Rnf111 has a pivotal role in regulating development of definitive hematopoietic stem and progenitor cells through the Smad2/3-Gcsfr/NO axis in zebrafish

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Supplementary methods

Zebrafish mutant generation

For generating Rnf111 knockout zebrafish by CRISPR/Cas9 technology, guide RNA (gRNA) was designed by ZiFiTTargeter software (http://zifit.partners.org/ZiFiT). The sequence of target site was as follows: 5'-GGCGGCTGCTGAGCAGCTAC-3'. Cas9 RNA and gRNA were co-injected into one-cell stage zebrafish embryos to obtain F0 generation. For identifying the genotypes, genomic DNA extracted from adult tail or embryos was amplified and DNA products (262 bp) were digested with AluI. Samples from the mutant strand were not digested into two fragments, while those from wildtype strand were fully cleaved. The PCR primers were as follows: Forward primer: TCCTCTTCCTCCTCCCGTAA; Reverse primer: TCTGCTCTCATCTGATTGGAGA.

Whole-mount in situ hybridization (WISH)

Plasmid constructs containing a portion of the coding sequence were firstly linearized by certain restriction endonuclease. Then T3 or T7 polymerase (Ambion, Life Technologies, Carlsbad, CA, USA) were used for synthesizing digoxigenin (DIG)-labeled RNA probes. Whole-mount mRNA in situ hybridization (WISH) was performed according to the procedure described previously.[1] Alkaline phosphatase-coupled anti-digoxigenin Fab fragment antibody (Roche, Basel, Switzerland) was used to recognize the digoxigenin-labeled probes. And then BCIP/NBT staining (Vector Laboratories, Burlingame, CA, USA) was carried out to detect the signaling.

Morpholinos and mRNA microinjection

Zebrafish embryos at the single-cell stage were utilized for morpholinos (MO) and mRNA injection. Morpholino oligonucleotides were designed by and ordered from Gene Tools. The MO sequence of *rnf111* was 5'-GCCGAAATCTCACTCTTCATGGCGA-3' and the MO sequence of *gcsfr* was 5'-AAGCACAAGCGAGACGGATGCCAT-3', which is the same as reported previously.[2] Transcribing linearized plasmids with mMachine SP6 kit (Invitrogen, Thermo Fisher, USA) produced capped mRNA samples, which were subsequently purified and diluted to 100 ng/ul (*rnf111*, *rnf111* RING MU, *rnf111* SIM MU, *gcsfb* and *gcsfr* RNA) or 50 ng/ul (*cebpb* RNA) for injection at one-cell stage of embryos.

Quantitative real-time PCR

Reverse transcription was performed using RevertAid First Strand cDNA Synthesis Kit (Life Technologies) in accordance with the manufacturer's instructions. Gene expression levels were

comparatively assessed using reverse transcription polymerase chain reaction (PCR) (Applied Biosystems, Foster City, CA, USA). Each sample was tested in triplicate and housekeeping gene *β-actin* was chosen for performing the normalization of gene expression using the ΔΔCt method.[3] The results were analyzed with GraphPad Prism software. The final results were expressed as the mean ± S.D.

Immunofluorescence assay

Tg (*cmyb*:EGFP)[4] embryos (3 days post-fertilization, dpf) were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Following a sequence of dehydration and rehydration steps, the embryos underwent treatment with Proteinase K (10 ug/ml; Sigma-Aldrich) for 30 min at room temperature (RT) and then acetone treatment for 10 minutes at -20°C. Subsequently, the embryos were permeabilized through incubation in a solution containing 0.1% Triton X-100 + 0.1% sodium citrate in PBS for 15 minutes at RT. After blocking for 1 hour at RT with blocking solution (2 mg/ml BSA+ 10% FBS + 0.3% Triton-X100 + 1%DMSO in PBST), the embryos were simultaneously stained with mouse anti-green fluorescent protein (GFP) (Invitrogen, Carlsbad, CA, USA) and rabbit anti-p-Smad2 (Ser465/467)/ Smad3 (Ser423/425) (Cell signaling) primary antibodies during an overnight incubation at 4°C. The secondary antibodies used for detection included Alexa Fluor 488-conjugated anti-mouse (Invitrogen) and Alexa Fluor 594-conjugated anti-rabbit (Invitrogen).

Bromodeoxyuridine (Brdu) and enhanced green fluorescent protein (EGFP) double immunostaining.

