

## **Supplementary Information**

### **Nlrc3 signaling is indispensable for hematopoietic stem cell emergence via Notch signaling in vertebrates**

Shuyang Cai. et al

#### **Corresponding authors:**

\*He Huang

Professor/MD/Ph.D.

Bone Marrow Transplantation Center, The First Affiliated Hospital, School of Medicine,

E-mail: [huanghe@zju.edu.cn](mailto:huanghe@zju.edu.cn)

\*Pengxu Qian

Investigator

School of Medicine, Zhejiang University

Email: [axu@zju.edu.cn](mailto:axu@zju.edu.cn)

\*Jiahui Lu

Professor

Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai University of

Traditional Chinese Medicine

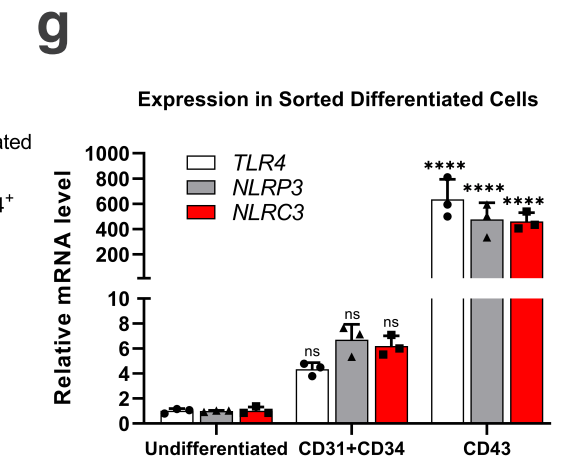
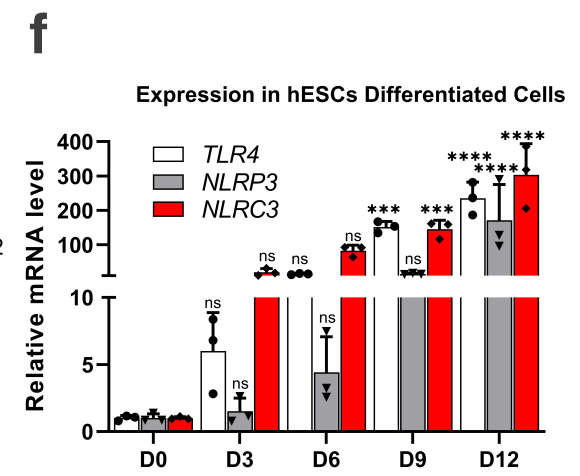
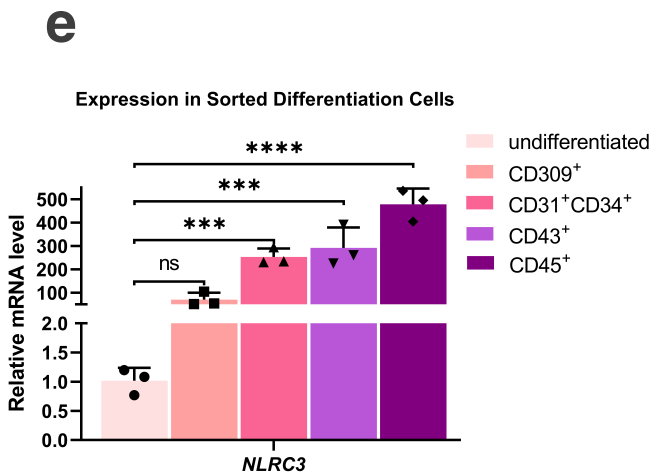
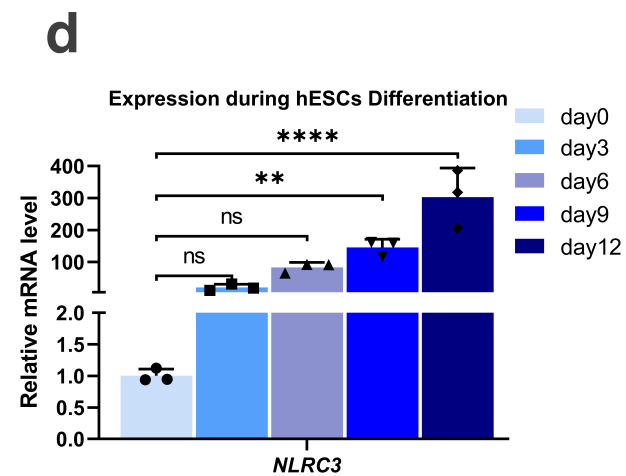
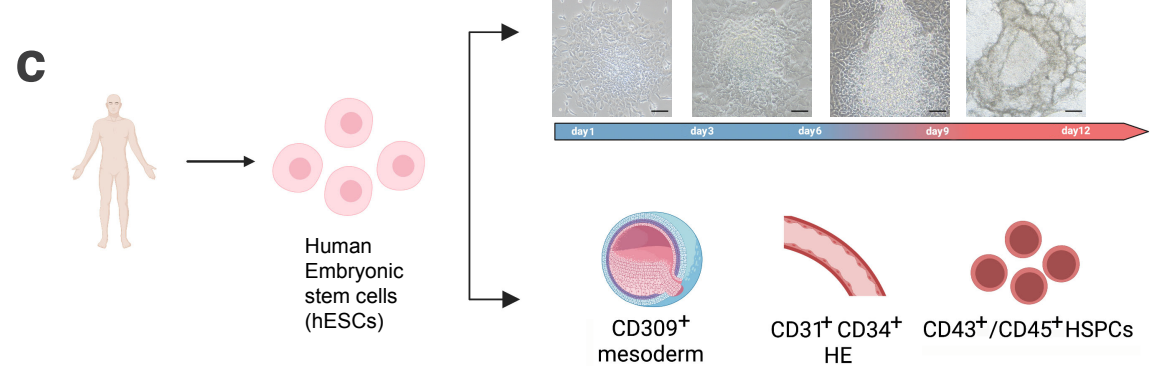
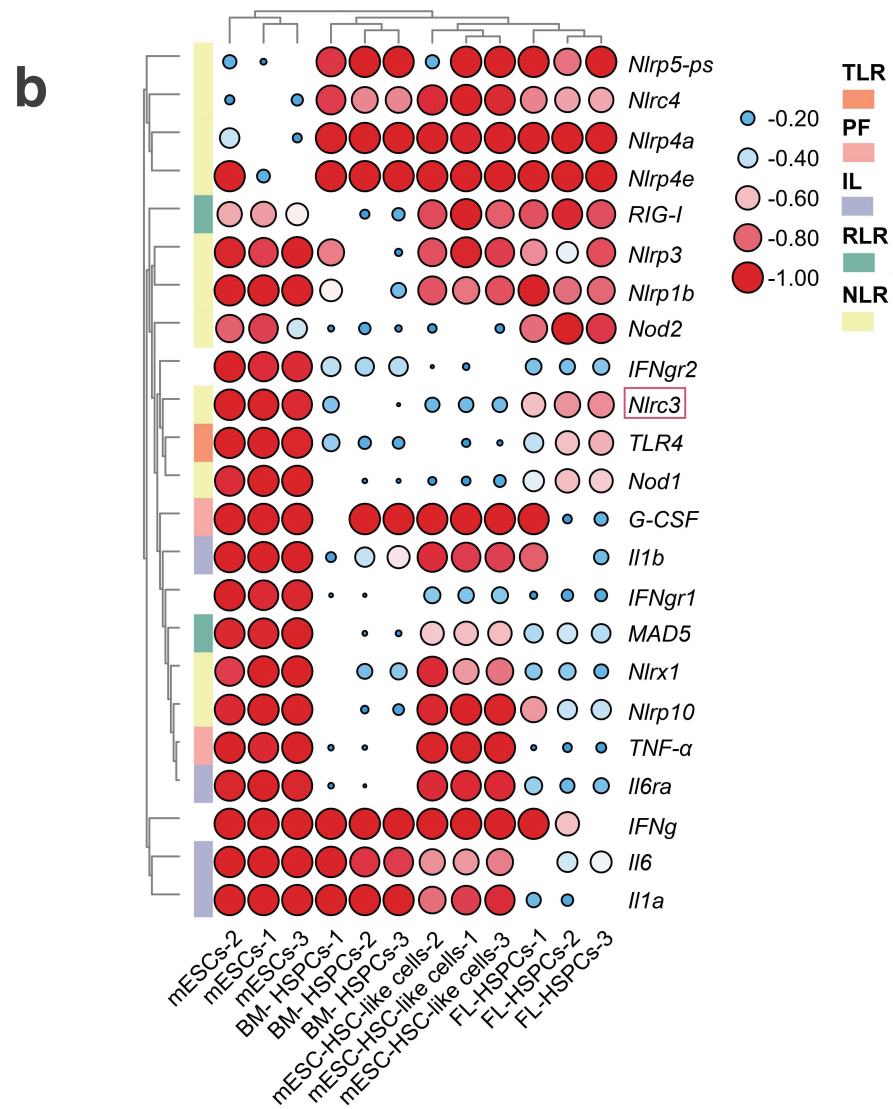
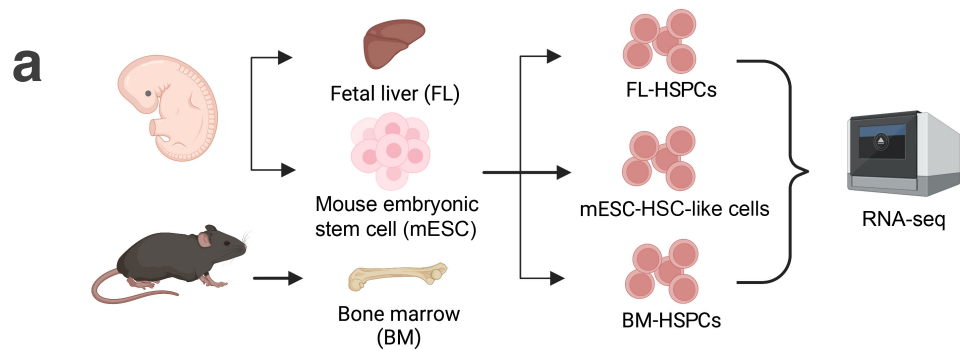
E-mail: [lujiahui\\_sci@126.com](mailto:lujiahui_sci@126.com)

#### **The PDF file includes:**

Supplementary Figure 1 to 10

Supplementary Table 1 to 5

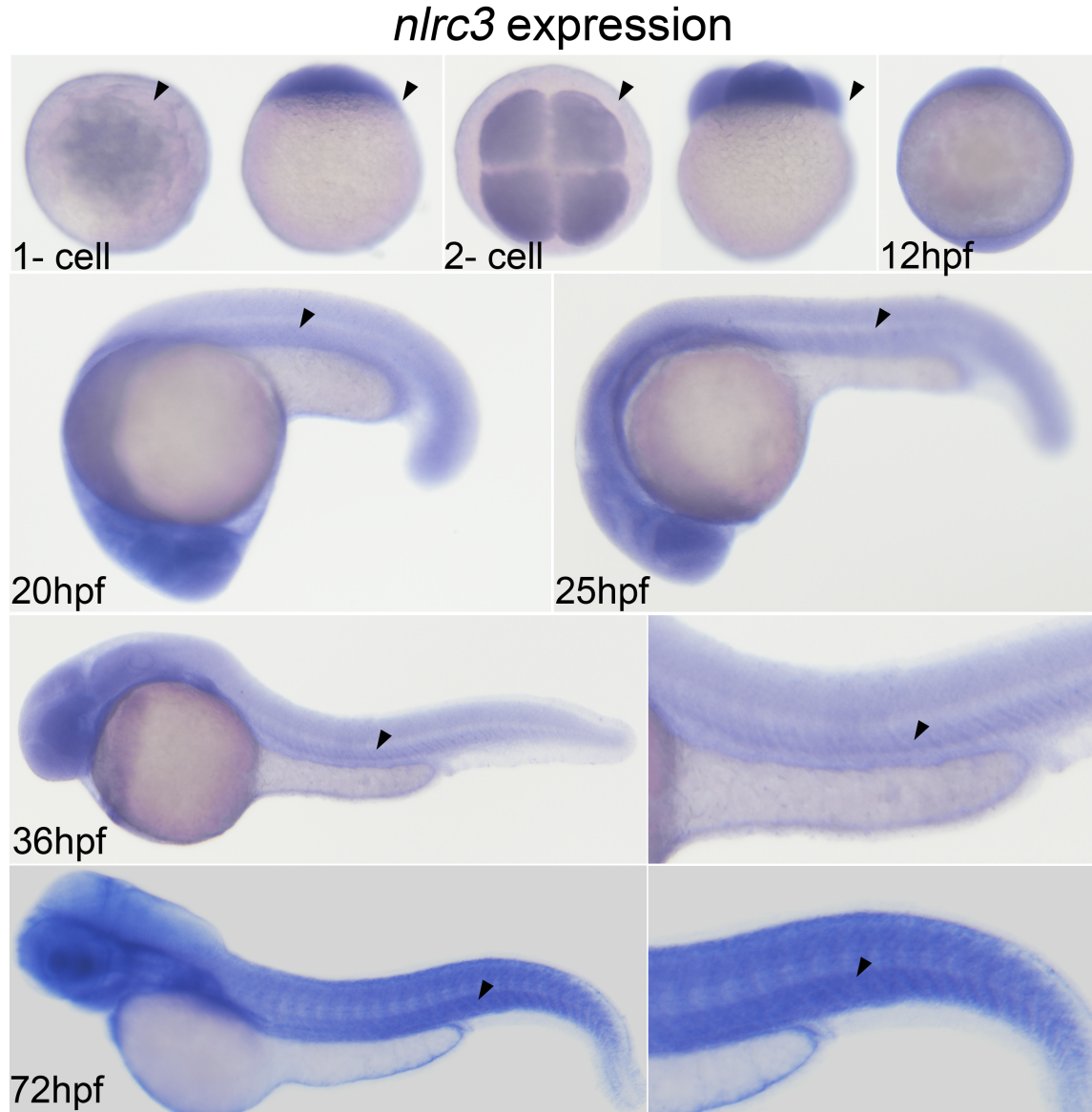
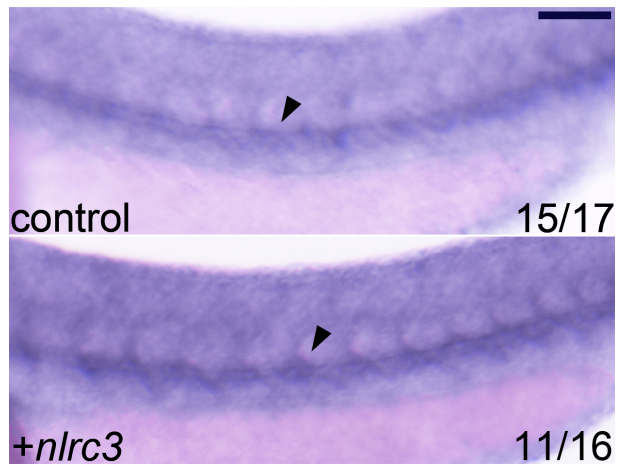
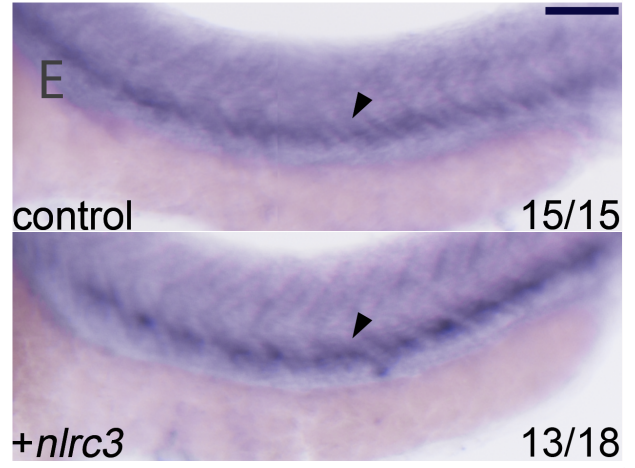
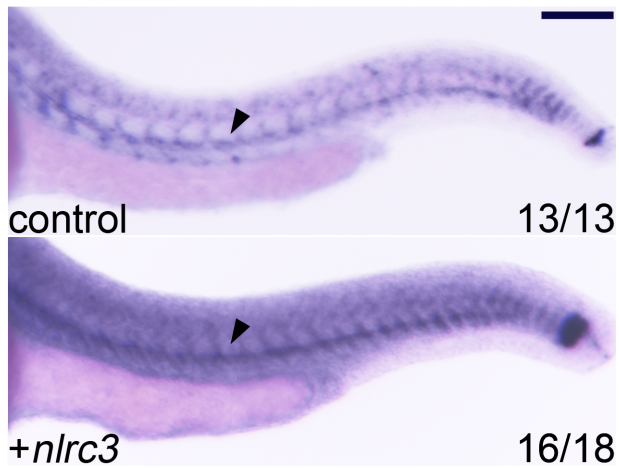
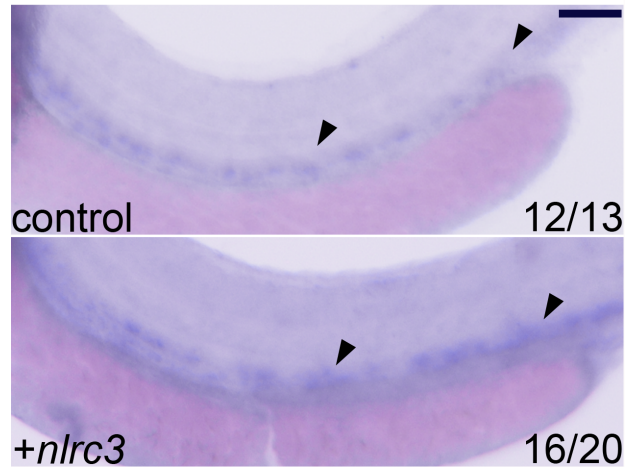
#### **Supplementary Figures**





