dnmt3bb.1 (arrows). (g) A Notch-Runx- c-myb pathway regulates HSPC emergence in the zebrafish. (h–j) In situ hybridization of 36 hpf runx1 morpholino-injected (h), control (i), or runx1 mRNA-injected (j) zebrafish trunks probed for dnmt3bb.1, showing that dnmt3bb.1 expression is reduced by runx1 knockdown (asterisks) and increased by runx1 overexpression (arrows). (k,l) In situ hybridization of 36 hpf wild type (k) or mind bomb mutant (l) zebrafish trunks probed for dnmt3bb.1, showing that dnmt3bb.1 expression seen in wild-type siblings (arrows in panel k) is strongly reduced in Notch-deficient mind bomb mutants (asterisks in panel l). (m) Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of dnmt3bb.1 transcript levels in 36 hpf (i) control, (ii) runx1W84X mutant, (iii) runx1 mRNA injected, (iv) mind bomb (mib) wild type sibling (wild type sibling of mib mutants), and (v) mib mutant zebrafish embryos. dnmt3bb.1 levels are normalized to the reference gene elf1a and to levels in controls. All graphs show mean ± standard error of the mean (SEM) and are representative of three biological replicates. Scale bars = 100 μm in b, h–l and 50 μm in c, f. DOI: http://dx.doi.org/10.7554/eLife.11813.003
Figure 1—figure supplement 1. Dmnt3bb.1 expression analysis and homology. (a) Phylogenetic tree showing zebrafish Dmnt3bb.1 is the closest homolog of human DNMT3B. (b–d) WISH showing non-hematopoietic expression of dnmt3bb.1, b, cmyb, c, and dnmt3bb.1 and cmyb, d, in the heads of 36 hpf zebrafish. (e) relative levels of dnmt3bb.1 in embryos at different developmental stages.

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Figure 2. Dnmt3bb.1 is necessary for hematopoietic gene expression. (a,b) Transmitted light images of 72 hpf control (a) and dnmt3bb.1<sup>y258</sup> (b) mutant zebrafish, showing absence of developmental delay or gross morphological abnormalities in dnmt3bb.1<sup>y258</sup> mutants. (c–h) Whole mount in situ hybridization of 36 hpf (c,d), 72 hpf (e,f) and 96 hpf (g,h) wild type (WT) sibling (c,e,g) or dnmt3bb.1<sup>y258</sup> (d,f,h) mutant animals, probed for cmyb. Cmyb is expressed in dnmt3bb.1 mutant at 36 hpf (arrows) but this expression is strongly reduced by 72 and 96 hpf (arrowheads). (i–l) Whole mount in situ hybridization of 72 hpf tails probed for l-plastin (i,j) and 5 dpf heads probed for rag1 (k,l) from WT sibling (i,k) or dnmt3bb.1 (j,l) mutant animals. Expression of both l-plastin and rag1 is present in controls (arrows) but strongly reduced in dnmt3bb.1 mutant animals (arrowheads). (m) Quantitative RT-PCR analysis of cmyb transcript levels in control (blue columns) or dnmt3bb.1 (red columns) morpholino injected animals at 36, 48, and 72 hpf. Transcript levels are normalized to the reference gene elf1a and to levels in 36 hpf control morphants. (n) Quantitative RT-PCR analysis of 3 dpf l-plastin and 5 dpf rag1 transcript levels in control (blue columns) or dnmt3bb.1 (red columns) morpholino-injected animals. Transcript levels are normalized to the reference gene elf1a and to levels in controls. All graphs in panels i and j show mean ± SEM, are representative of three biological replicates. Scale bars = 150 µm in a,b, 50 µm in c,d, 100 µm in e–l. DOI: http://dx.doi.org/10.7554/eLife.11813.005
Figure 2—figure supplement 1. TALEN-mediated Dnmt3bb.1 mutation. (a) Schematic diagram showing the position of the TALEN target site within exon 6 of dnmt3bb.1. (b) TALEN pair binding sites and spacer between them in the WT dnmt3bb.1 locus. The spacer region includes a Sac I restriction enzyme site. (c) dnmt3bb.1^y258 mutation with an 11 nucleotide deletion resulting in loss of the Sac I site. (d) The dnmt3bb.1^y258 mutation causes a frame shift after amino acid 166 that results in a truncated polypeptide of 172 amino acids lacking the DNA methylase domain as well as other important functional domains. (e) A representative genotyping gel containing PCR products digested with SacI, amplified from genomic DNA prepared from individual 72 hpf WT, dnmt3bb.1^y258 heterozygous, and dnmt3bb.1^y258 homozygous mutant embryos after blind scoring for cmyb expression by in situ hybridization. Eight out of 32 embryos scored showed weak expression of cmyb at 72 hpf, and all eight were homozygous mutants while the rest were wild type or heterozygotes.