The 3 dpf transgenic Tg (*runx1*: EGFP)[5] and Tg (*cmyb*:EGFP) embryos were firstly incubated with 10 mM Bromodeoxyuridine (Brdu) (Sigma) for 30 minutes, followed by a 2-hour incubation in egg water. Subsequently, the embryos were fixed in 4% PFA overnight at 4˚C. Then, after dehydrated and rehydrated at RT, the embryos were digested with proteinase K (10 ug/ml; Sigma-Aldrich) at 30°C for 30 min and treated with acetone at -20°C for 30 min. After blocked with blocking solution (PBS+ 0.3%TritonX-100 + 1% DMSO + 10 mg/ml bovine serum albumin + 10% normal goat serum) for 2 hours, the embryos were sequentially immunostained by rabbit anti-GFP primary antibody (1:500; Invitrogen) and Alexa Fluor 488-conjugated anti-rabbit secondary antibodies (1:500; Invitrogen). Then, the embryos were treated with 2 N HCl for 1 hour and further stained with mouse anti-Brdu (1:50; Roche) and rabbit anti-GFP antibodies simultaneously. Finally, Alexa Fluor 594-conjugatedanti-mouse and Alexa Fluor 488-conjugated anti-rabbit (1:500;

Invitrogen) were used as secondary antibodies.

Cell culture and luciferase reporter assay

HEK293T cells were seeded in 24-well plates and cultured in DMEM (Life technologies, Grand Island, NY, USA) with10% Fetal Bovine Serum (Life technologies, Grand Island, NY, USA) at 37°C with 5% CO_2 atmosphere. Effectene Transfection Reagent (QIAGEN) was used for plasmid transfections according to the manufacturer's instructions. For the luciferase reporter assay, cells were harvested 36-48 hours following transfection and then detected using the Dual Luciferase Reporter Assay Kit (Promega, Maddison, WI, USA).

Immunohistochemistry

The 3 dpf embryos were fixed in 4% PFA overnight at 4°C. After dehydration and rehydration, the embryos were treated with Proteinase K (10 ug/ml; Sigma-Aldrich) for 30 min at RT and then treated with acetone for 10 minutes at -20°C. Then, the embryos were incubated with the rabbit anti-p-Smad2 (Ser465/467)/ Smad3 (Ser423/425) primary antibodies and alkaline phosphatase conjugated anti-rabbit secondary antibodies at 4°C overnight. BCIP/NBT staining was performed to detect the signaling.

Western blot

For Western blot analysis, embryos were deyolked and homogenized in lysis buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor PhosSTOP™ (Roche) as previously reported.[6] Total proteins were separated by 10% SDS-PAGE (Sigma-Aldrich, St. Louis, MO, USA) and transferred onto nitrocellulose membranes (GE Healthcare Life sciences, Pittsburgh, PA, USA). The membrane was blocked by 5% non-fat milk at RT for 1 hour, followed by overnight incubation with primary antibodies at 4 °C overnight. After incubated with secondary antibody at RT for 2 hours, SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rochford, IL, USA) was used to detect the signal. The antibodies used in western blot analysis were as follows: Rabbit anti-p-Smad2 (Ser250) (Beyotime, Shanghai), Rabbit anti-p-Smad3 (Ser423/425) (Beyotime, Shanghai), rabbit anti-Smad2 antibody (Abmart, Shanghai), rabbit anti-Smad3 antibody (Abmart, Shanghai), mouse anti-Smad7 monoclonal antibody (R&D Systems, USA) and mouse anti-GAPDH Monoclonal antibody (Proteintech Group, USA).

Chromatin immunoprecipitation PCR (ChIP-PCR)

GFP and Smad2-GFP RNA were injected to embryos at one cell stage and harvested at 48 hpf.

Following homogenization and fixation, SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology) was used for immunoprecipitating cross-linked chromatin with anti-GFP antibody (Abcam). The immunoprecipitated samples were analyzed by quantitative PCR using primer pairs listed in the supplementary table.

Chemical treatment

Embryos were treated with 10 μM S nitroso N-acetylpenicillamine (SNAP) (Sigma-Aldrich) at bud stage as previously reported.[7] Definitive endoderm 2 inducer (IDE2) (Abcam) at the concentration of 1uM was used to activate the phosphorylation of Smad2 from 16 hpf to 3 dpf. Nitric oxide synthase 2 (NOS2)-specific inhibitor 1400W (Selleck) was used to treat embryos at a concentration of 1mM from 1 dpf to 3 dpf.