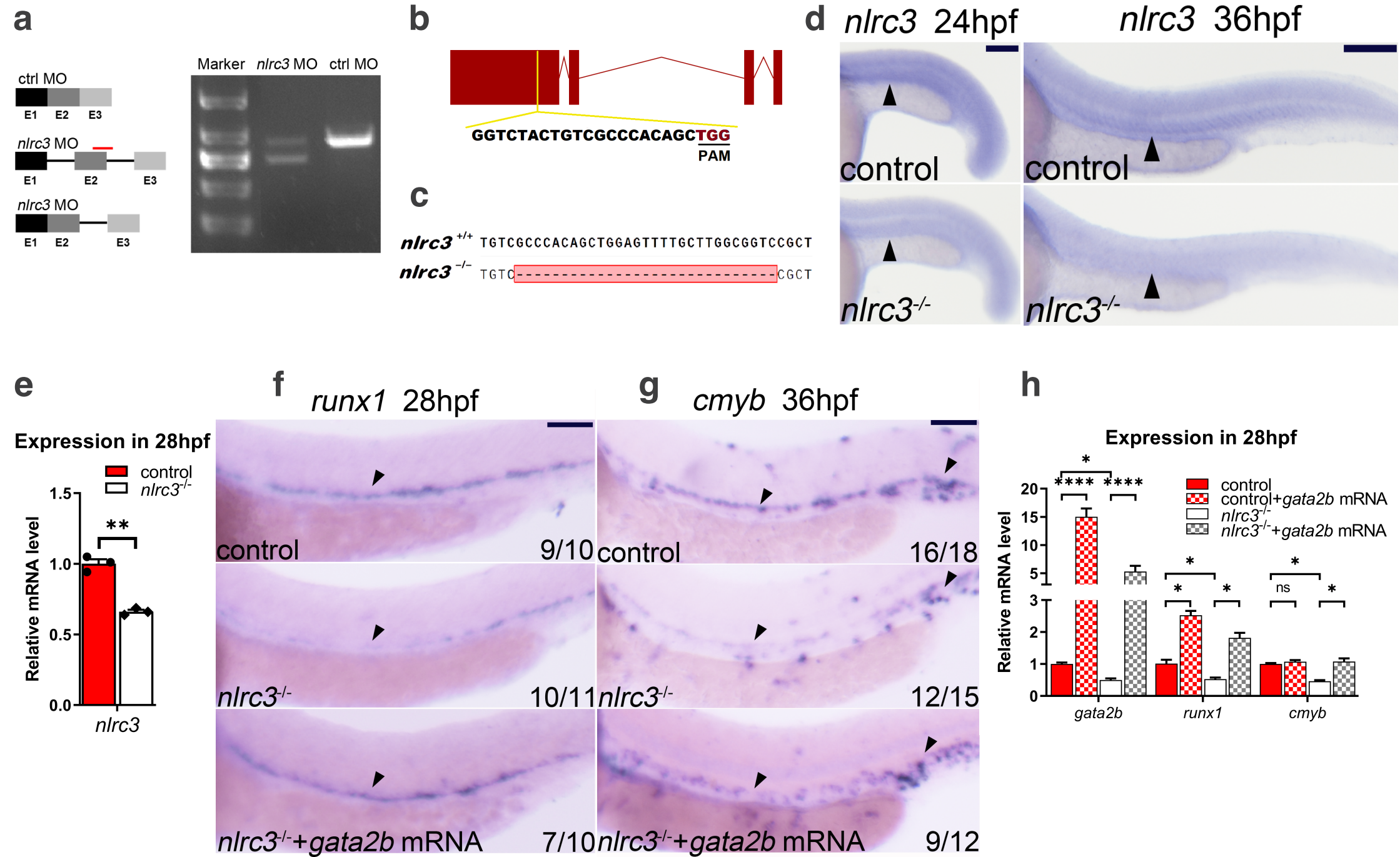
**Supplemental Figure 1. *NLRC3* is highly expressed in induced HSPC differentiated from mouse ESCs or human ESCs.**

**a** Schematic overview of mouse embryonic stem cells (ESCs) sorted for culture in various differentiation systems. **b** Bubble plot of sequencing data demonstrating the expression of nucleotide-binding domain leucine-rich repeat (NLR) family members including *Nlrc3* and related inflammatory genes as mouse ESCs matured into hematopoietic differentiation in various cell types, including undifferentiated ESCs, ESCs derived LSKCD201<sup>+</sup> HSC-like cells, fetal liver (FL) derived HSPCs and bone marrow (BM) derived HSPCs. **c** Schematic overview of the chemical defined system to induce HSPCs from hESCs. **d** Dynamic analysis of *NLRC3* expression by qPCR during hematopoietic differentiation of hESCs. Relative expression is normalized to the expression level of undifferentiated hESCs. ns = 0.9740, 0.1579, \*\*P = 0.0084, \*\*\*\*P < 0.0001. **e** qPCR analysis of *NLRC3* expression in undifferentiated hESCs, mesoderm (APLNR<sup>+</sup>), HE (CD31<sup>+</sup> and CD34<sup>+</sup>), and HSPCs (CD43<sup>+</sup> and CD45<sup>+</sup>) derived from hESCs. Relative expression normalized to the expression level in undifferentiated hESCs. ns = 0.4688, \*\*\*P = 0.0007, 0.0002, \*\*\*\*P < 0.0001. **f** Dynamic expression of *NLRC3*, *NLRP3*, and *TLR4* by qPCR during hematopoietic differentiation of hESCs. Relative expression is normalized relative to undifferentiated hESCs. ns = 0.9999, 0.9918, 0.9734, 0.0986, 0.9907, > 0.9999, \*\*\*P = 0.0004, 0.0007, \*\*\*\*P < 0.0001. **g** qPCR analysis of *NLRC3*, *NLRP3*, and *TLR4* in undifferentiated hESCs, HE (CD31<sup>+</sup> and CD34<sup>+</sup>), and HSPCs (CD43<sup>+</sup>) derived from hESCs. Relative expression is normalized to the expression levels of undifferentiated hESCs. ns = 0.9983, 0.9949, 0.9958, \*\*\*\*P < 0.0001. Error bars, mean ± s.d., the exact p-values mentioned above are listed from left to right, by using one-way ANOVA – Sidak test in **d**, **e**, two-way ANOVA with Tukey's post hoc test in **f**, **g**. *n* = 3 biological replicates in **d**, **e**, **f**, **g**. Source data are provided as a Source Data file.

