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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Abstract

The ubiquitination or SUMOylation of hematopoietic-related factors plays pivotal roles in hematopoiesis. RNF111, known as a ubiquitin ligase, is a newly discovered SUMO-targeted ubiquitin ligase involved in multiple signaling pathways mediated by transforming growth factor (TGF)-β family members. However, its role in hematopoiesis remains unclear. Herein, a heritable Rnf111 mutant zebrafish line was generated by CRISPR/Cas9-mediated genome editing. Impairment of hematopoietic stem and progenitor cells (HSPC) of definitive hematopoiesis was found in Rnf111-deficient mutants. Ablation of Rnf111 resulted in decreased phosphorylation of Smad2/3 in HSPC. Definitive endoderm 2 inducer (IDE2), which specifically activates TGF-β signaling and downstream Smad2 phosphorylation, could restore definitive hematopoiesis in Rnf111-deficient embryos. Further molecular mechanism studies revealed that Gcsfr/NO signaling was an important target pathway of Smad2/3 involved in Rnf111-mediated HSPC development. In conclusion, our study demonstrated that Rnf111 contributes to the development of HSPC by maintaining Smad2/3 phosphorylation and activation of the Gcsfr/NO signaling pathway.

Introduction

In vertebrates, hematopoiesis occurs in multiple waves.[1,2] The earliest wave, namely primitive hematopoiesis, mainly produces erythroid and myeloid cells.[2] The second wave of hematopoiesis - intermediate hematopoiesis - occurs in the posterior blood island and gives rise to erythromyeloid progenitors.[3] The last wave, also called definitive hematopoiesis, generates hematopoietic stem cells (HSC) that are multipotent and can give rise to all lineages of blood cells for the whole life span.[4] The hematopoietic regulatory network of zebrafish (*Danio rerio*) is highly conserved with that of mammals. Almost all transcription factors and key genes involved in hematopoietic regulation in mammals have orthologues in zebrafish.[5] In zebrafish, definitive hematopoietic stem and progenitor cells (HSPC) emerge from the ventral wall of the dorsal aorta, an area functionally equivalent to the mammalian aorta-gonad-mesonephros,[6,7] at approximately 33 to 35 hours post-fertilization (hpf) and then the nascent HSPC migrate to the caudal hematopoietic tissue, which is analogous to the mammalian fetal liver.[8] Beginning at around 4 days post-fertilization (dpf), HSPC migrate through caudal hematopoietic tissue to the adult hematopoietic tissue-thymus and kidney marrow, which is similar to the process by which HSC migrate through fetal liver and home to bone marrow in mammals.[8]

The development of HSC is precisely regulated by a variety of transcription factors and signaling pathways. Dysregulation of this process leads to serious developmental defects or diseases. It has been reported that the ubiquitination and SUMOylation of hematopoietic-related factors also play important roles in the hematologic system.[9-11] Arkadia (also known as RING finger 111, RNF111) is a nuclear E3 ubiquitin ligase (UbL) that ubiquitinates intracellular effectors and regulators of transforming growth factor (TGF)-β/nodal-related signaling, leading to their proteasome-dependent

degradation. The characteristic RING finger domain of RNF111 in its C-terminus is required for degradation of the three major negative regulators of TGF-β signaling - Smad7, c-Ski and SnoN - in a small ubiquitin-like modifier (SUMO)-independent manner.[12-15] RNF111 specifically regulates induced regulatory T-cell differentiation through facilitating the degradation of SKI/SnoN proteins.[16] Knockdown of RNF111 promotes the differentiation of C2C12 myoblasts through reducing myostatin/TGF-β signaling.[17] RNF111 is also involved in atrial fibrillation-induced myocardial fibrosis through mediating poly-ubiquitination and degradation of Smad7.[18] Besides, RNF111 has been recognized as a SUMO-targeted ubiquitin ligase (STUbL) in the past several years. STUbL recognizes polysumoylated proteins through N-terminal SUMO-interacting motifs (SIM), mediating the ubiquitination degradation of target sumoylated substrates.[19] RNF111 was shown to ubiquitinate polysumoylated promyelocytic leukemia in a SIM-dependent manner and was required for subsequent degradation of the polyubiquitinated product in promyelocytic leukemia nuclear bodies.[20,21] Meanwhile, RNF111 has been discovered to enhance the ubiquitylation of SUMOylated xeroderma pigmentosum C protein, a pivotal DNA damage recognition factor of nucleotide excision repair, to facilitate the DNA damage response.[22] However, the physiological function of RNF111 in hematopoiesis currently remains unknown.