Supplementary references

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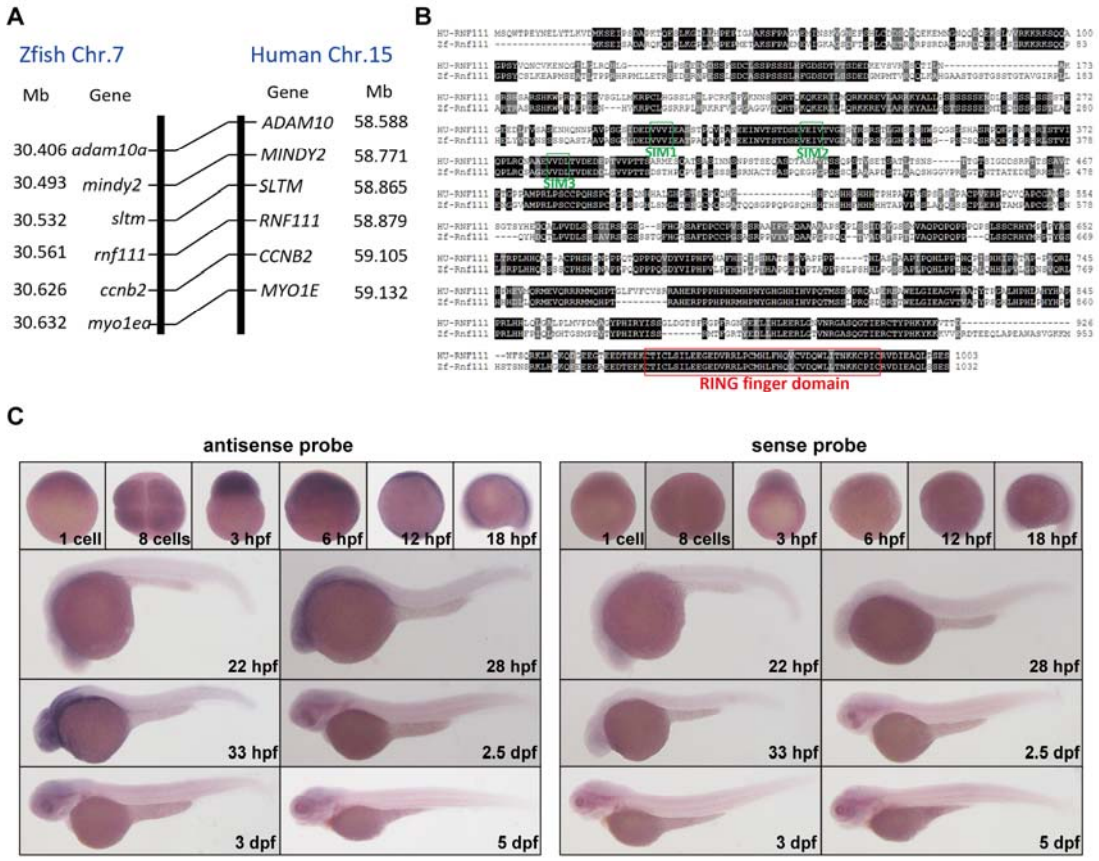

Figure S1.* Conservation analysis of zebrafish *rnf111*. (A) Synteny analysis of human *RNF111* and zebrafish *rnf111* loci. (B) The alignment of multiple sequences indicates the evolutionary conservation of human RNF111 and zebrafish Rnf111 proteins. The sequence in the green box represents the SUMO-interacting motifs (SIM). The sequence in the red box represents the RING finger domain. (C) WISH result showed that *rnf111* is a maternal gene, expressing ubiquitously including hematopoietic tissues. SIM: SUMO-interacting motifs; hpf: hours post-fertilization; dpf: days post-fertilization; WISH: whole-mount in situ hybridization.