**a****b***efnb2* 28hpf**d***runx1* 26hpf**c***dlc* 30hpf**e***cmyb* 36hpf

**Supplemental Figure 2. The expression of *nlrc3* pattern in zebrafish.**

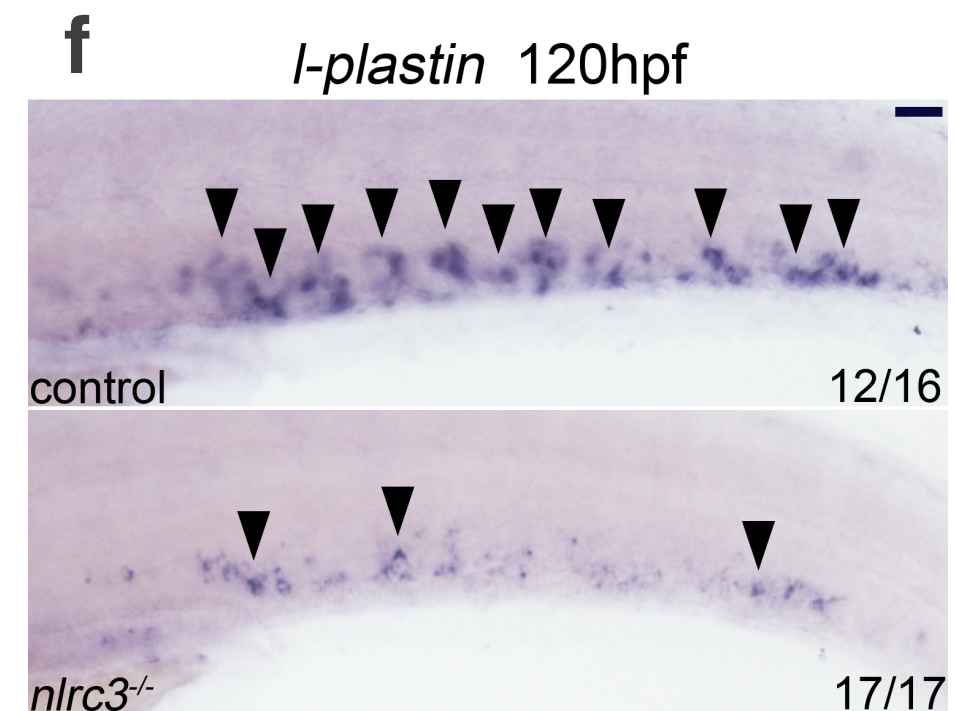
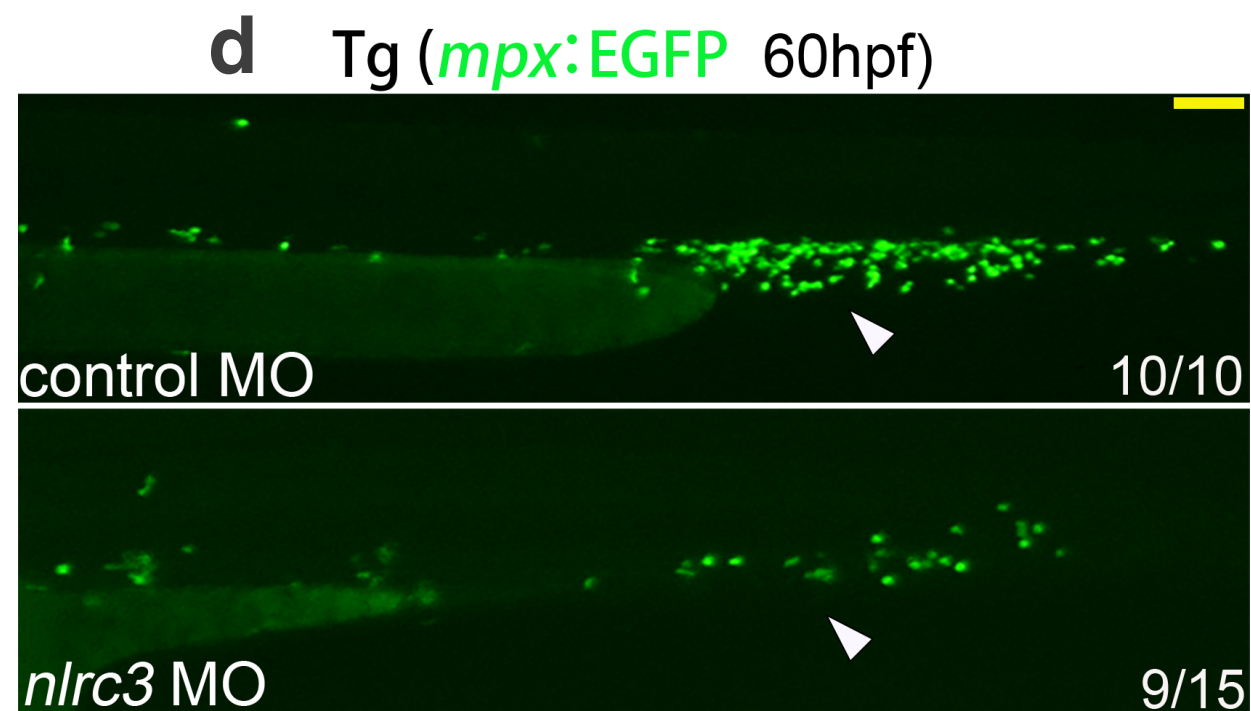
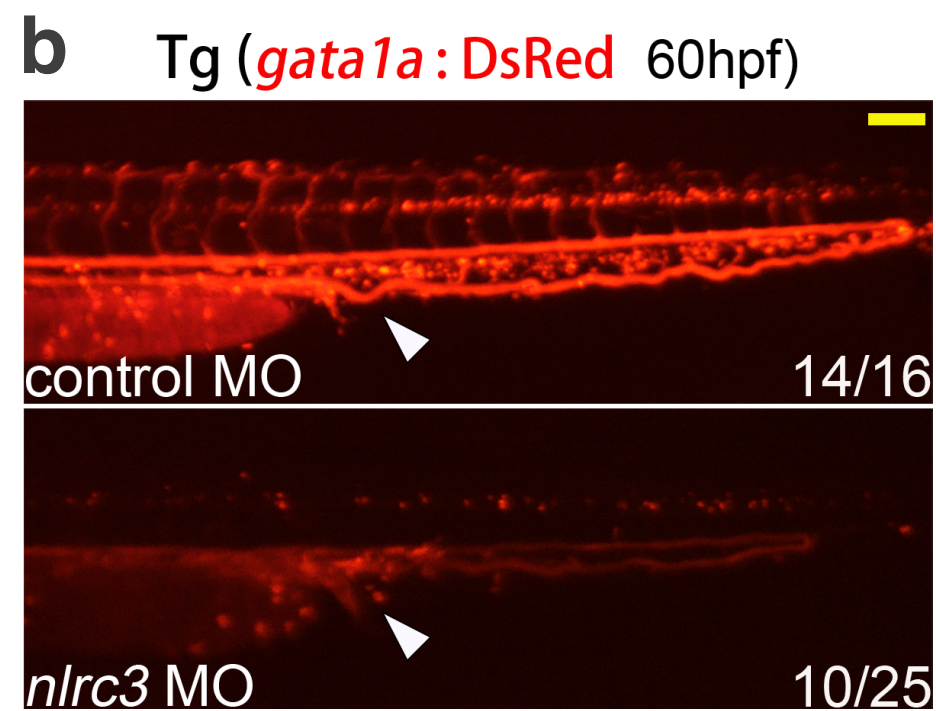
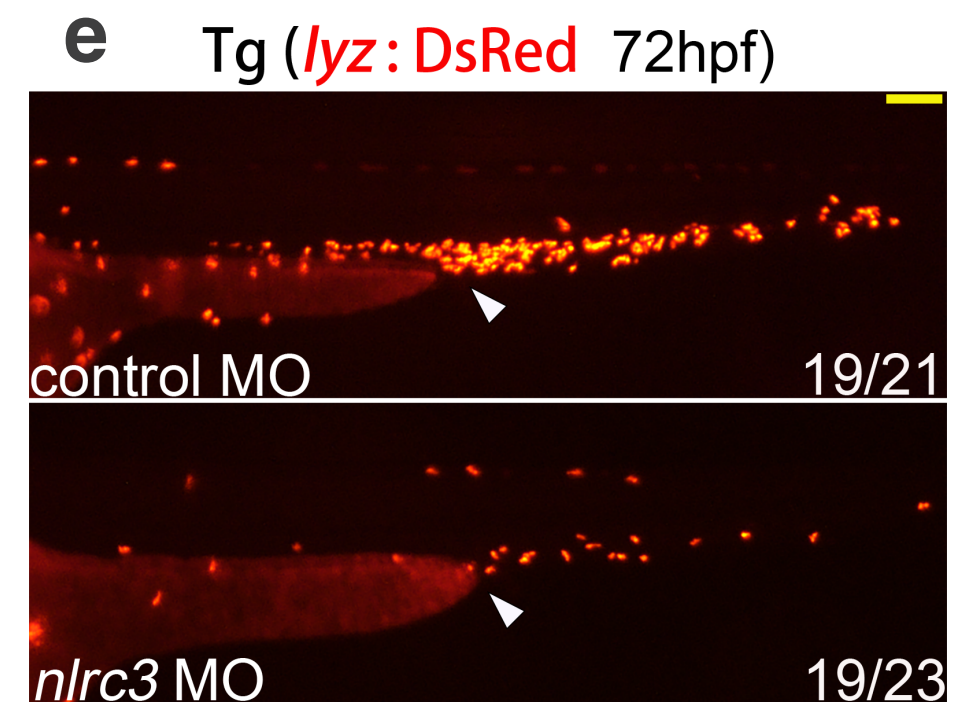
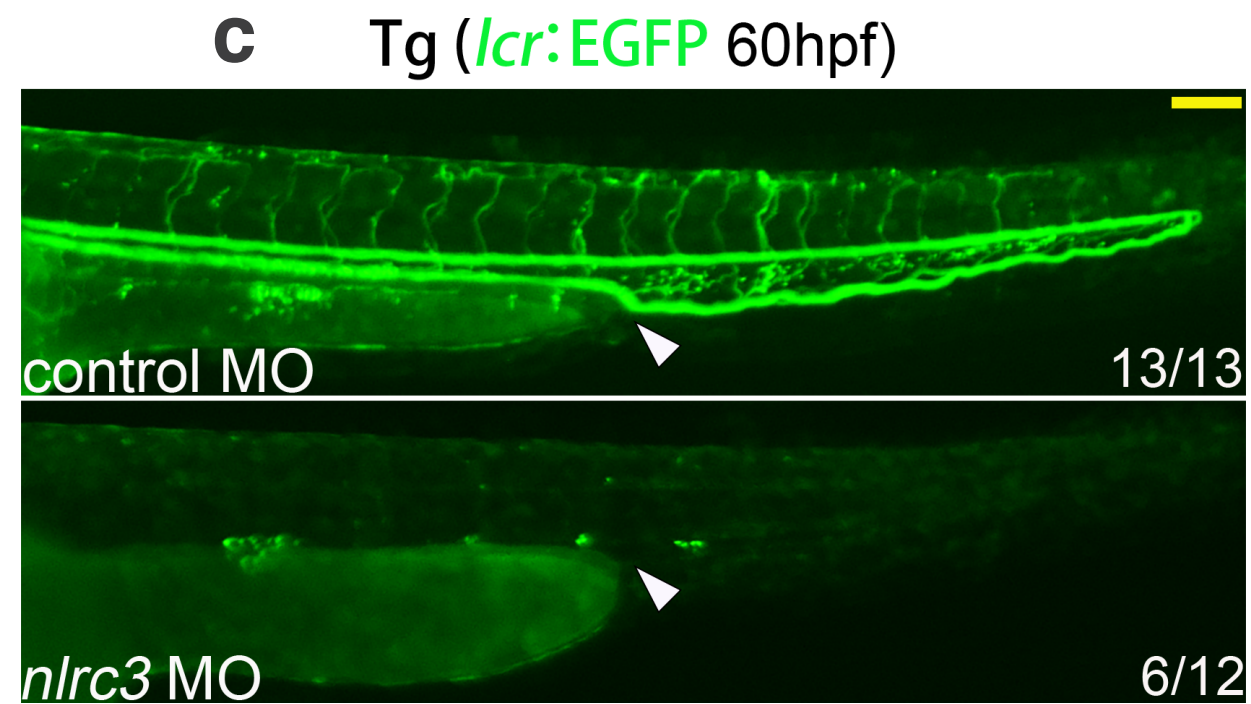
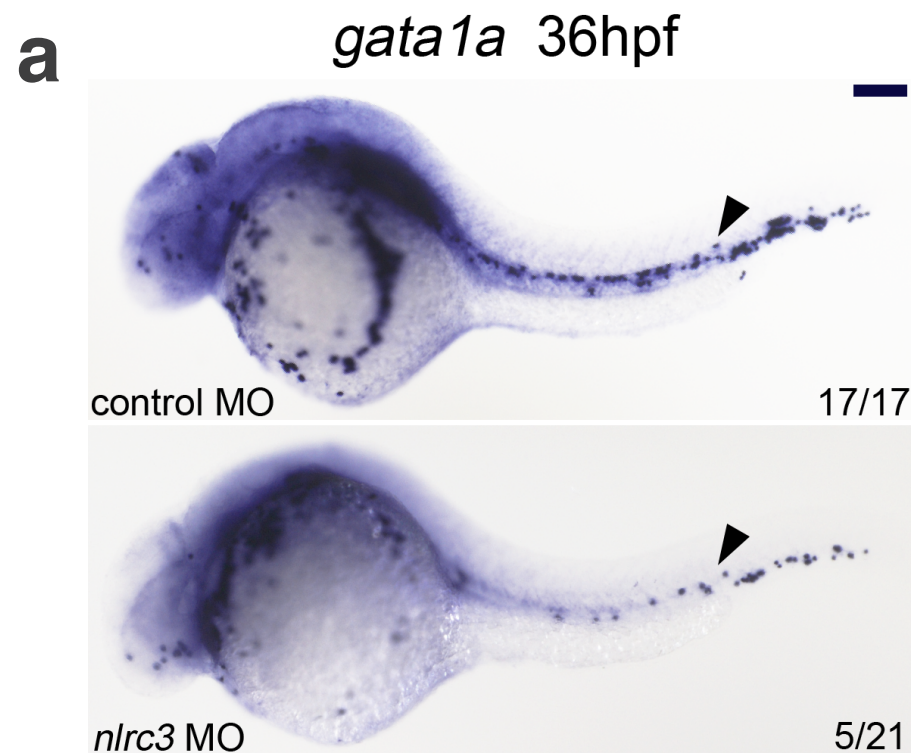
**a** Expression pattern of *nlrc3* during zebrafish embryogenesis: The stage examined by whole-mount in situ hybridization (WISH) is shown in each panel: 1 - cell stage, 4 - cell stage, 12 hours postfertilization (hpf), 20 hpf (Scale bars: 200  $\mu$  m), 25 hpf, 36 hpf and 72 hpf. **b** Expression of arterial marker *efnb2* in control embryos and control embryos with co-expression of *nlrc3* probe at 28 hpf by WISH. **c** Expression of arterial marker *dlc* in control embryos and control embryos with co-expression of *nlrc3* probe at 30 hpf by WISH. **d** Expression of HSPC marker *runx1* in control embryos and control embryos with co-expression of *nlrc3* probe at 26 hpf by WISH. **e** Expression of HSPC marker *cmyb* in control embryos and control embryos with co-expression of *nlrc3* probe at 36 hpf by WISH. Scale bars, 100  $\mu$  m in **b**, **c**, **d**, **e**. Source data are provided as a Source Data file.



**Supplemental Figure 3. Generation and validation of *nlrc3* morphants and mutants.**

**a** Schematic diagram of splice morpholino knockdown target in second exon of *nlrc3*. **b** The panel showing the target site in the second exon of *nlrc3* designed for the CRISPR–Cas9 mutants. **c** The panel revealing the 29 bp deletion in the *nlrc3* target site. **d** The expression of *nlrc3* in the dorsal aorta (DA) during EHT stage of the WT and *nlrc3* mutants. **e** Expression of *nlrc3* in control and *nlrc3* mutants at 28 hpf by qPCR. \*\*P = 0.0012. **f** Expression of HSPC marker *runx1* in control embryos, *nlrc3* mutants, *nlrc3* mutants with overexpression of *gata2b* mRNA at 28 hpf by WISH. **g** Expression of HSPC marker *cmyb* in control embryos, *nlrc3* mutants, *nlrc3* mutants with overexpression of *gata2b* mRNA at 36 hpf by WISH. **h** Expression of *gata2b*, *runx1* and *cmyb* in control, control with overexpression of *gata2b* mRNA, *nlrc3* mutants, and *nlrc3* mutants with overexpression of *gata2b* mRNA at 28 hpf by qPCR. ns = 0.9997, \*P = 0.0418, 0.0261, 0.0213, 0.0204, 0.0133, 0.0155, \*\*\*\*P < 0.0001. Error bars, mean  $\pm$  s.d., the exact p-values mentioned above are listed from left to right, by using two-tailed, unpaired student's *t*-test in **e**, **h**, *n* = 3 biological replicates in **e**, **h**. Source data are provided as a Source Data file.