To determine the role of RNF111 in hematopoiesis, we generated a heritable Rnf111 zebrafish mutant line by CRISPR/Cas9-mediated knockout technology. The Rnf111-deficient embryos showed defects in definitive hematopoiesis, manifested by a reduction in HSPC, whereas primitive hematopoiesis was unaffected. Mechanistic studies indicated that Rnf111 plays an important role in the development of HSPC through maintaining the phosphorylation of Smad2/3 and activating the granulocyte colony-stimulating factor receptor/nitric oxide (Gcsfr/NO) signaling pathway.

Methods

Zebrafish maintenance and mutant generation

Zebrafish were maintained and staged under standard conditions as described before.[23] All animal-related procedures were approved by the Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. The methods for generating Rnf111 knockout zebrafish and identifying the genotypes are described in the *Online Supplementary Methods*. Heritable fish with 4-base deletions were preserved and utilized for subsequent phenotypic analysis.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization (WISH) was carried out with probes including *cmyb*, *scl*, *mpx*, *hbae1*, *runx1* and *rag1*, which have already been used and reported before,[24] and the *gcsfr*, *rnf111* and *gata2b* probes, which were newly cloned into pGEM-T Easy vector (Promega). Details of the procedure are described in the *Online Supplementary Methods*.

Morpholinos and mRNA microinjection

The sequences of the morpholinos and details about mRNA microinjection are provided in the *Online Supplementary Methods*.

Quantitative real-time polymerase chain reaction

The procedures for the reverse transcription and quantitative polymerase chain reaction (PCR) are described in the *Online Supplementary Methods*. Each sample was tested in triplicate. The *Online Supplementary Table* details the primers employed for real-time quantitative PCR.

Immunofluorescence assay, bromodeoxyuridine and enhanced green fluorescent protein double immunostaining and confocal fluorescent imaging

The 3 dpf transgenic Tg (*cmyb*:EGFP)[25] embryos were used for immunofluorescence assays. Double staining with bromodeoxyuridine (Brdu) and enhanced green fluorescent protein (EGFP) was carried out on the 3 dpf Tg (*runx1*: EGFP)[26] and Tg (*cmyb*:EGFP) embryos. Details of the procedures are described in the *Online Supplementary Methods*. An FV 1000 confocal microscope (Olympus, Tokyo, Japan) was used for observation and taking images.[24]

Immunohistochemistry, cell cultures and assays

The embryos were treated with 4% paraformaldehyde overnight at 4°C, followed by incubation with Sudan black (Sigma-Aldrich) solution for 25 minutes. Subsequently, they were thoroughly washed in 70% ethanol to facilitate the detection of the granules of granulocytes.

HEK293T cells were used for plasmid transfections according to the manufacturer's instructions. Detailed procedures are provided in the *Online Supplementary Methods*.

The 3 dpf embryos were subjected to immunohistochemical studies according to procedures described in the *Online Supplementary Methods*.

The preparation of samples, information about antibodies and other relevant details on western blot analysis and chromatin immunoprecipitation (ChIP) PCR are described in the *Online Supplementary Methods*.

Chemical treatment

The concentrations of drugs and duration of treatments are listed in the *Online Supplementary Methods*.

DAF-FM assay

Embryos at 28 hpf, 2 dpf and 3 dpf were exposed to 5 μM DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) for 2 hours in the dark at 28.5°C, rinsed in fish water and then observed under a fluorescence microscope.[27] The NO fluorescence intensity was quantified by

ImageJ software and the relative intensity was calculated and presented as mean ± standard deviation.

Statistical analysis

All experiments were independently replicated at least three times. SPSS software (version 20) was used for the statistical analysis. Comparisons between two groups were conducted using a Student unpaired two-tailed *t* test, while one-way analysis of variance (ANOVA) was employed for the comparison of multiple groups. Statistical significance was established at a threshold of $P<0.05$.