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Figure 2—figure supplement 2. Dnmt3bb.1 morpholino phenocopies TALEN-induced mutant defects. (a) Schematic diagram showing the splice acceptor morpholino target site at the dnmt3bb.1 intron 3-Exon 4 boundary (red line) and positions of primers used to detect splicing changes (blue arrows). (b) RT-PCR gel image showing loss of correctly spliced dnmt3bb.1 transcripts in splice acceptor morpholino-injected animals. (c–f) WISH of 36 hpf trunks (c,d) and 72 hpf tails (e,f) from control (c,e) and dnmt3bb.1 (d,f) morpholino injected animals, probed for cmyb. cmyb is expressed in dnmt3bb.1 morphants at 36 hpf (arrows) but the expression is lost by 72 hpf (arrowheads). (g–j) WISH of 72 hpf tails probed for l-plastin (g,i) and 5 dpf heads probed for rag1 (h,j) from control (g,h) or dnmt3bb.1 (i,j) morpholino-injected animals. (k–n) WISH of 5 dpf embryos probed for gata1 expression in control (k,m) and dnmt3bb.1 (l,n) morpholino injected embryos. m,n, higher magnification images of highlighted areas from k,l. (o) Quantitative RT-PCR analysis at 5 dpf lck, ikaros and eGFP transcript levels in WT siblings (red columns), dnmt3bb.1 mutants (green columns), control morpholino (blue columns) or dnmt3bb.1 (yellow columns) morpholino-injected animals. Transcript levels are normalized to the reference gene elf1a and to levels in controls. (p,q) WISH of 5 dpf embryos probed for ikaros expression in control (p) and dnmt3bb.1 (q) morpholino injected embryos. Expression of i- plastin, rag1, gata1 and ikaros is present in controls (arrows) but reduced in dnmt3bb.1 morpholino-injected animals (arrowheads). (r,s) Confocal images of 5 dpf Tg(lck:gfp) embryos injected with control (q) or dnmt3bb.1 morpholinos. Scale bars 50 μm in d, 100 μm in f,i,j,n,p, 25 μm in r.

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Figure 2—figure supplement 3. HSPC apoptosis but no gross morphological and vascular defects in dnmt3bb.1 morphants. (a,b) Immunohistochemical staining of the trunks of 48 hpf control (a) and dnmt3bb.1 (b) morpholino-injected Tg(cmyb:gfp) transgenic zebrafish with anti-GFP (green) and anti-caspase3 (red) antibodies, showing increased numbers of caspase3-positive HSPC in dnmt3bb.1-deficient animals. (c) Quantitation of the percentage of GFP (cmyb) positive HSPCs that are also caspase3-positive in 48 hpf control or dnmt3bb.1 morpholino-injected Tg(cmyb:gfp) transgenic zebrafish. Columns show mean ± SEM, and statistical significance calculated by paired t-test is shown (p = 0.0012). (d,e) Transmitted light images of whole 36 hpf control (d) and dnmt3bb.1 (e) morpholino-injected zebrafish, showing absence of developmental delay or gross morphological abnormalities in dnmt3bb.1 morphants. (f,g) Confocal images of trunk EGFP fluorescence in 36 hpf control (f) or dnmt3bb.1 (g) morpholino-injected Tg(fli1a:egfp)y1 transgenic zebrafish, showing normal trunk vessel patterning in dnmt3bb.1 morphants. (h,i) Whole mount in situ hybridization of the trunks of 36 hpf control (h) or dnmt3bb.1 (i) morpholino-injected zebrafish probed for vecdn, showing normal expression in dnmt3bb.1 morphants. All images are lateral views, rostral to the left. Scale bars 25 μm in b, 100 μm in e and 50 μm in g,i.