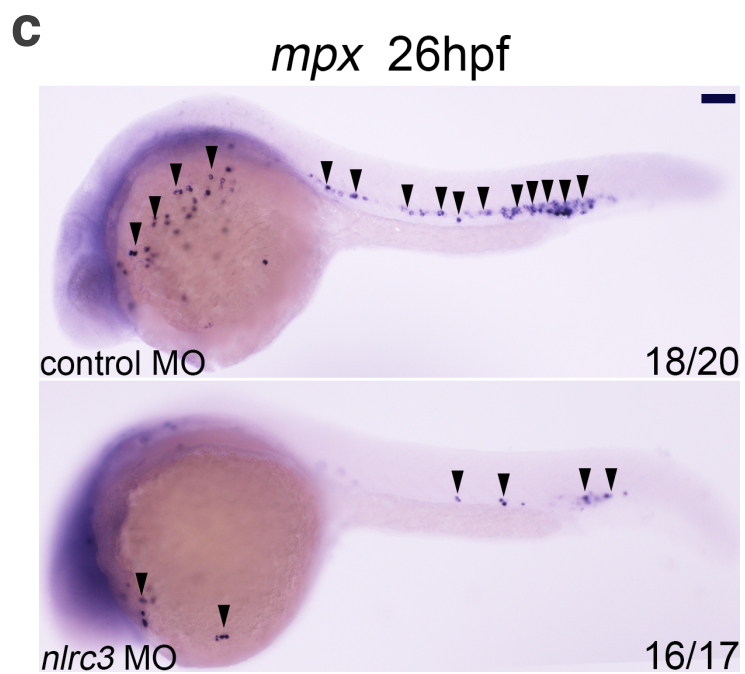
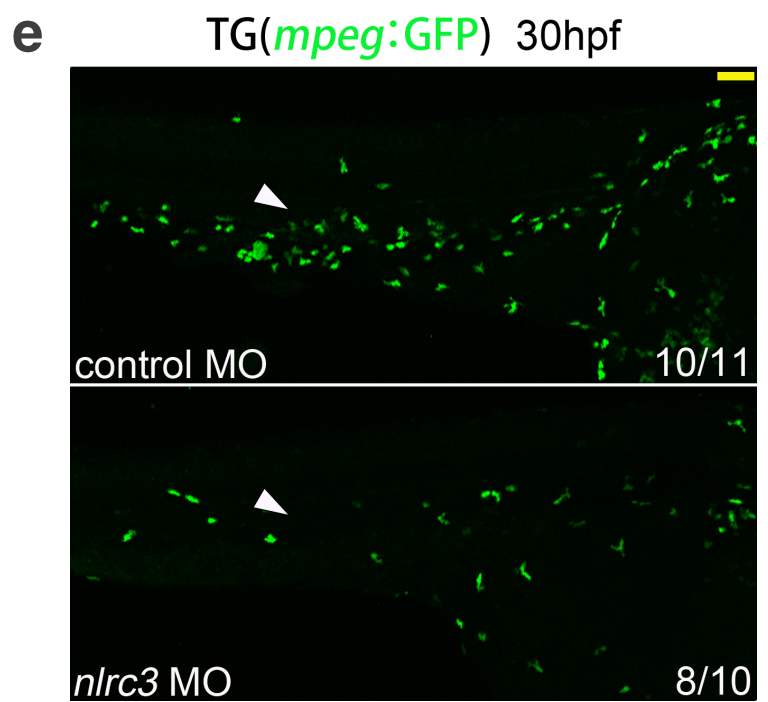
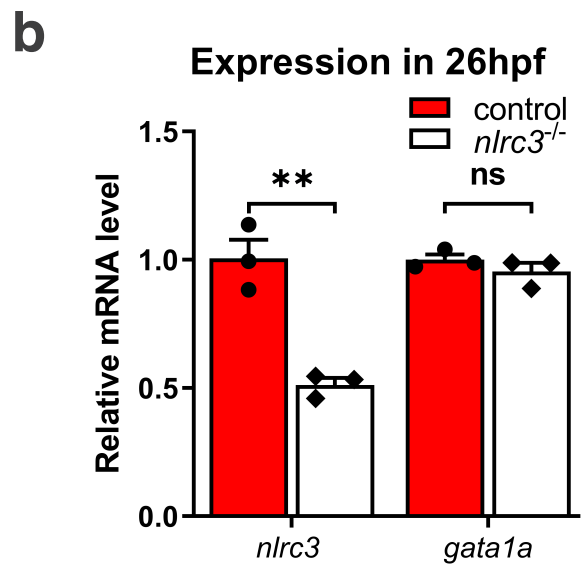
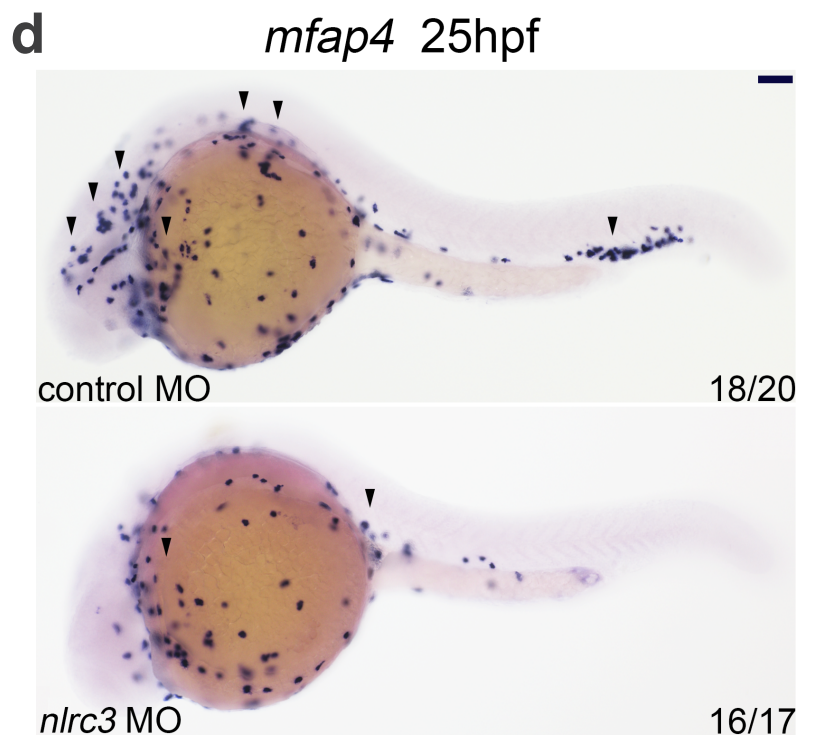






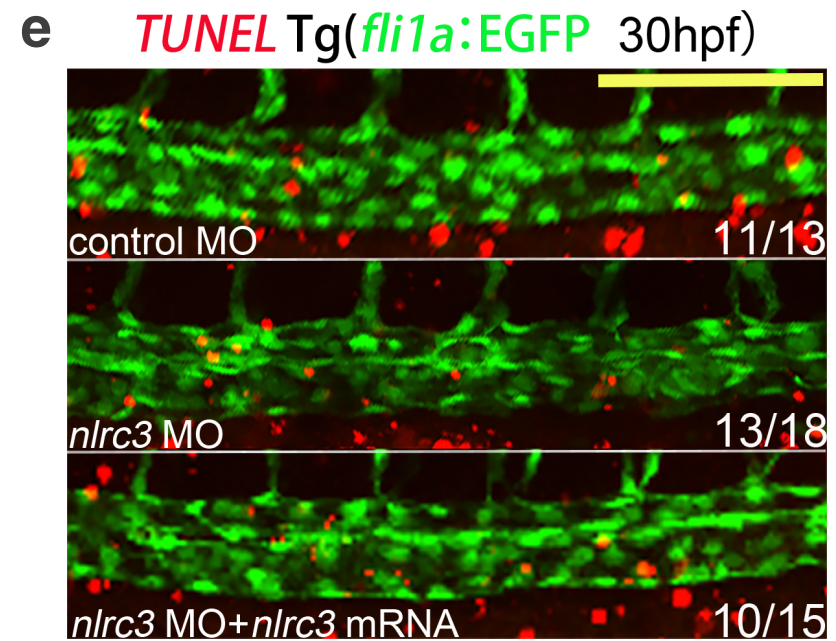
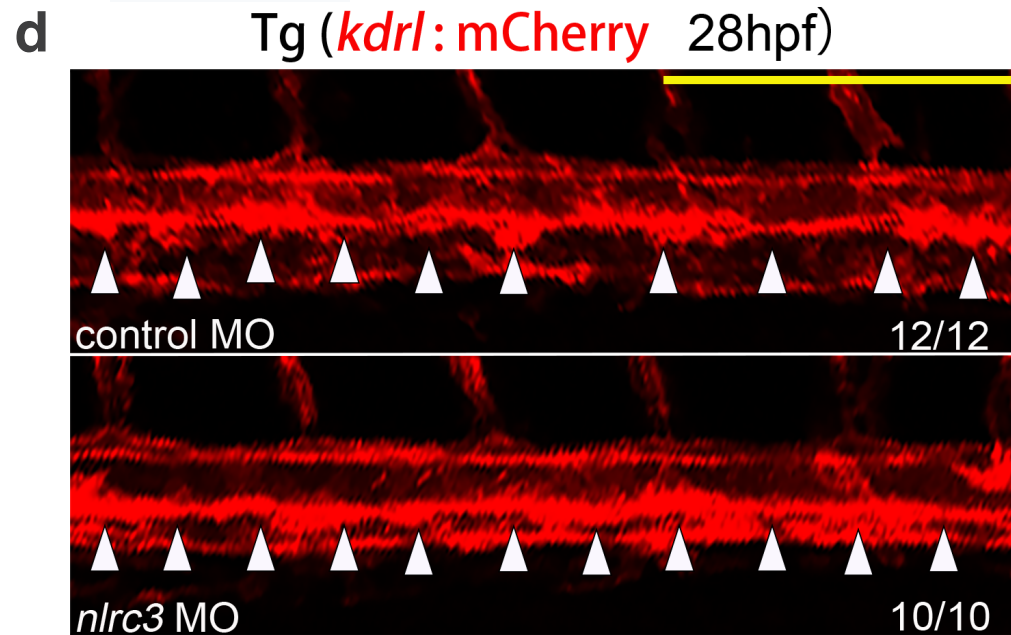
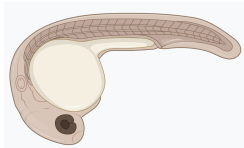
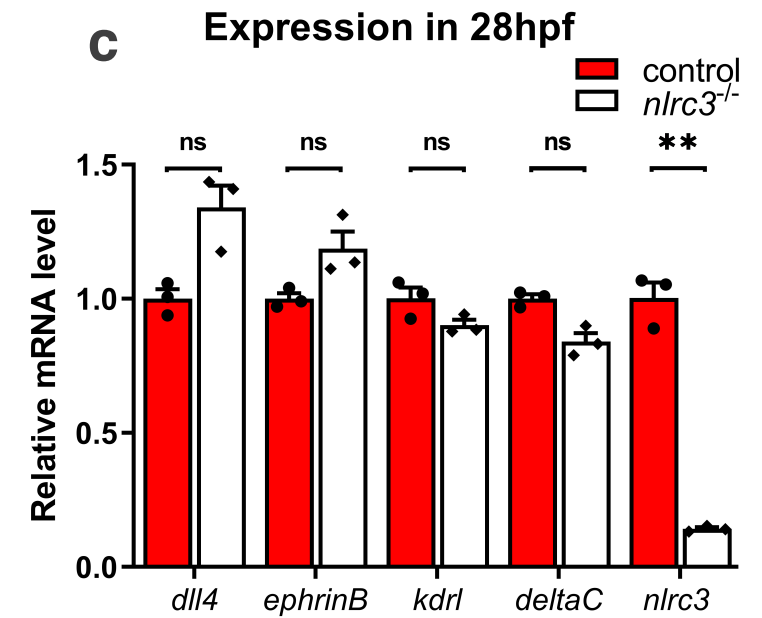
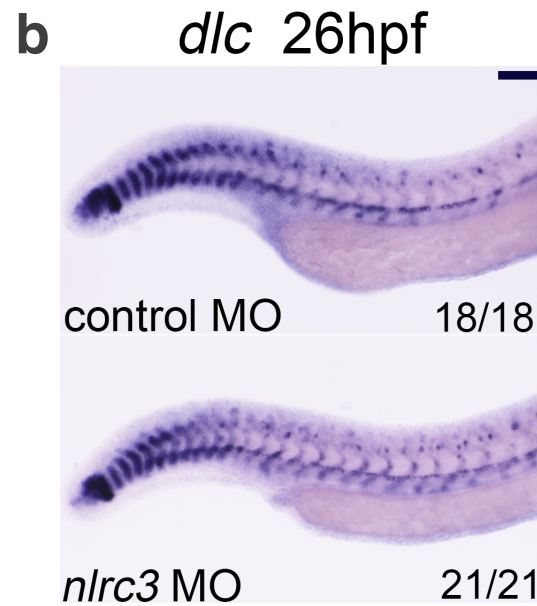
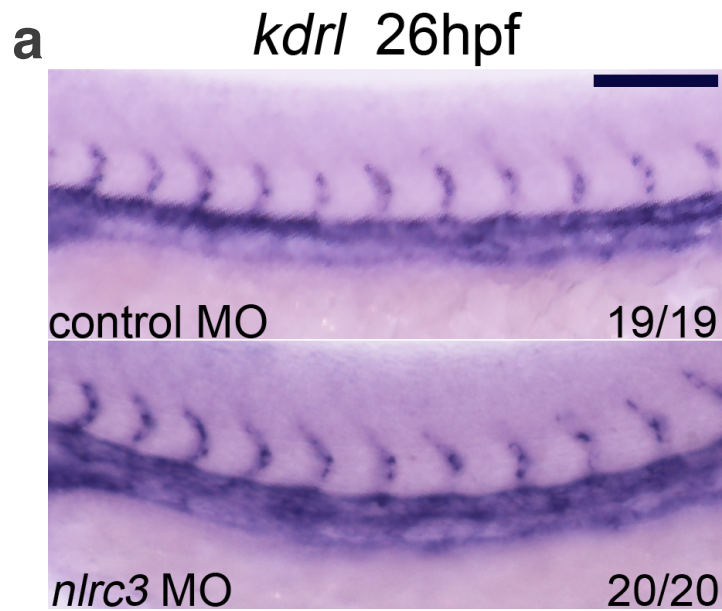
**Supplement Figure 4. *Nlrc3* signaling is imperative for HSPC differentiation in zebrafish.**

**a** Expression of erythroid marker *gata1a* in control embryos and *nlrc3* morphants at 36 hpf by WISH. **b** Snapshot of erythroid marker Tg (*gata1a*:DsRed) in control embryos and *nlrc3* morphants at 60 hpf. **c** Snapshot of erythroid marker Tg (*lcr*:EGFP) in control embryos and *nlrc3* morphants at 60 hpf. **d** Snapshot of myeloid marker Tg (*mpx*:EGFP) in control embryos and *nlrc3* morphants at 60 hpf. **e** Snapshot of myeloid marker Tg (*lyz*:DsRed) in control embryos and *nlrc3* morphants at 72 hpf. **f** Expression of myeloid marker *l-plastin* in control embryos and *nlrc3* mtants at 120 hpf by WISH. Each sample was composed of at least 5 embryos and Scale bars, 100  $\mu$ m in **a, b, c, d, e, f**. Source data are provided as a Source Data file.