Results

Evolutionary conservation of Rnf111 and generation of a heritable Rnf111-mutant zebrafish line

Zebrafish Rnf111 contains all the structural motifs, including nuclear localization signals, a RING finger domain, SUMO-interacting motifs (SIM) and Axin1 interaction domain (AID), which were originally described for mouse RNF111.[28] Zebrafish *rnf111* has a highly degree of synteny with the *RNF111* locus in human (*Online Supplementary Figure S1A*) and its protein shares a 53.46% sequence similarity with human RNF111 (*Online Supplementary Figure S1B*). Subsequently, both antisense and sense probes were used to detect the mRNA expression pattern of *rnf111* in zebrafish embryos with a WISH assay. The results showed that *rnf111* is a maternal gene that exhibits ubiquitous expression, with high expression in the nervous system and relatively weak expression in hematopoietic tissues (*Online Supplementary Figure S1C*), consistent with the single-cell gene expression data on the Daniocell website.

To address the roles of Rnf111 in hematopoiesis, a Rnf111 mutant line was generated using the CRISPR/Cas9 system. The target site was in exon 5 and four nucleotides were deleted (Figure 1A, B), which led to a frameshift and formed a truncated protein containing only 552 amino acids (Figure 1C). The wild-type and mutant coding sequences were cloned into PCS2+ vector and transfected into 293T cells, and a shorter protein corresponding to the truncated mutant was detected by western blot analysis (Figure 1D). To further confirm the function of the truncated protein, the CAGA12-luc transcriptional reporter construct,[15,29] stimulated by TGF-β and activin, was used to test whether the truncated protein can promote activation of TGF-β-inducible DNA elements detectable by dual luciferase reporter assay. As expected, Rnf111 mutants failed to promote the luciferase reporter gene expression (Figure 1E), indicating that the *rnf111*-mutated allele was indeed loss-of-function.