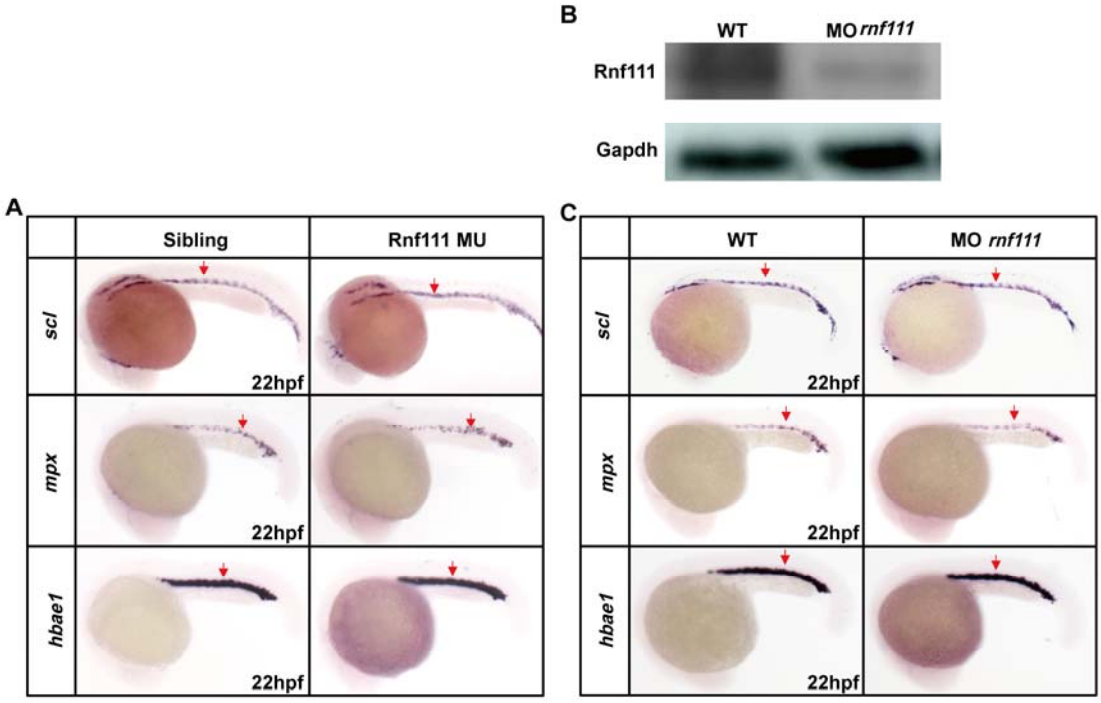

Figure S2.* The phenotypes of primitive hematopoiesis in Rnf111 deficient embryos. (A) WISH analysis of *scl*, *mpx* and *hbae1* at 22 hpf in Rnf111 mutants. (B) Western blot of Rnf111 indicated the protein level of Rnf111 was sharply decreased at 2 dpf in *rnf111* morphants. (C) WISH analysis of *scl*, *mpx* and *hbae1* at 22 hpf in *rnf111* morphants. All experiments were independently replicated at least three times. MU: Rnf111 mutants with deletion of 4bp nucleotides; hpf: hours post-fertilization; WT: wildtype; MO *rnf111*: *rnf111* morphants; WISH: whole-mount in situ hybridization; dpf: days post-fertilization.

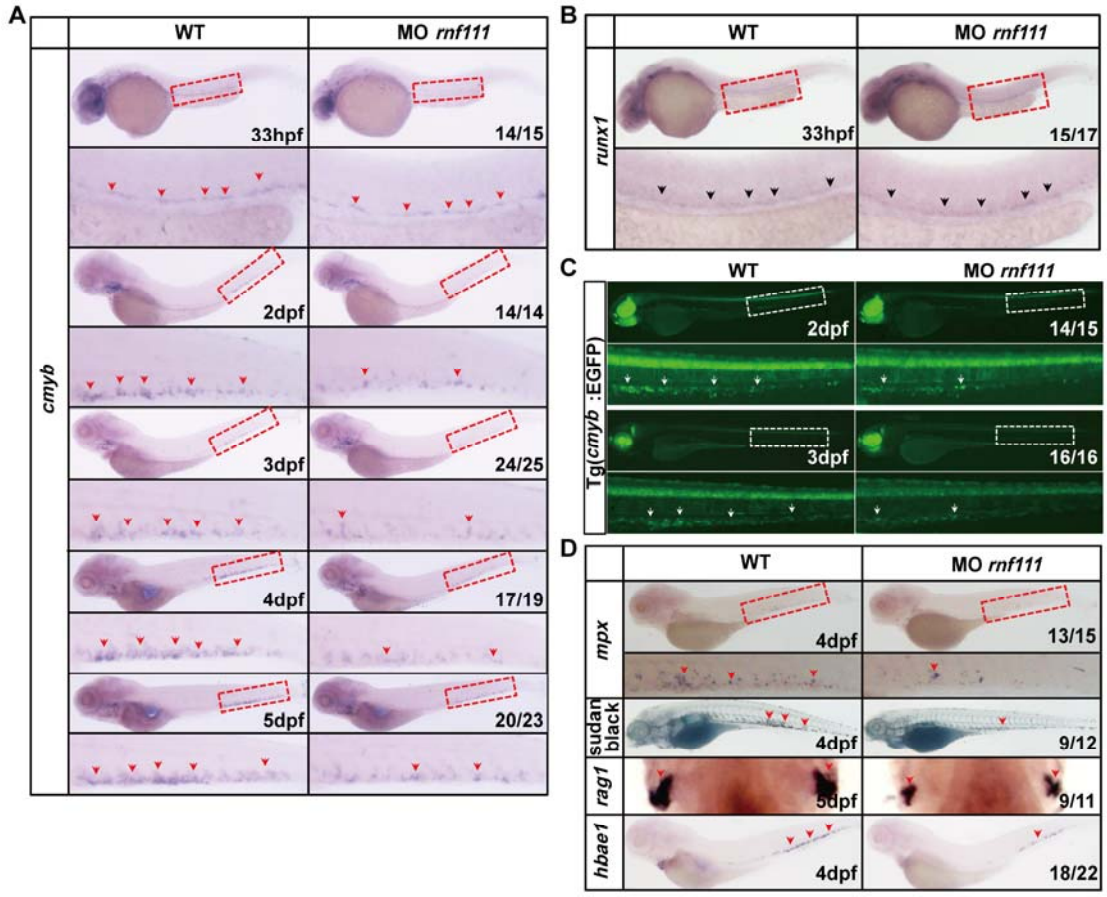

Figure S3.* Impairment of definitive hematopoiesis in *rnf111* morphants. (A) WISH analysis of *cmyb* expression from 33 hpf to 5 dpf. In *rnf111* morphants, *cmyb* expression was normal at 33 hpf and decreased from 2 dpf until all subsequent stages of development. Red arrows indicate *cmyb*-positive HSPC in the AGM or CHT. (B) WISH analysis of *runx1* expression at 33 hpf. Black arrows indicate *runx1*-positive HSPC in the AGM. (C) Fluorescent images of Tg (*cmyb*: EGFP) embryos. White arrows indicate GFP-positive HSPC in the CHT. (D) WISH analysis of key hematopoietic markers and Sudan Black B staining in *rnf111* morphants and wildtype embryos. Expressions of myeloid-specific marker *mpx*, lymphocyte maker *rag1*, mature erythrocyte marker *hbae1* and Sudan Black signal were decreased in *rnf111* morphants. All experiments were independently replicated at least three times. WT: wildtype; MO *rnf111*: *rnf111* morphants; hpf: hours post-fertilization; dpf: days post-fertilization; Tg: transgenic; EGFP: enhanced green fluorescent protein; WISH: whole-mount in situ hybridization; GFP: green fluorescent protein; HSPC: hematopoietic stem and progenitor cell; CHT: caudal hematopoietic tissue; AGM: aorta–gonad–mesonephros.