**Supplement Figure 5. *Nlrc3* signaling was indispensable for primitive neutrophils and macrophages but not erythropoiesis.**

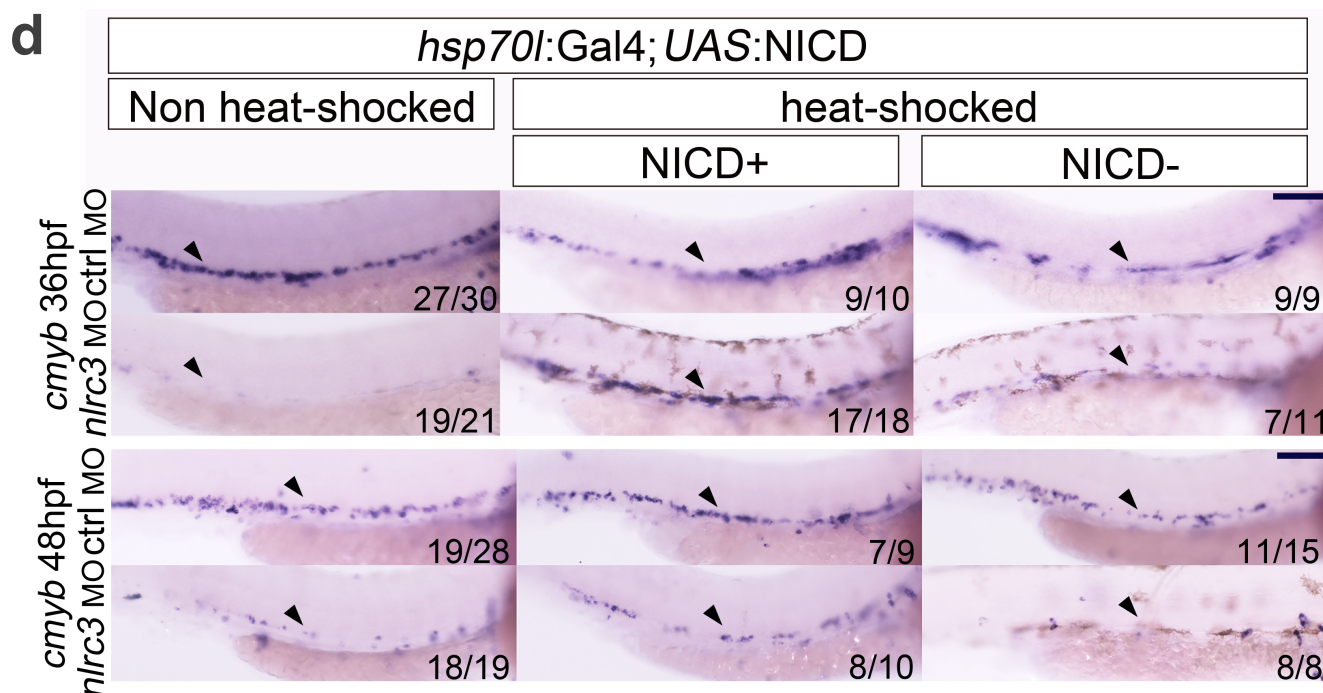
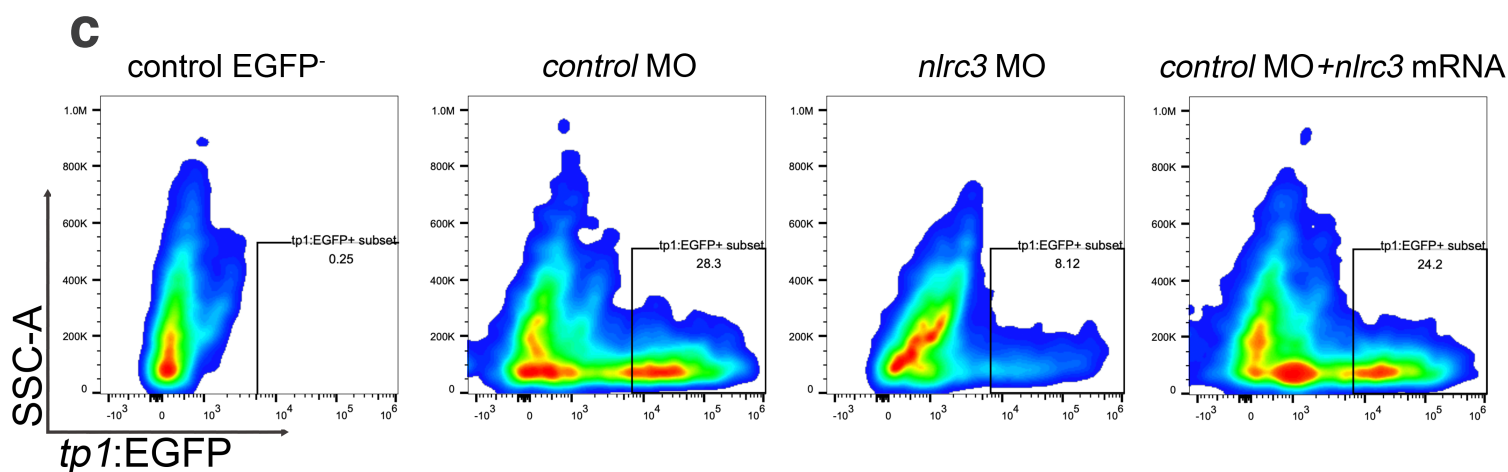
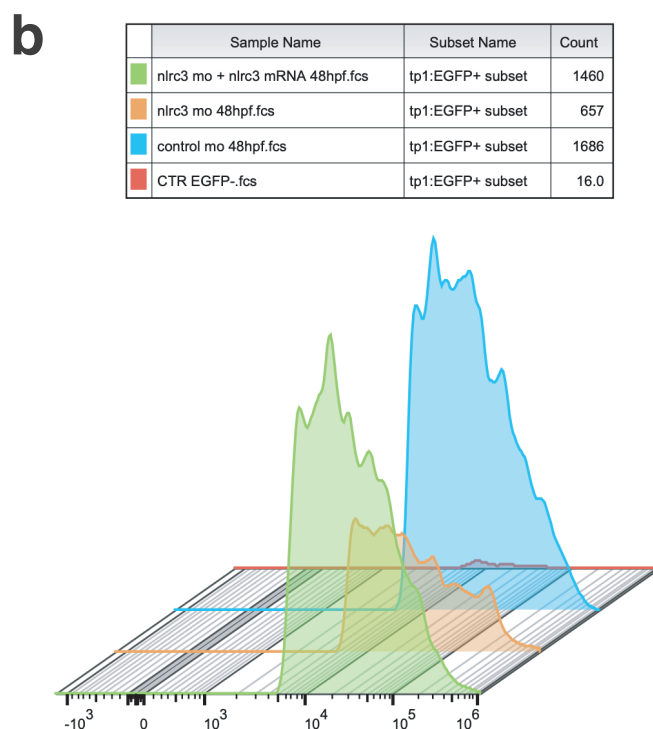
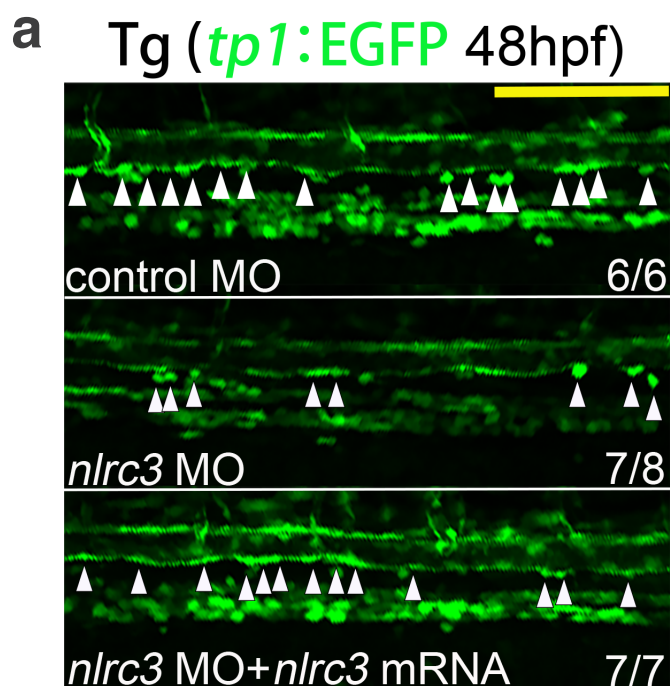
**a** Expression of erythroid marker *gata1a* in control embryos and *nlrc3* morphants at 26 hpf by WISH. **b** Expression of erythroid gene *gata1a* in control embryos and *nlrc3* mutants at 26 hpf by qPCR. ns = 0.4808, \*\*P = 0.0075. Error bars, mean  $\pm$  s.d., by using two-tailed, unpaired student's *t*-test. *n* = 3 biological replicates. **c** Expression of myeloid marker *mpx* in control embryos and *nlrc3* morphants at 26 hpf by WISH. **d** Expression of macrophage marker *mfap4* in control embryos and *nlrc3* morphants at 25 hpf by WISH. **e** Confocal imaging showing the macrophage marker *mpeg*:GFP in control embryos and *nlrc3* morphants at 30 hpf. Scale bars, 100  $\mu$ m and each sample was composed of at least 5 embryos in **a**, **c**, **d**, **e**. Source data are provided as a Source Data file.



**Supplement Figure 6. *Nlrc3*-deficient embryos displayed normal vasculogenesis and apoptosis.**

**a** Expression of endothelial marker *kdrl* in control embryos and *nlrc3* morphants at 26 hpf by WISH. **b** Expression of arterial marker *dlc* in control embryos and *nlrc3* morphants at 26 hpf by WISH. **c** Expression of endothelial and arterial gene *dll4*, *ephrinB2*, *kdrl*, and *deltaC* in control embryos and *nlrc3* mutants at 28 hpf by qPCR. ns = 0.0931, 0.1147, 0.2372, 0.0742, \*\*P = 0.0043. Error bars, mean  $\pm$  s.d., by using two-tailed, unpaired student's *t*-test. *n* = 3 biological replicates. **d** Confocal imaging showing the endothelial marker Tg (*kdrl*:mCherry) in control embryos and *nlrc3* morphants at 28 hpf. **e** Confocal imaging showing the maximum projections of the AGM region of 30 hpf Tg (*fli1a*:EGFP) embryos injected in control embryos and *nlrc3* morphants and assayed for TUNEL (red). Each sample was composed of at least 5 embryos. Scale bars, 100  $\mu$ m in **a**, **b**, **d**, **e**. Illustrations created with BioRender.com. Source data are provided as a Source Data file.

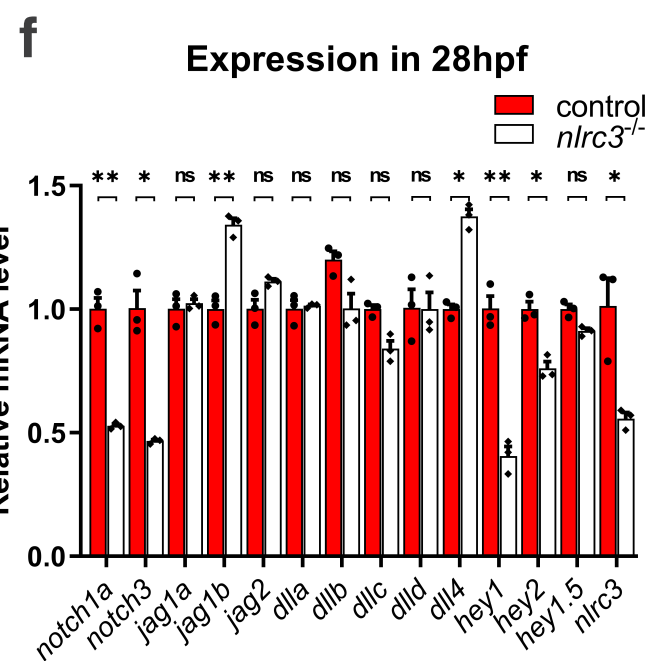
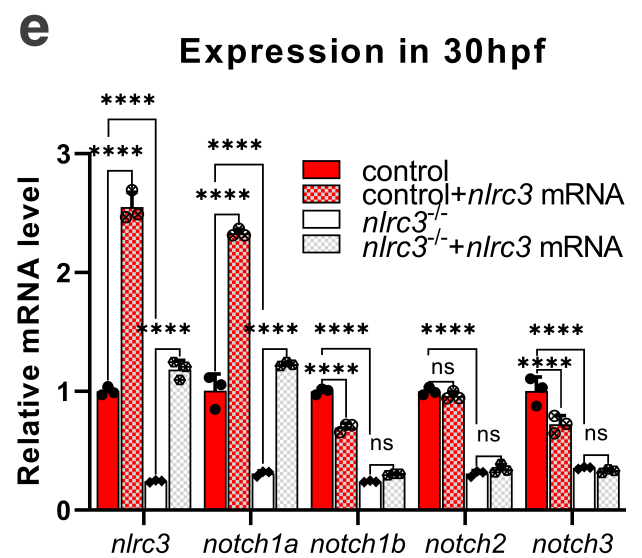
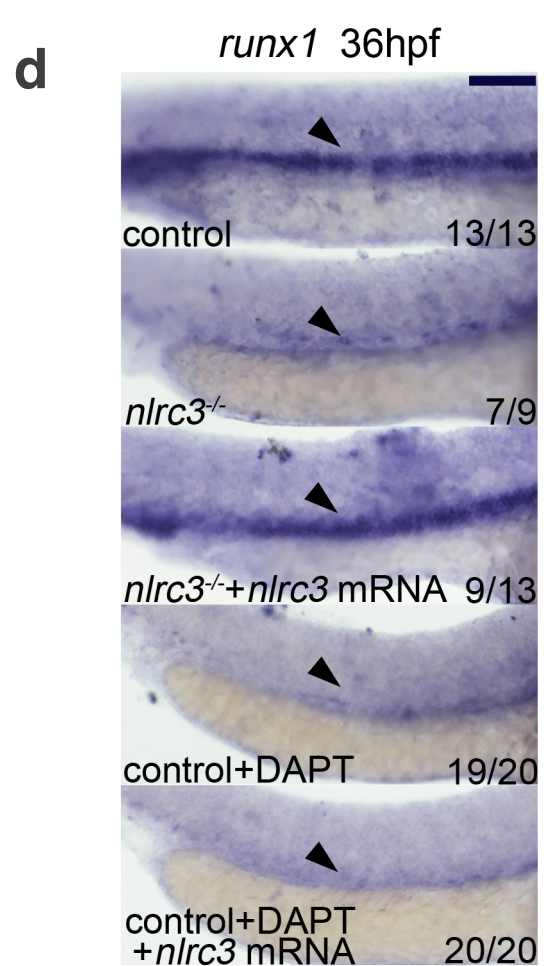
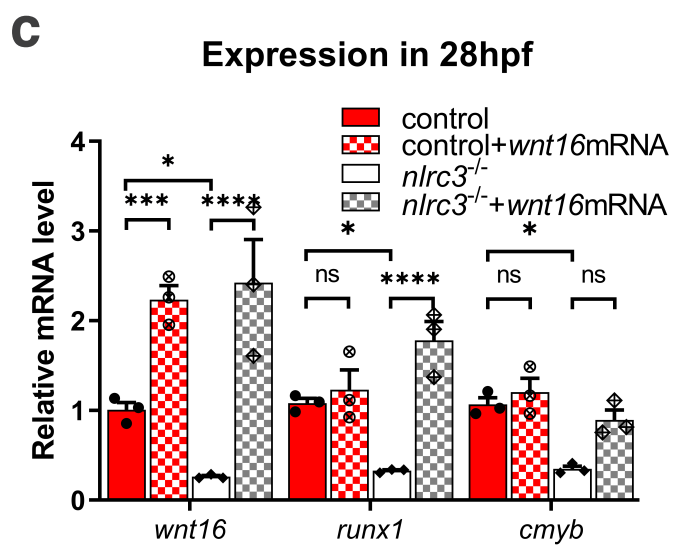
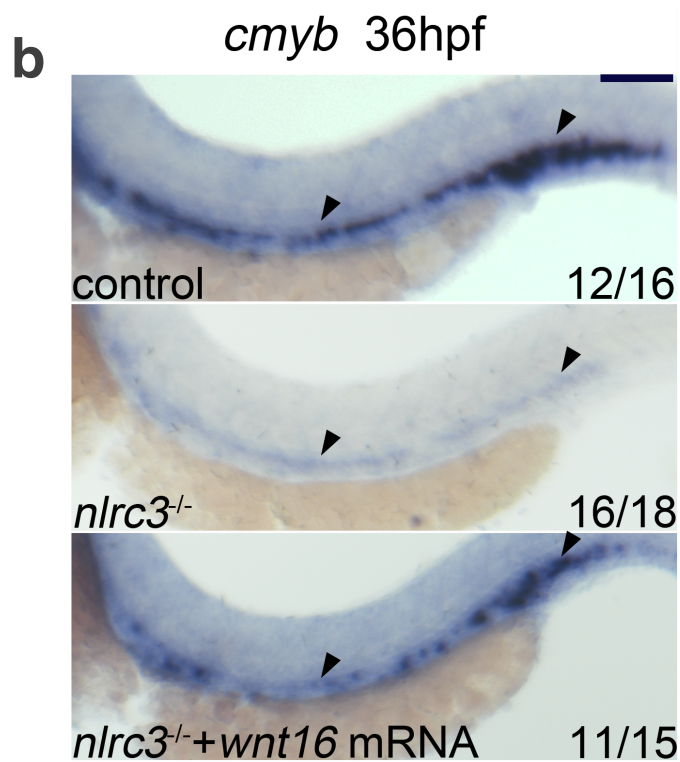
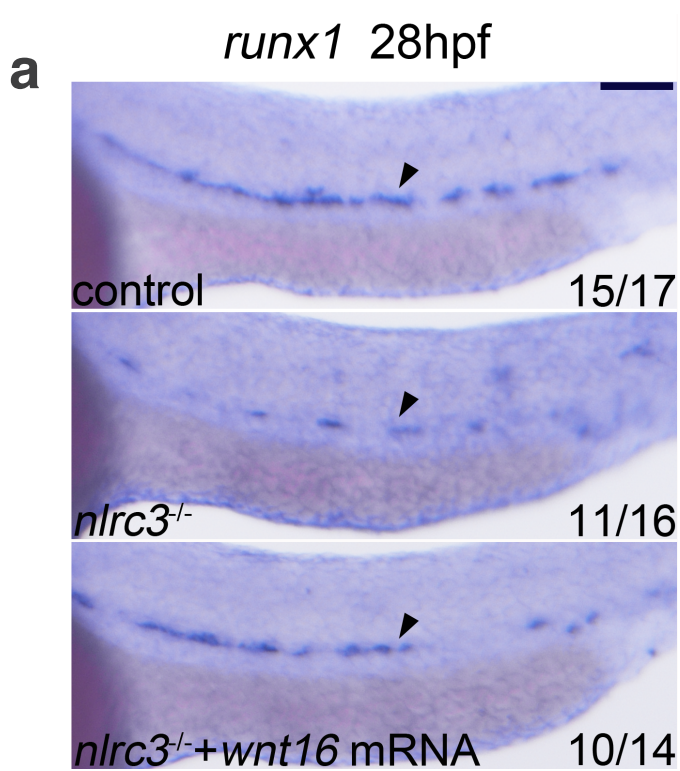