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Figure 3. Dnmt3bb.1s necessary for epigenetic regulation of hematopoietic gene expression. (a) Schematic diagram showing the experimental procedure for the isolation of GFP/mApple double-positive HSPCs from cmyb:GFP, kdrl:mApple double transgenic zebrafish and use of DNA and RNA from these HSPC for next-gen sequencing projects. (b) RNAseq analysis showing differentially expressed endothelial and hematopoietic genes in dnmt3bb.1 deficient HSPCs. (c) qRT-PCR analysis of selected genes from the RNAseq analysis. (d) Ingenuity Pathway Analysis (IPA) of the RNAseq data showing top pathways up- or down-regulated in dnmt3bb.1-deficient HSPCs. (e) Top five HSC-expressed genes with the most significantly reduced DNA methylation and expression from bisulfite and RNAseq analysis, respectively, as well as the top 2 endothelial genes. (f,g) Bisulfite sequencing analysis of DNA methylation at the cmyb intron 1 (f) and runx1 exon 3 (g) CpG islands in DNA isolated from control (Ctrl MO, top) and dnmt3bb.1 (‘Dnmt3bb.1 MO’, bottom) morpholino-injected HSPCs, showing strongly reduced methylation of the cmyb CpG island but not the runx1 CpG island in dnmt3bb.1-deficient animals.

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The following source data is available for figure 3:

**Source data 1.** Hematopoietic and Vascular genes with reduced methylation and altered expression in dnmt3bb.1 morpholino-injected animals.

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Figure 3—figure supplement 1. Analysis of RNA seq and DNA methylation. (a) Schematic diagram depicting the workflow for global RNAseq and DNA methylation (RRBS) analysis of control or dnmt3bb.1 deficient HSPCs. (b) RNA seq analysis showing upregulation of cell death genes in dnmt3bb.1-deficient HSPCs. (c,d) Bisulfite sequencing of CpG islands from hematopoietic (c) and endothelial (d) genes identified in the RNAseq analysis including hematopoietic genes gata3, lmo2, erg, bmp4 and runx1, c, and endothelial genes dll4, etv2, cldn5b and cdh5, d. None of the genes showed changes in methylation of their CpG islands.

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Figure 4. Measurement of DNA methylation by bisulfite sequencing of gene body CpG islands from all known hematopoietic genes. (a) Schematic diagram showing the method used to collect blood from 5–7 dpf embryos for bisulfite analysis. (b–k) Bisulfite sequencing of blood cell genomic DNA from 5–7 dpf control (top) and dnmt3bb.1 (bottom) morpholino-injected animals. The results are shown for ten different sequences identified in a genome-wide screen for gene body CpG islands methylated in blood cells: cmyb (b), runx1 (c), CCAT/enhancer binding protein 1-Exon 1 (d), Kruppel-like factor 4-Exon 3 (e), Nuclear receptor co-repressor-2-Exon 37 (f), RALBP1 associated domain containing 2-Exon 2 (g), short stature homebox-Exon 5 (h), solute carrier family 12-Exon 1 (i) and solute carrier family 8–5’ UTR (j), lamin a-Exon 7 (k). Open circles represent unmethylated and filled circles represent methylated cytosine residues from CpG dinucleotides.

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Figure 5. Dnmt3bb.1g is sufficient for cmyb gene expression in the endothelium. (a) Schematic diagram of the I-Sce1(kdrl:dnmt3bb.1-gfp) transgene used for the pan-endothelial expression of a dnmt3bb.1-gfp fusion protein. (b) Camera lucida drawing of a 24 hpf zebrafish embryo with red, blue and green boxes noting the approximate regions of the head, trunk and trunk/tail shown in in situ hybridization images in panels c-h. (c,e) Double whole-mount in situ hybridization of the head (e) and trunk (c) of 36 hpf I-Sce1(kdrl:dnmt3bb.1-gfp) transgene-injected zebrafish probed for gfp (blue) and cmyb (red). (d,f) fluorescence images of in situ hybridization corresponding to panels c and e, respectively. In panels c-e, endothelial cells expressing both the gfp transgene and cmyb are noted with arrows, while normal trunk HSC expressing only cmyb but not gfp are noted with arrowheads. (g, h) Double whole-mount in situ hybridization of the trunk/tail of a 36 hpf runx1 mutant (g) and a 36 hpf runx1 mutant injected with I-Sce1(kdrl:dnmt3bb.1-gfp) transgene (h), probed for cmyb (blue) and gfp (red). The cmyb gene is not expressed in runx1 mutants, but injection of dnmt3bb.1-gfp fusion protein into runx1 mutants results in the appearance of cmyb/gfp double-positive cells. Scale bars 25 μm in e,f and 100 μm in h.