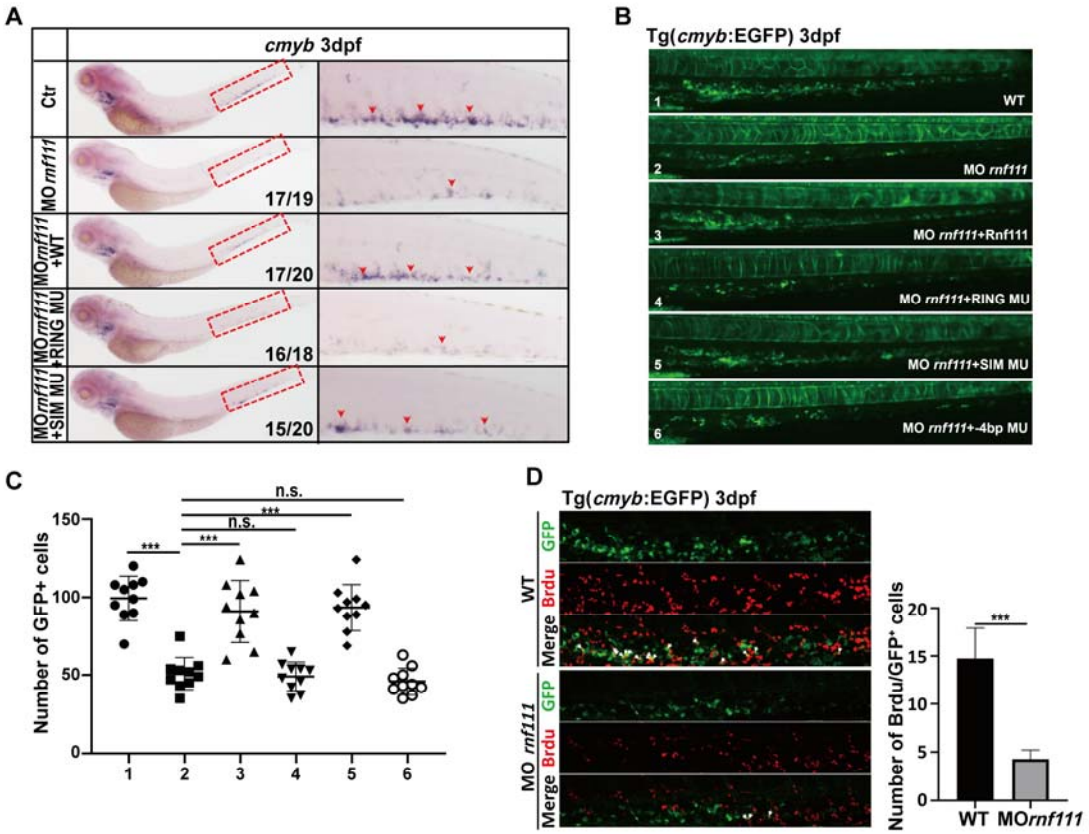

Figure S4.* Analysis of rescue efficiency of different Rnf111 mRNAs in *rnf111* morphants and Brdu assay in Tg(*cmyb*:EGFP) embryos at 3 dpf. (A) WISH analysis of rescue efficiency of Rnf111 WT, Rnf111 RING mutant and Rnf111 SIM mutant RNA. Red arrows indicate *cmyb*-positive HSPC. (B) Fluorescent images of rescue efficiency of Rnf111 RNAs on HSPC in *rnf111* morphants. (C) Statistical analysis of *cmyb*-GFP+ cell numbers in corresponding groups listed in Figure S4B. The data are presented as mean ± S.D. with ***P < 0.001 and n.s.: no significant difference. (D) Double immunostaining of *cmyb*-EGFP and anti-Brdu in the CHT region of Tg (*cmyb*-EGFP) line at 3 dpf. White arrows indicate Brdu and *cmyb*-EGFP double positive cells. All experiments were independently replicated at least three times. dpf: days post-fertilization; Ctr: control; MO *rnf111*: *rnf111* morphants; WT: wildtype; RING MU: Rnf111 RING mutant with the RING domain mutated; SIM MU: Rnf111 SIM mutant with three SUMO-interacting motifs all mutated; SIM: SUMO-interacting motif; HSPC: hematopoietic stem and progenitor cell; GFP: green fluorescent protein; Brdu: Bromodeoxyuridine; CHT: caudal hematopoietic tissue.