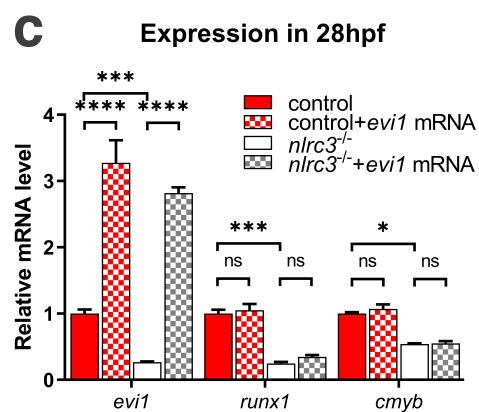
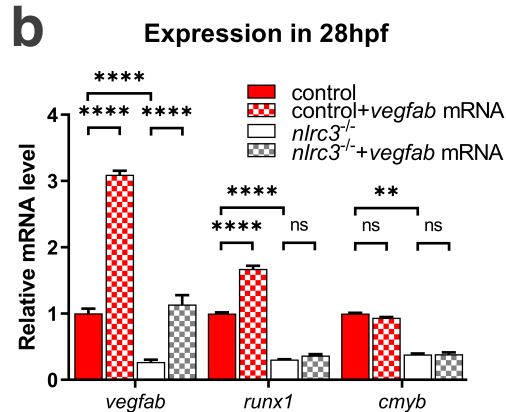
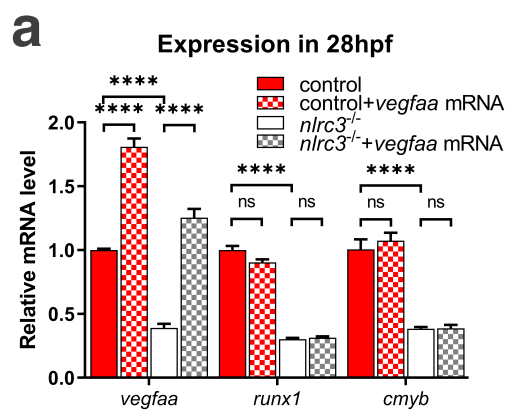
**Supplement Figure 7. Notch signaling was in downstream of *nlrc3* during HSPC production.**

**a** Confocal imaging showing the Tg (*tp1*:EGFP) embryos at 48 hpf in the floor of the DA in control embryos, *nlrc3* morphants and *nlrc3* morphants with overexpression of *nlrc3* mRNA. **b** Representative histogram of Tg (*tp1*:EGFP) cells in control embryos, *nlrc3* morphants and *nlrc3* morphants with overexpression of *nlrc3* mRNA at 48 hpf. **c** Flow cytometry results showed the changes of Tg (*tp1*:EGFP) cells in control embryos, *nlrc3* morphants and *nlrc3* morphants with overexpression of *nlrc3* mRNA at 48 hpf. **d** *hsp70*: Gal4; *UAS*:NICD-myc embryos injected with control embryos and *nlrc3* MOs were heat shocked at 18 hpf and WISH for *runx1* was performed at 36 and 48 hpf. Error bars represents means  $\pm$  SD; Each sample was composed of at least 5 embryos. Scale bars, 100  $\mu$ m in **a** , **d**. Source data are provided as a Source Data file.

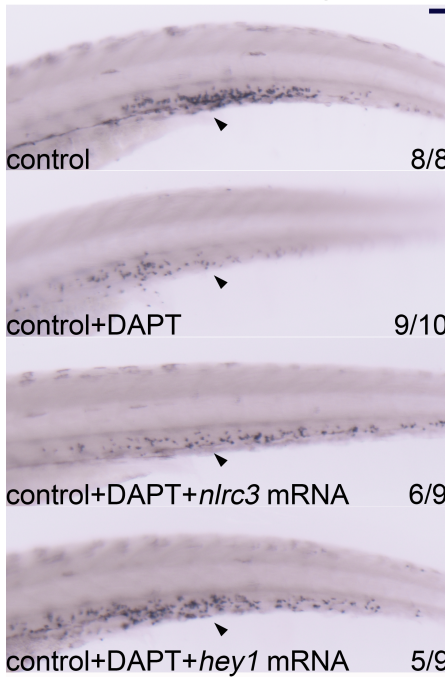


**Supplement Figure 8. *Nlrc3* regulated HSPC production via activating the Notch signaling.**

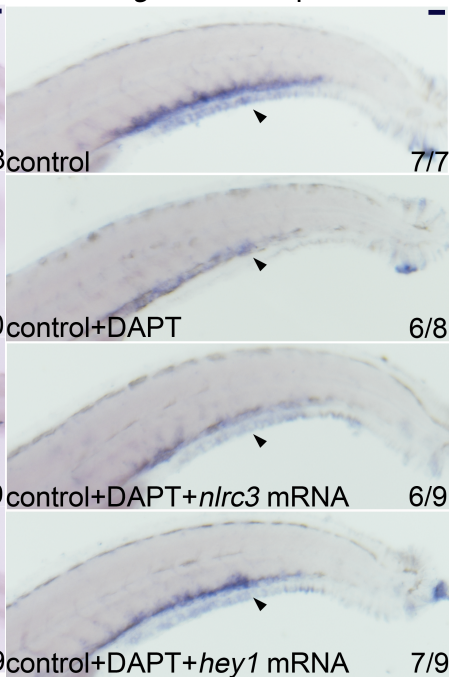
**a** Expression of HSPC marker *runx1* in control embryos, *nlrc3* mutants, *nlrc3* mutants with overexpression of *wnt16* mRNA at 28 hpf by WISH. **b** Expression of HSPC marker *cmyb* in control embryos, *nlrc3* mutants, *nlrc3* mutants with overexpression of *wnt16* mRNA at 36 hpf by WISH. **c** Expression of *wnt16*, *runx1* and *cmyb* in control, control with overexpression of *wnt16* mRNA, *nlrc3* mutants, and *nlrc3* mutants with overexpression of *wnt16* mRNA at 28 hpf by qPCR. ns = 0.9358, 0.9488, 0.1737, \*P = 0.0366, 0.0343, 0.0457, \*\*\*P = 0.0004, \*\*\*\*P < 0.0001. **d** Expression of HSPC marker *runx1* in control embryos, *nlrc3* mutants, *nlrc3* mutants with overexpression of *nlrc3* mRNA, treatment group with DAPT and treatment group with DAPT and with overexpression of *nlrc3* mRNA at 36 hpf by WISH. **e** Expression of NOTCH genes in control embryos, control with overexpression of *nlrc3* mRNA, *nlrc3* mutants and *nlrc3* mutants with overexpression of *nlrc3* mRNA at 30 hpf by qPCR. ns = 0.5877, 0.8516, 0.8594, 0.9470, \*\*\*\*P < 0.0001. **f** Expression of NOTCH genes in control embryos and *nlrc3* morphants at 28 hpf by qPCR. ns = 0.7233, 0.0518, 0.7827, 0.0730, 0.0742, 0.9558, 0.0771, \*P = 0.0120, 0.0198, 0.0121, 0.0417, \*\*P = 0.0071, 0.0028, 0.0046. Error bars, mean  $\pm$  s.d., by using two-tailed, unpaired student's *t*-test in **f**, two-way ANOVA with Tukey's post hoc test in **c**, **e**, *n* = 3 biological replicates in **c**, **e**, **f**. Scale bars, 100  $\mu$ m in **a**, **b**, **d**. Source data are provided as a Source Data file.



**d** Sudan Black 96hpf



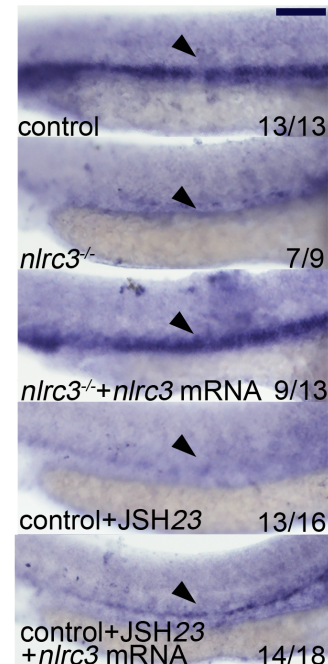
**e** *gata1a* 96hpf



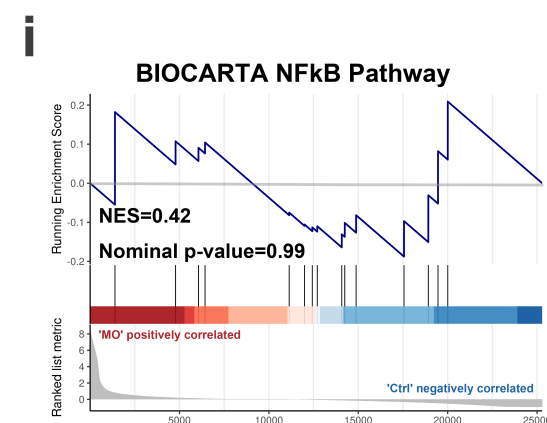
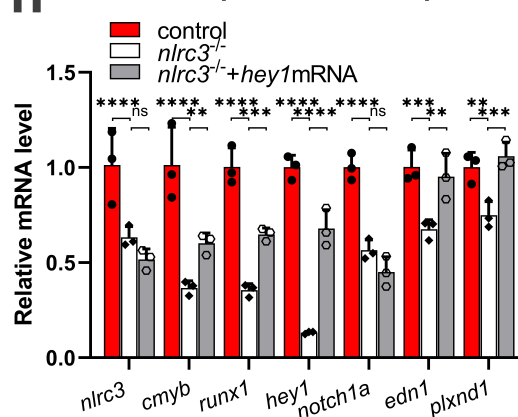
**f** *rag1* 96hpf