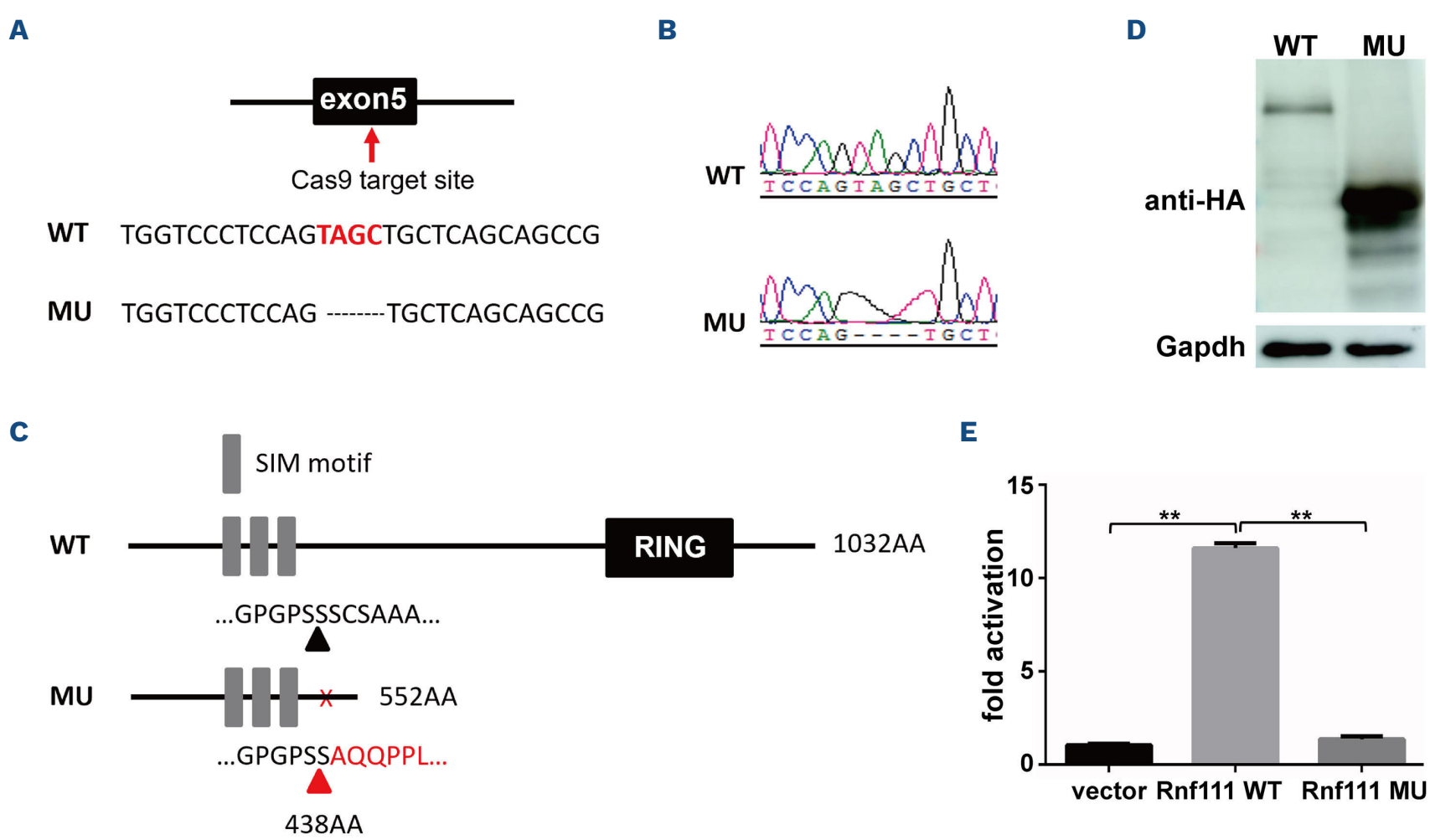

Figure 1. Generation of the Rnf111 mutant line. (A) The Cas9 target site was in the fifth exon; 4 base pair (bp) nucleotides were deleted. (B) The wild-type and mutant sequencing peak maps. (C) Schematic representation of wild-type and mutant Rnf111 proteins. (D) Western blot of HA-tagged wild-type and mutant Rnf111 proteins expressed in HEK293T cells. (E) CAGA12-luciferase transcriptional reporter assay of wild-type and mutant Rnf111. The data are presented as mean ± standard deviation with $**P<0.01$. WT: wild-type; MU: Rnf111 mutants with deletion of 4 bp; SIM: SUMO-interacting motifs.

Meanwhile, we found that the homozygous mutants cannot grow into adult fish and died within 8 to 10 days, which was consistent with the fate of *Rnf111* knockout mice.

Ablation of Rnf111 impairs the development of definitive hematopoietic stem and progenitor cells

We analyzed the hematopoietic phenotypes of Rnf111 mutated embryos. Firstly, a series of markers involved in primitive hematopoiesis, such as the hematopoietic precursor cell marker *scl*,[30] mature erythrocyte marker *hbae1*[31] and myeloid-specific marker *mpx*[32] were examined at 22 hpf (*Online Supplementary Figure S2A*); no overt changes were observed, suggesting that primitive hematopoiesis was not affected in Rnf111 mutants. Then WISH analysis of the definitive wave was carried out. The expression of HSPC markers *cmyb*[33] and *runx1*[1] was normal at 36 hpf, but the expression of *cmyb* was decreased in Rnf111 mutants from 2 dpf (Figure 2A, B), suggesting that definitive HSPC was impaired. Moreover, the expression of the mature erythrocyte marker *hbae1*, myeloid-specific marker *mpx* and Sudan black staining, and lymphoid specific marker *rag1*[34] were all diminished in Rnf111 mutants (Figure 2C), which further indicated that the deficiency of HSPC occurred in the Rnf111 mutants.

To further confirm the phenotype of hematopoiesis, a translation-blocking antisense morpholino oligonucleotide, which can sharply knock down the level of Rnf111 protein (*Online Supplementary Figure S2B*), was used to inhibit the function of Rnf111. The phenotypes of both primitive and definitive waves of *rnf111* morpholino-injected embryos (*rnf111* morphants) were detected by WISH using markers for each blood lineage involved in hematopoietic development. Consistent with the results in Rnf111 mutants, the *scl*, *mpx* and *hbae1* markers were normal in primitive hematopoiesis (*Online Supplementary Figure S2C*). The expression of definitive HSPC markers *cmyb*[33] and *runx1* in rnf111 morphants was comparable with that in wild-type embryos at 33 hpf (*Online Supplementary Figure S3A, B*), which indicated that deficiency of Rnf111 had no effect on the generation of HSPC. The number of HSPC was decreased from 2 dpf in all subsequent developmental stages (*Online Supplementary Figure S3A*), suggesting that definitive HSPC was impaired. We then injected a morpholino to Tg (*cmyb*:EGFP) embryos (a stable zebrafish transgenic line expressing EGFP under the control of the *cmyb* promoter).[25] As expected, the number of EGFP-positive cells was significantly decreased in the caudal hematopoietic tissue at 2 dpf and 3 dpf (*Online Supplementary Figure S3C*). Finally, the markers of downstream lineages were all diminished in *rnf111* morphants (*Online Supplementary Figure S3D*). These results further confirmed that HSPC were specifically affected in Rnf111-deficient embryos.