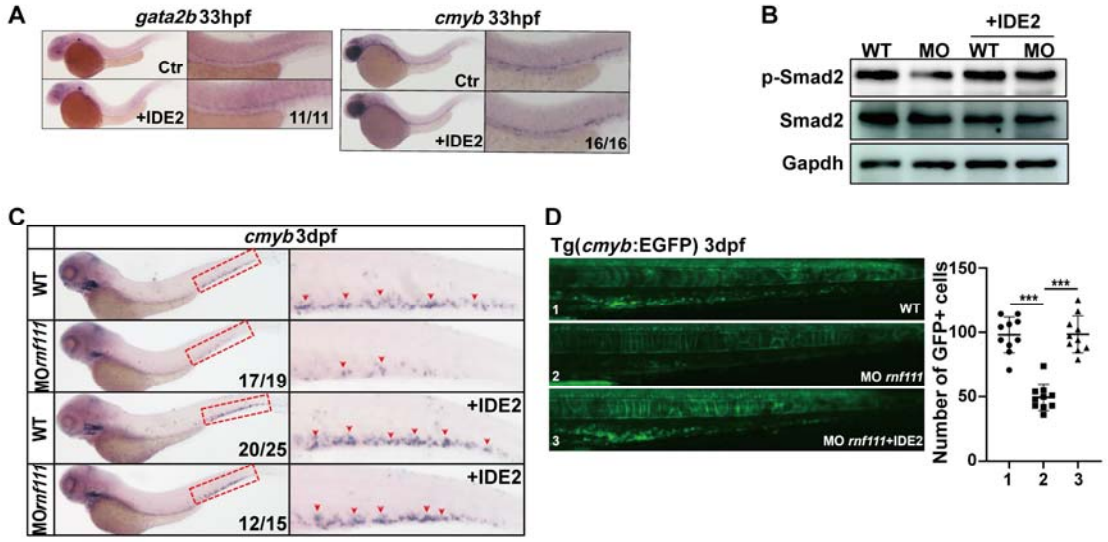

Figure S5.* Rescue efficiency of IDE2 in *rnf111* morphants. (A) WISH assay of HE marker *gata2b* and HSPC marker *cmyb* in IDE2 treated WT embryos from 10 hpf to 33 hpf. (B) Western blot analysis of phosphorylation of Smad2 with or without IDE2 treatment in *rnf111* morphants. (C) WISH analysis of rescue efficiency of IDE2 in *rnf111* morphants. Red arrows indicate *cmyb*-positive HSPC in the CHT. (D) Fluorescent images of rescue efficiency of IDE2 on HSPC in *rnf111* morphants and statistical analysis of *cmyb*-GFP^{+} cell number in different groups. The data are presented as mean ± S.D. with ***P < 0.001. All experiments were independently replicated at least three times. hpf: hours post-fertilization; Ctr: control; IDE2: definitive endoderm 2 inducer; WT: wildtype; MO *rnf111*: *rnf111* morphants; WISH: whole-mount in situ hybridization; HE: hemogenic endothelium; HSPC: hematopoietic stem and progenitor cell; CHT: caudal hematopoietic tissue.