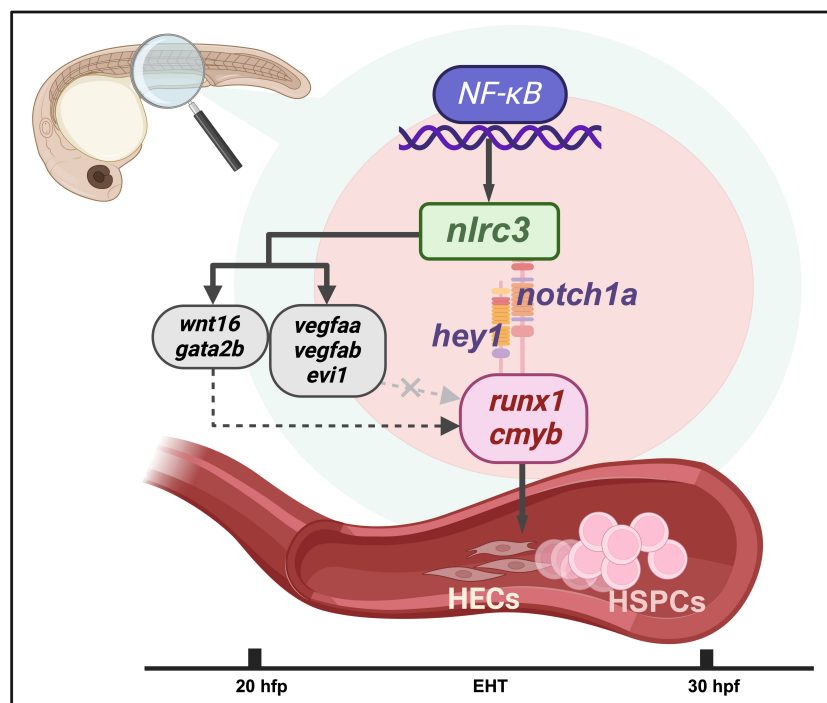
**g** *runx1* 36hpf



**h** Expression in 48hpf



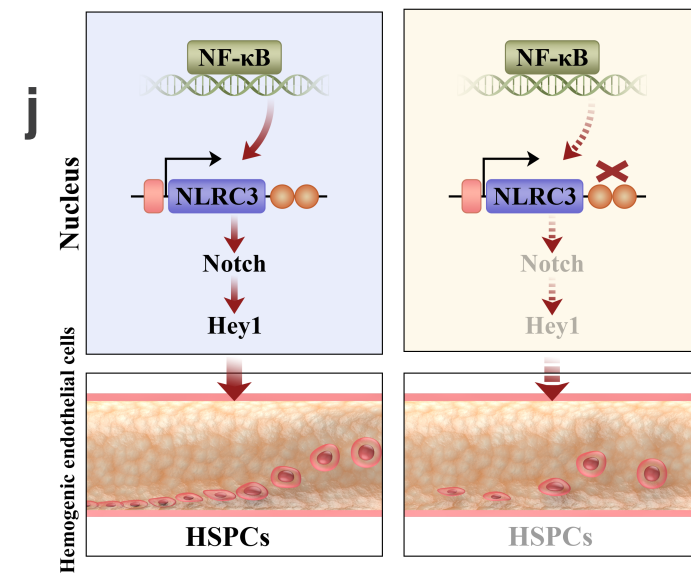
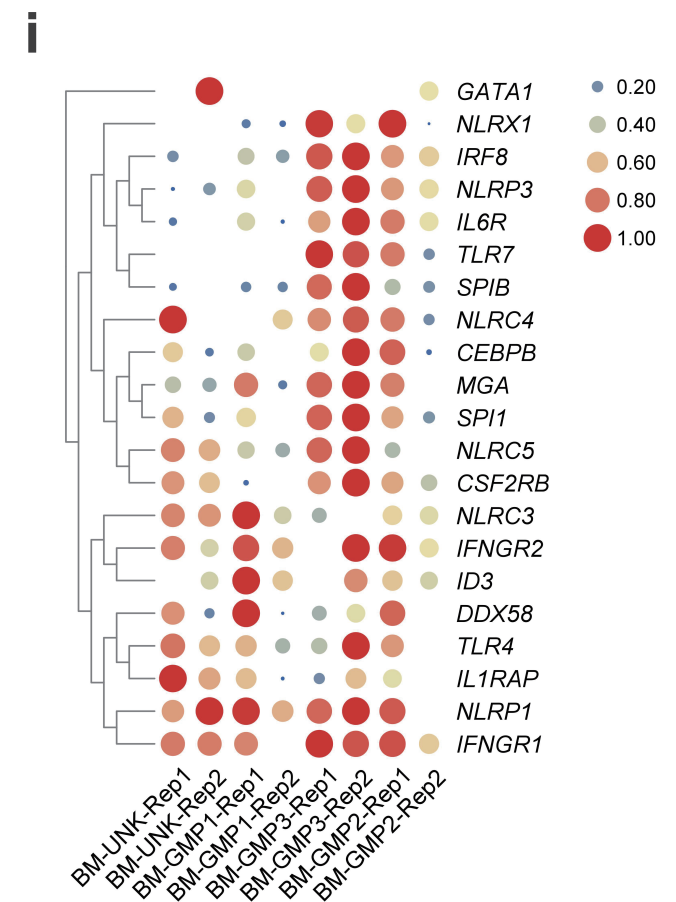
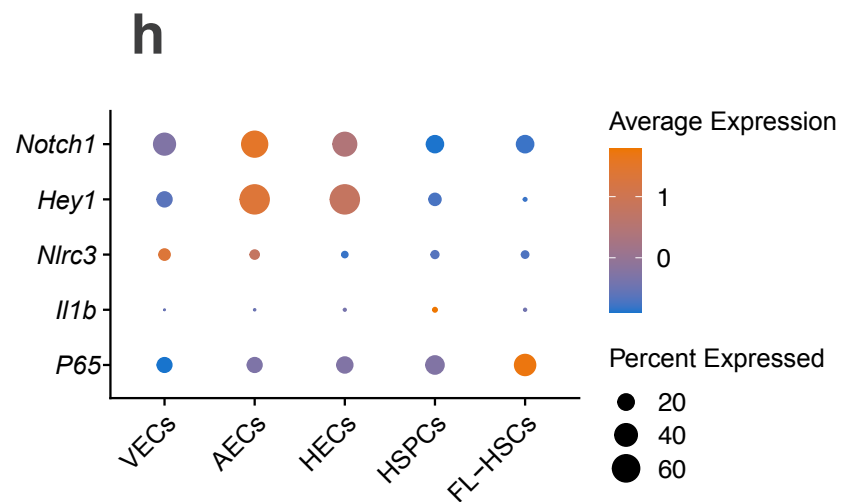
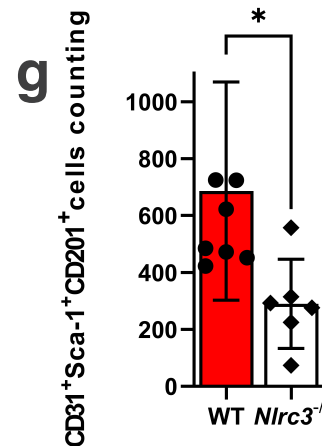
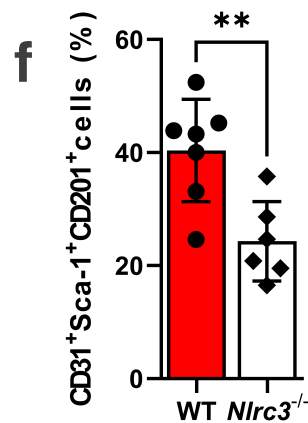
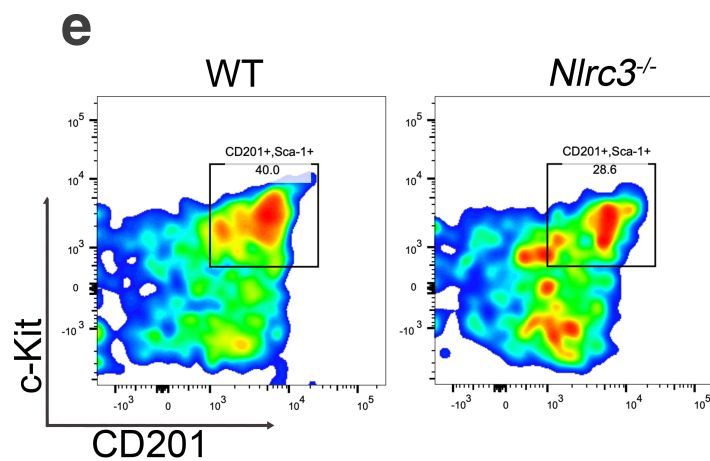
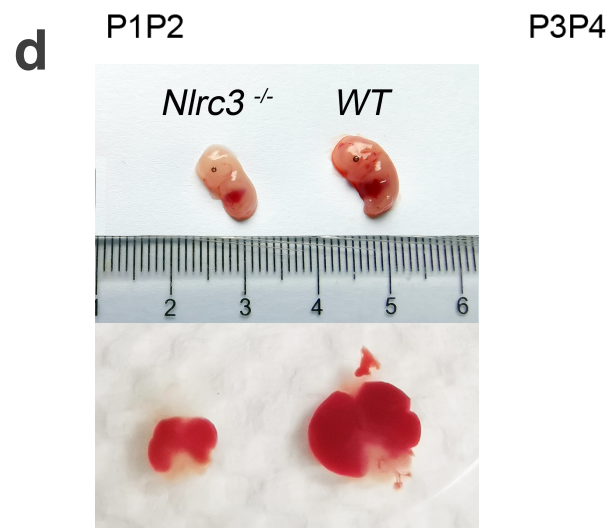
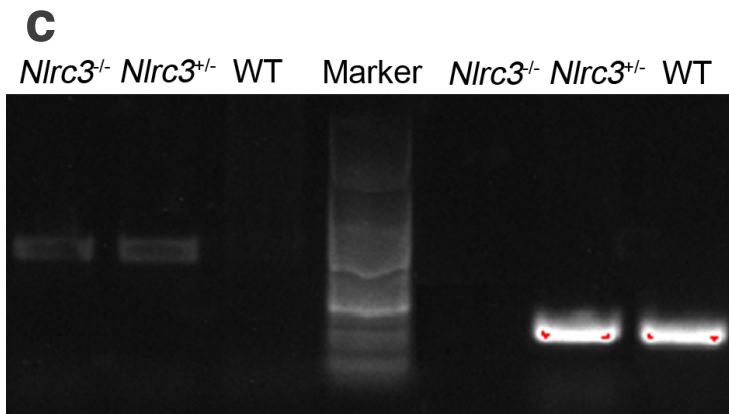
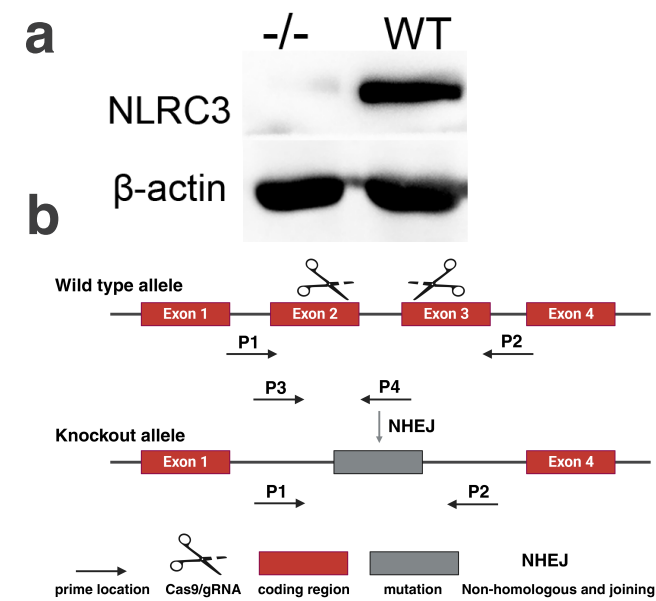
**j**



**Supplement Figure 9. *Nlrc3* signaling was in the downstream of NF- $\kappa$ B signaling pathway and upstream of *hey1* for regulating HSPC emergence.**

**a** Expression of *vegfaa*, *runx1*, and *cmyb* in control, control with overexpression of *vegfaa* mRNA, *nlrc3* mutants, and *nlrc3* mutants with overexpression of *vegfaa* mRNA at 28 hpf by qPCR. ns = 0.4042, 0.9966, 0.6902, > 0.9999, \*\*\*\*P < 0.0001. **b** Expression of *vegfab*, *runx1*, and *cmyb* in control, control with overexpression of *vegfab* mRNA, *nlrc3* mutants, and *nlrc3* mutants with overexpression of *vegfab* mRNA at 28 hpf by qPCR. ns = 0.8504, 0.8351, > 0.9999, \*\*\*\*P < 0.0001. **c** Expression of *evil*, *runx1*, and *cmyb* in control, control with overexpression of *evil* mRNA, *nlrc3* mutants, and *nlrc3* mutants with overexpression of *evil* mRNA at 28 hpf by qPCR. ns = 0.9903, 0.9143, 0.9685, 0.9999, \*P = 0.0346, \*\*\*P = 0.0005, 0.0004. **d** Expression of *Sudan Black* in control embryos, treatment group with DAPT, treatment group with DAPT and with overexpression of *nlrc3* mRNA, treatment group with DAPT and with overexpression of *hey1* mRNA at 96 hpf. **e** Expression of *gata1a* in control embryos, treatment group with DAPT, treatment group with DAPT and with overexpression of *nlrc3* mRNA, treatment group with DAPT and with overexpression of *hey1* mRNA at 96 hpf. **f** Expression of *rag1* in control embryos, treatment group with DAPT, treatment group with DAPT and with overexpression of *nlrc3* mRNA, treatment group with DAPT and with overexpression of *hey1* mRNA at 96 hpf by WISH. **g** Expression of HSPC marker *runx1* in control embryos, *nlrc3* mutants, *nlrc3* mutants with overexpression of *nlrc3* mRNA, treatment group with JSH23, treatment group with JSH23 with overexpression of *nlrc3* mRNA at 36 hpf by WISH. **h** Expression of HSPC genes, Notch genes and genes in the downstream of *hey1* in control embryos, *nlrc3* mutants and *nlrc3* mutants with overexpression of *hey1* mRNA at 48 hpf by qPCR. ns = 0.2636, 0.2743, \*\*P = 0.0074, 0.0014, 0.0037, \*\*\*P = 0.0007, 0.0002, 0.0004, \*\*\*\*P < 0.0001. **i** Enrichment plot of the NF- $\kappa$ B pathway relative to differentially regulated genes in the *nlrc3* morphant group compared to the control group by GSEA (Gene Set Enrichment Analysis). Normalized enrichment score = NES. P = 0.99. The statistical tests were one-sided and adjustments were made for multiple comparisons. **j** Model figure of *nlrc3* and its upstream and downstream pathways regulating zebrafish EHT. Error bars, mean  $\pm$  s.d., by using two-way ANOVA with Tukey's post hoc test,  $n = 3$  biological replicates in **a**, **b**, **c**. **h**. Scale bars, 100  $\mu$ m in **d**, **e**, **f**, **g**. Illustrations created with BioRender.com. Source data are provided as a Source Data file.