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The following source data is available for figure 5:

Source data 1. Mosaic expression of dnmt3bb.1:gfp in endothelial cells induces cmyb expression.
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Figure 6. Dnmt3bb.1 is sufficient for HSPC and downstream lineage gene expression. (a) Schematic diagram of the Tol2(hsp70:dnmt3bb.1-gfp) transgene used for ubiquitous heat shock-inducible expression of dnmt3bb.1-gfp fusion protein. (b) Camera lucida drawing showing the experimental protocol employed for early embryonic induction of dnmt3bb.1. One cell-stage embryos are injected with Tol2(hsp70:dnmt3bb.1-gfp) transgene +/- cmyb morpholino (MO), raised to 3 hpf (mid-blastula stage), heat shocked, allowed to further develop to 6 hpf (shield stage), then either fixed for whole mount in situ hybridization (WISH) or collected to prepare RNA for RT-qPCR. (c–j) Whole mount in situ hybridization of 6 hpf control Tol2(hsp70:gfp) transgene-injected (c–e), Tol2(hsp70:dnmt3bb.1-gfp) transgene-injected (f–j), or Tol2(hsp70:dnmt3bb.1-gfp) transgene plus cmyb morpholino (MO)-injected (i,j) zebrafish embryos, probed for cmyb (c,f), rag1 (d,g,i), or l-plastin (e,h,j). (k) Quantitative RT-PCR analysis of cmyb, l-plastin and rag1 expression in control and dnmt3bb.1 overexpressing embryos. (l) Schematic drawing showing the experimental protocol employed for early embryonic induction of dnmt3bb.1-gfp and subsequent transplantation.
of cells into the circulation. One cell-stage embryos were injected with Tol2(hsp70:egfp) or Tol2(hsp70:dnmt3bb.1-egfp) transgenes, raised to 3 hpf (mid-blastula stage), heat shocked, and allowed to develop to 5 hpf, at which point the embryos were dissociated and cell suspensions was prepared. Dissociated cells were injected into the circulation of 48 hpf host larvae using a borosilicate needle without filament. (m) Camera lucida drawing of a 72 hpf zebrafish embryo with a green box noting the approximate regions containing the thymus. (n–p) Confocal images of a 5 dpf Tg(lck:GFP) host animal showing transplanted cells colonizing the thymus. Transmitted light image (n), green fluorescent lck:gfp positive host thymus (n,o), and red fluorescent rhodamine-dextran positive donor cells populating the thymus (p). (q) Quantitation of the number of control GFP and dnmt3bb.1-GFP expressing cells in the thymus and heart/aortic arch region. Images in panels c-f,r,s are dorsal views and panels n-p are lateral views. Scale bars = 150 μm in c-j, 25 μm in n, 10 μm in o.

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Figure 6—figure supplement 1. Dnmt3bb1 induces hematopoietic but not endothelial gene expression in early embryos. (a-l) Whole mount in situ hybridization of 6 hpf control Tol2(hsp70:gfp) (a,c,e,g,i,k) or Tol2 (hsp70:dnmt3bb1:gfp) (b,d,f,h,j,l) transgene-injected zebrafish embryos, probed for hbae1 (a,b), ikaros (c,d), lck (e,f), rag2 (g,h), cdh5 (i,j) and etsrp (k,l) All panels show animal pole views. Scale bar 150 μm in l.
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Figure 7. Dnmt3bb.1 induces methylation of cmyb exon 1 CpG island DNA in early embryos. (a,b) Bisulfite sequencing of genomic DNA from 6 hpf control Tol2(hsp70:gfp) transgene- or Tol2(hsp70:dnmt3bb.1-gfp) transgene-injected embryos to assess methylation of the cmyb intron 1 CpG island (a) or cmyb 5′ CpG island (b). Open circles represent unmethylated cytosine residues and filled circles represent methylated cytosine residues from CpG dinucleotides. Methylation of the intron 1 CpG island is detected in dnmt3bb.1-gfp expressing embryos, but not in control gfp-expressing embryos. (c–e) Chromatin immunoprecipitation (CHIP) using anti-GFP or anti-methylcytosine antibodies to detect Dnmt3bb.a-GFP (or TCF-GFP control) fusion protein binding or cytosine methylation in CpG islands (CGI), respectively. (c) Schematic diagram showing the experimental procedure used for CHIP analysis, (d) Anti-GFP CHIP with either no added chromatin (negative control; column 1) or with chromatin from either hsp:dnmt3bb.1-GFP injected embryos (column 2) or from hsp:TCF-GFP injected embryos (column 3; CHIP positive control). Column 4 is 10% of input chromatin from hsp:dnmt3bb.1-GFP injected embryos. (e) Anti-methylcytosine CHIP with either no added chromatin (negative control; column 1) or using chromatin from hsp:GFP injected embryos (column 2; negative control for dnmt3bb.1-gfp methylation) or from hsp:dnmt3bb.1-GFP injected embryos.
Figure 7 continued