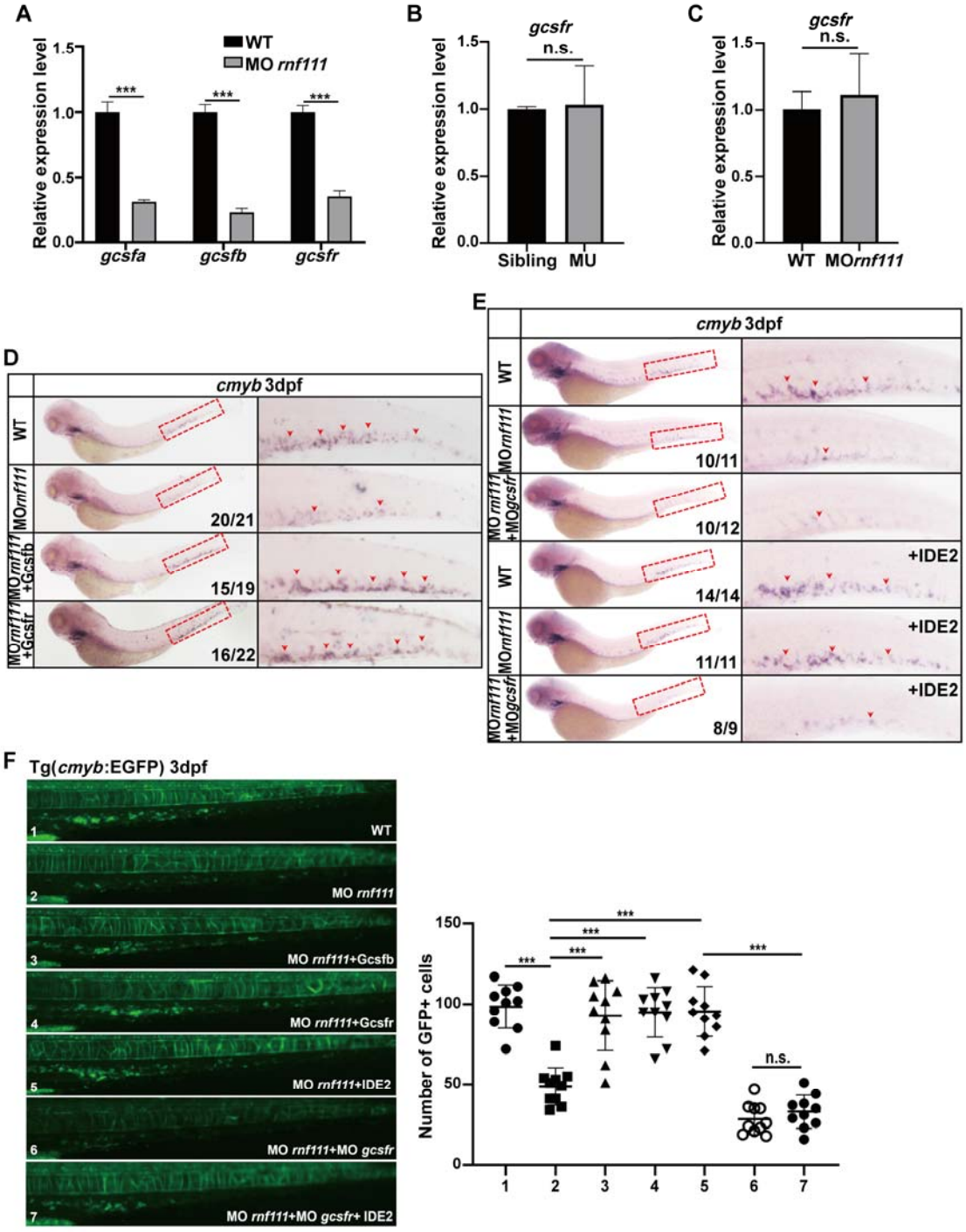

Figure S6.* Analysis of the function of Gcsfr involved in Rnf111 regulating HSPC development.

(A) RT-qPCR of *gcsfa*, *gcsfb* and *gcsfr* at 3 dpf. (B) RT-qPCR of *gcsfr* in siblings and mutants at 36 hpf. (C) RT-qPCR of *gcsfr* in AGM region of WT and *rnf111* morphants at 31 hpf. (D) Rescue assay of *gscfb* and *gcsfr* RNA in *rnf111* morphants. (E) Compared with *rnf111* MO + IDE2 group, *gcsfr* MO blocked the rescue effect of IDE2 on HSPC in *rnf111* morphants. (F) Fluorescent images of rescue efficiency of different treatment on HSPC in *rnf111* morphants and statistical analysis of

cmyb-GFP^{+} cell number in different groups. The data are presented as mean ± S.D. with ***P < 0.001 and n.s.: no significant difference. All experiments were independently replicated at least three times. Red arrows indicate *cmyb*-positive HSPC in the CHT. WT: wildtype; MO *rnf111*: *rnf111* morphants; dpf: days post-fertilization; MO: morpholino; IDE2: definitive endoderm 2 inducer; WISH: whole-mount in situ hybridization; RT-qPCR: real-time quantitative polymerase chain reaction; HSPC: hematopoietic stem and progenitor cell; CHT: caudal hematopoietic tissue.