**Supplement Figure 10. *Nlrc3* signaling was conserved in the regulation of vertebrate hematopoiesis.**

**a** Protein level of *Nlrc3* in mouse embryonic mutants at E10.5. **b** Schematic illustration of *Nlrc3* knockout mice construction and verifying the mutant type. **c** DNA fragments of mutant types are separated and visualized by gel electrophoresis. **d** The photo of E14.5 fetal mice and fetal liver of *Nlrc3*<sup>-/-</sup> and wild-type. **e** Flow cytometry results showing the number of CD31<sup>+</sup>Sca-1<sup>+</sup>CD201<sup>+</sup> cells in the AGM of *Nlrc3* embryos decreased. **f** Percentage of CD31<sup>+</sup>Sca-1<sup>+</sup>CD201<sup>+</sup> cells. \*\*P = 0.0026. **g** Quantification of CD31<sup>+</sup>Sca-1<sup>+</sup>CD201<sup>+</sup> cells. \*P = 0.0370. **h** Bubble plot of published RNA-Seq data demonstrating the expression of *Nlrc3*, Notch and NF-κB genes in the clusters of venous endothelial cells (VECs), arterial endothelial cells (AECs), pre-hemogenic endothelial cells (pre-HECs), hemogenic endothelial cells (HECs), HSPCs, fetal liver (FL)-derived HSPCs in the mouse embryos. **i** Bubble plot of published scRNA-Seq data demonstrating the expression of *Nlrc3*, inflammatory factors, myeloid-promoting cytokines, and myeloid genes in three distinct types of granulocyte-macrophage progenitors (GMPs) derived from human bone marrow-derived CD34<sup>+</sup> cells. **j** Schematic illustration of the role of the NLCR3 signal during EHT in vertebrates. Error bars, mean ± s.d., by using two-tailed, unpaired student's *t*-test. *n* = 8, 6 in **f**, **g**. Illustrations created with Photoshop. Source data are provided as a Source Data file.

Table S1. The Morholinos used in this work		
MO	sequence (5'-3')	Reference
<i>nlrc3-spMO</i>	TTATCCTGGGTACTGAATATACCTT	This work
Control-MO	GeneTools	*Espin-Palazon R, Stachura DL, Campbell CA, et al. Cell. 2014;159(5):1070-1085.

Table S2. The primers in qPCR assay		
species	Primer name	qPCR primer sequence (5'→3')
zebrafish	<i>nlrc3</i> -F	ACTGCCACGCCTTGTTATCT
	<i>nlrc3</i> -R	AAGTGTGGTGAGGGTTCGGT
	<i>runx1</i> -F	GCCTCTCTGCAGAACTTTCC
	<i>runx1</i> -R	GACGGCAGAGTAGGGAAGT
	<i>cmyb</i> -F	TGATGCTTCCCAACACAGAG
	<i>cmyb</i> -R	TTCAGAGGGAATCGTCTGCT
	<i>Ikaros</i> -F	AGAAGGGTAACCTGCTCCGACAC
	<i>Ikaros</i> -R	GGGCTTCCAACCGAATGAGT
	<i>il7r</i> -F	TACACCAAACATCCCACA
	<i>il7r</i> -R	TCACTCACTGACGCACTT
	<i>rag1</i> -F	AGAGGACAGTGGGTAAAGA
	<i>rag1</i> -R	ATGGGTTCAGGTGTTGGTT
	<i>lck</i> -F	AGATGAATGGTGTGACCAGTGTA
	<i>lck</i> -R	GATCCTGTAGTGCTTGATGATGT
	<i>gata1a</i> -F	TCTGAGCCTTCTCGTTGGG
	<i>gata1a</i> -R	CTCTGGACGCTGGTGGAATA
	<i>l-plastin</i> -F	TGTCTGTGCCCACACCAT
	<i>l-plastin</i> -R	GGCGGAGGCAGAGTTCAG
	<i>pu.1</i> -F	AGGAGTGTATGAGAGACCACATCAG
	<i>pu.1</i> -R	ATTTCGCAGAAGGTCAAGCA
	<i>csf1r</i> -F	TCCGGGACTCTCGAGTTCTCT
	<i>csf1r</i> -R	CTTCACCTTCTTTCCGCACATA
	<i>lyz</i> -F	GTGAAAATGGACGGGCTGAA
	<i>lyz</i> -R	CTTTGTTTGCGCTGCTCACA
	<i>dll4</i> -F	ACTCTTCTGCTACGGTATGTTT
	<i>dll4</i> -R	CAATGCTGGTTGAAGGTTTT
	<i>ephrinB2a</i> -F	CAAGGACAGCAAATCGAATG
	<i>ephrinB2a</i> -R	TGAGCCAATGACTGATGAGG
	<i>deltaC</i> -F	ATTATTGCACCGAACCCA
	<i>deltaC</i> -R	AAAATAGACCGCCCCAAC
	<i>kdr1</i> -F	ACTTGCCCAGATTATGGTGATG
	<i>kdr1</i> -R	TCAGCCTATCGTAGAGGTATCTTGTG

<i>il1b</i> -F	AGAATGAAGCACATCAAACC
<i>il1b</i> -R	AATCCACCACGTTCACTTC
<i>p65</i> -F	CATTCCCTACGGCTAAACGA
<i>p65</i> -R	AGAAAAAGGAGGTGGGTGGA
<i>nfkb1a</i> -F	AGTCATGCCAGAGAGCGAAT
<i>nfkb1a</i> -R	CAGAGCCGGATGTCATCATA
<i>nfkb1b</i> -F	CAGCTCGGCGCAGATATAA
<i>nfkb1b</i> -R	TCGATGAGAAGTTTGACCATGT
<i>nfkb1</i> -F	CGCAAGTCCTACCCACAAGT
<i>nfkb1</i> -R	ACCAGACTGTGAGCGTGAAG
<i>nfkb2</i> -F	CATATGTCCACACAATCAAGAC
<i>nfkb2</i> -F	AGCCACCATAATGATCTGGAA
<i>notch1a</i> -F	CGGGCCTGACGGATTAC
<i>notch1a</i> -R	GGACTCCAGCAGACGTTTAGC
<i>dla</i> -F	GCAACTGAGGTGTAATGGGC
<i>dla</i> -R	CACCTAAGGTTTCGAGCAGC
<i>dlb</i> -R	GATGTTGCAGTCTCGCGTCC
<i>dlb</i> -F	TGTTGCAGTTGGTGGCTTCG
<i>dlc</i> -F	ACGAGCAGTGTGTGTAA
<i>dlc</i> -R	TGTTATTCTCTGTTGACTGG
<i>dld</i> -F	AGTACTGCACAGAACC
<i>dld</i> -R	TCTTGGTTACAGAAGAG
<i>dll4</i> -F	GCATTTTTTCACGACCGTTTT
<i>dll4</i> -R	CGGAAGAAAGTCCTGCAGTC
<i>jag1a</i> -F	TAAATGTCCAGAAGATTACG
<i>jag1a</i> -R	ACACACTGGAAGAGATTAGC
<i>jag1b</i> -F	CTAGTTGCTTGTGTTAAAC
<i>jag1b</i> -R	AAGGCAGTCTACAGTGTTC
<i>jag2</i> -F	GCTCTGGATCTACCGACGTT
<i>jag2</i> -R	GTCCCAAGCTTCAAGGATGA
<i>hey1</i> -F	TTTGATGCTCACGCTCTGGC
<i>hey1</i> -R	ACCTGCTGAGATGGGACAAG
<i>hey2</i> -F	ATTGATGTGGGCAGCGAGAA
<i>hey2</i> -R	TGGGATGTGGTGGATGTGGA
<i>her15.1</i> -F	CACGTCACCTATTCTCATCG
<i>her15.1</i> -R	TCCACAGGAGTTCAACATTG
<i>edn1</i> -F	TGCGTCTACTTTTGCCACCT
<i>edn1</i> -R	CCCTGTCTGACGCTGCTTTA
<i>hand2</i> -F	GACGCCAAAGAAGAAAGGCG
<i>hand2</i> -R	TCAGCTCCAATGCCCAAACA
<i>hsp70l</i> -F	CCGCTGACTAGGGCACAT
<i>hsp70l</i> -R	GACGGCATCTTTATTACATTA
<i>β actin</i> -F	GCTGTTTTCCCCTCCATTGTT

	<i>β actin</i> -R	TCCCATGCCAACCATCACT
	<i>efl1a</i> -F	GAGAAGTTCGAGAAGGAAGC
	<i>efl1a</i> -R	CGTAGTATTGCTGGTCTCG

species	Primer name	qPCR primer sequence (5'→3')
Human	<i>TLR4a</i> -F	AGACCTGTCCCTGAACCCTAT
	<i>TLR4a</i> -R	CGATGGACTTCTAAACCAGCCA
	<i>NLRC3</i> -F	GTGCCGACCGACTCATCTG
	<i>NLRC3</i> -R	GTCCTGCACTCATCCAAGC
	<i>NLRP3</i> -F	GATCTTCGCTGCGATCAACAG
	<i>NLRP3</i> -R	CGTGCAATTATCTGAACCCAC
	<i>GAPDH</i> -F	TCCCACTCTTCCACCTTCGATGC
	<i>GAPDH</i> -R	GGGTCTGGGATGGAAATTGTGAGG
species	Primer name	qPCR primer sequence (5'→3')
MOUSE	<i>Notch1</i> -F	GATGGCCTCAATGGGTACAAG
	<i>Notch1</i> -R	TCGTTGTTGTTGATGTCACAGT
	<i>Notch2</i> -F	GAGAAAAACCGCTGTCAGAAATGG
	<i>notch2</i> -R	GGTGGAGTATTGGCAGTCCTC
	<i>Hey1</i> -F	GCGCGGACGAGAATGGAAA
	<i>Hey1</i> -R	TCAGGTGATCCACAGTCATCTG
	<i>GAPDH</i> -F	AAGCGCCCTTGTGAGGAAAC
	<i>GAPDH</i> -R	GGTAGTTGTTCGGTGAATTGGAC
	<i>Hey2</i> -F	GATGGCCTCAATGGGTACAAG
	<i>Hey2</i> -R	TCGTTGTTGTTGATGTCACAGT
	<i>Hes5</i> -F	AGTCCCAAGGAGAAAAACCGA
	<i>Hes5</i> -R	GCTGTGTTTCAGGTAGCTGAC
	<i>Runx1</i> -F	ATGCCTGATCGTACCGAGAAG
	<i>Ruxn1</i> -R	GTCGTTGGCGTAAATGAGCTG
	<i>GAPDH</i> -F	TCCCACTCTTCCACCTTCGATGC
	<i>GAPDH</i> -R	GGGTCTGGGATGGAAATTGTGAGG

Table S3. The zebrafish transgenic strain used in this work		
Tg( <i>runx1</i> :EGFP; <i>kdrl</i> :mCherry)	Double-positive cells are marked the emerging HSPCs from haemogenic endothelium.	PMID: 28869969
Tg( <i>cmyb</i> :EGFP; <i>kdr</i> :mCherry)	Item	Item
Tg( <i>CD41</i> :GFP)	Positive cells mark the HSPCs and platelets in the CHT.	PMID: 25416946
Tg( <i>tp1</i> :EGFP)	EGFP cells reflect Notch activity.	PMID: 19595765
Tg( <i>flil1a</i> :EGFP)	Positive cells mark zebrafish blood vessels.	PMID: 12167406

Tg( <i>kdr</i> :mCherry)	Positive cells mark zebrafish blood vessels.	PMID: 28869969
Tg( <i>lck</i> :EGFP)	EGFP cells mark T-cells.	PMID: 30855581
Tg( <i>mpeg</i> :GFP)	EGFP cells mark macrophages.	PMID: 31909502
Tg( <i>lyz</i> :DsRed)	DsRED expression under the neutrophil specific promoter lysozyme.	PMID: 33162327
Tg( <i>mpx</i> :EGFP)	EGFP expression under the neutrophil specific promoter myeloperoxidase.	Item
Tg( <i>gata1a</i> :DsRed)	dsRED expression under the specific promoter erythroid transcription factor Gata1.	Item
Tg( <i>lcr</i> :EGFP)	EGFP expression under the erythroid specific globin regulatory sequence locus control region.	Item

<b>Table S4. Deposited Data</b>		
<i>Nlr3</i> knockdown RNA-seq	This work	PRJCA009166
scRNA-seq in zebrafish	Xia J, et al. Proc Natl Acad Sci USA. 2021 Apr 6; 118(14): e2015748118.	GSE146404
scRNA-seq in mouse	Zhu Q, et al. <i>Blood</i> 2020 Aug 13;136(7):845-856.	GSE137117
scRNA-seq in human	Calvanese V, et al. Nature 2022 Apr;604(7906):534-540.	GSE162950
bulk RNA-Seq in human CD34 <sup>+</sup> bone marrow CD34 <sup>+</sup>	Buenrostro JD, et al. <i>Cell</i> 2018 May 31;173(6):1535-1548.e16.	GSE96811

<b>Table S5. The antibodies used in this work</b>	
anti-mouse TER-119 (clone: TER-119)	BD Biosciences #560509
anti-mouse CD31-APC (clone: mmab)	BD Biosciences #551262
anti-mouse CD45-FITC (clone: OX-1)	BD Biosciences #554877
anti-mouse CD41-PE/cy7 (clone: HIP8)	BD Biosciences #561424
anti-mouse c-Kit-PE/cy5 (clone: 2B8)	Biolegend #105810
anti-mouse Sca-1-APC (clone: D7)	Biolegend #108112
anti-mouse CD45.1-AF-700 (clone: A20)	BD Biosciences #110724,
anti-mouse CD150-FITC (clone: TC15-12F12.2)	Biolegend #115902
anti-mouse CD48-PE (clone: HM48-1)	Biolegend #103406
anti-mouse CD201-APC (clone: RCR-16)	Biolegend #141506
anti-mouse $\beta$ -ACTIN	Sangon Biotech #D191047
anti-mouse Anti-Nlr3	Abcam #ab77817