hsp:dnmt3bb.1-GFP -injected embryos (column 3). Column 4 is 10% of input chromatin from hsp:dnmt3bb.1-GFP injected embryos. Primers used for the CHIP experiments in both e and f were: (1) cmyb Intron 1 CpG Island DNA, site #1, (2) cmyb Intron 1 CpG Island DNA, site #2, (3) cmyb Exon 5 DNA (non-CpG island), (4) cmyb Intron 1 DNA (non-CpG island), (5) runx1 Exon 3 CpG island DNA, (6) ntla CpG island DNA, and (7) sox3 upstream TCF binding site DNA (non-CpG).

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Figure 8. Hematopoietic defects in adult \textit{dnmt3bb.1y258} Mutants. (a,b) Wild type sibling (a) and \textit{dnmt3bb.1y258} homozygous mutant (b) adult zebrafish. (c) Representative low-magnification sagittal section through a \textit{dnmt3bb.1y258} homozygous mutant adult zebrafish, showing the head kidney (marked an arrow and surrounded by a dashed line), a major site of hematopoiesis in adult zebrafish. (d,e) Representative higher magnification images of sagittal sections through the head kidneys of wild type sibling (d) and \textit{dnmt3bb.1y258} mutant (e) adult zebrafish, stained with hematoxylin/eosin. (f) Quantitation of the percentage of the hematopoietic (non-kidney tubule) area that is acellular in head kidney sections from wild type sibling (WT) and \textit{dnmt3bb.1y258} mutant (dnmt3bb.1y258) adult zebrafish. (g,h) Giemsa-stained blood smears from wild type sibling (g) and \textit{dnmt3bb.1y258} mutant (h) adult zebrafish. (i) Quantitation of the adult erythroid cell circularity in WT and \textit{dnmt3bb.1y258} mutant (see Materials and methods), measured from blood smears such as those in panels g and h. (j,k) Giemsa stained FACS-sorted HSPC (as in Figure 3a) from 36 hpf \textit{dnmt3bb.1} (j) or control (k) morpholino-injected zebrafish.

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Figure 8—figure supplement 1. Cmybinduces hematopoietic but not endothelial gene expression in early embryos. (a) Schematic diagram of the Tol2(hsp70:cmyb-2A-mCherry) transgene used for ubiquitous heat shock-inducible expression of cmyb. (b–q) WISH of 6 hpf control Tol2(hsp70:mCherry) (b,d,f,h,i,l,n,p) or Tol2(hsp70:cmyb-2A-mCherry) (c,e,g,k,m,o,q) transgene-injected zebrafish embryos, probed for hbae1 (b,c), rag1 (d,e), l-plastin (f, g), ikaros (h,i), lck (j,k), rag2 (l,m), cdh5 (n,o), and etsrp (p,q) All panels show animal pole views. Scale bar 150 μm in q.

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Figure 9. Model for regulation of HSPC cell fate during development. (a) Notch-Runx1 signaling controls specification of HSPCs in the ventral wall of the dorsal aorta during early embryogenesis. This signaling pathway initiates HSPC expression of both the key transcription factor Cmyb and the epigenetic regulator Dnmt3bb.1. (b) As development proceeds Runx1 expression is down-regulated and continued maintenance of active Cmyb expression in HSPC depends on Dnmt3bb.1–mediated Cmyb gene body DNA methylation, ensuring maintenance of HSPC cell fate during the Runx1-independent phase.

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