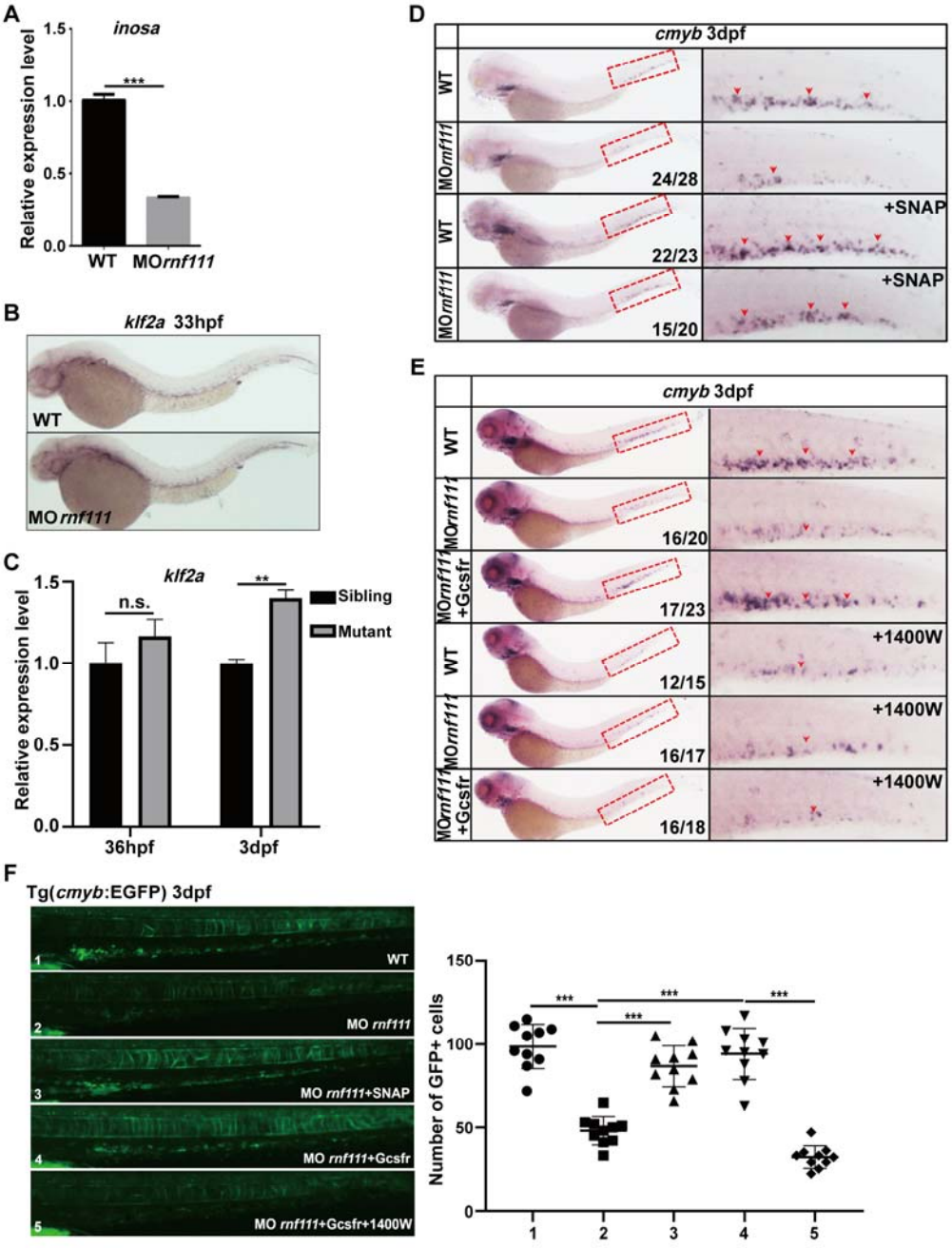

Figure S7.* Analysis of the function of NO signal involved in Rnf111 regulating HSPC development. (A) RT-qPCR result of *inosa* in *rnf111* morphants compared with WT embryos at 3 dpf. (B) WISH assay of *klf2a*. (C) RT-qPCR result of *klf2a* in siblings and mutants. (D) Rescue effect of NO agonist SNAP on *cmyb* expression was observed in *rnf111* morphants. (E) In the absence of 1400W, *gcsfr* RNA can rescue the HSPC defect in *rnf111* morphants. (F) Fluorescent images of rescue efficiency of different treatment on HSPC in *rnf111* morphants and statistical analysis of *cmyb*-GFP^{+} cell number in different groups. The data are presented as mean ± S.D. with **P < 0.01, ***P < 0.001 and n.s.: no significant difference. All experiments were independently

replicated at least three times. In contrast, the rescue effect of *gcsfr* RNA disappeared after 1400W treatment. WT: wildtype; MO *rnf111*: *rnf111* morphants; hpf: hours post-fertilization; dpf: days post-fertilization; SNAP: S nitroso N-acetylpenicillamine; 1400W: a NOS2-specific inhibitor; WISH: whole-mount in situ hybridization; RT-qPCR: real-time quantitative polymerase chain reaction; HSPC: hematopoietic stem and progenitor cell.

qPCR primers	zf-gcsfa-F	AACTACATCTGAACCTCCTG
	zf-gcsfa-R	GACTGCTCTTCTGATGTCTG
	zf-gcsfb-F	GGAGCTCTGCGCACCCAACA
	zf-gcsfb-R	GGCAGGGCTCCAGCAGCTTC
	zf-gcsfr-F	CGACTACAGACTCACTACAG
	zf-gcsfr-R	AGTATCAGCGTGGATGTTC
	zf-nos2a-F	CATCTCCCAGAAGACCCCAG
	zf-nos2a-R	GGGGCTAATTTGCTGACCTG
	zf-klf2a-F	ACCTTAACTGGGACGACTGG
	zf-klf2a-R	ATCCTTCCACCTGTTCTCCC
	zf-β-actin-F	GCTGTTTTCCCCTCCATTGTT
	zf-β-actin-R	TCCCATGCCAACCATCACT
	negative primer-F	CGCCAGACAGTGACAAAAGA
	negative primer-R	ATCAGCGGCTCACATAAAGG
	positive primer-F	TTCGGTTTGTGCTTGTGGTT
	positive primer-R	TTGTGTAGTTTGTGCCCAGC

Table S1. The sequence of primers used in quantitative polymerase chain reaction. qPCR: quantitative polymerase chain reaction; zf: zebrafish; F: forward primer; R: reverse primer.