The functional domain of ubiquitin ligase is required for Rnf111 to regulate hematopoietic stem and progenitor cell development

Rnf111 is a multifunctional protein acting as both an UbL and STUbL. In order to elucidate the functional contribu-

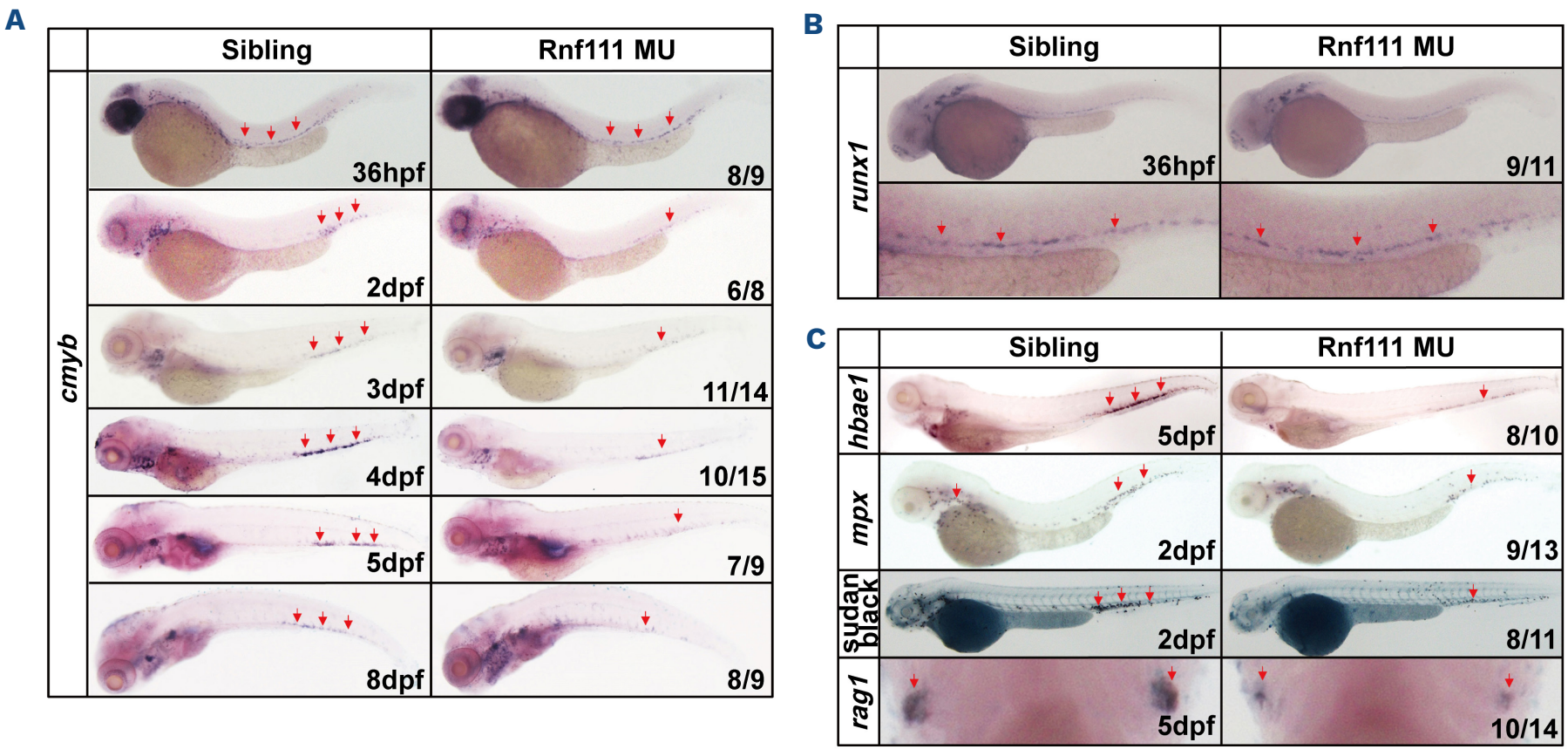

Figure 2. Impairment of definitive hematopoiesis in Rnf111 mutants. (A) Whole-mount *in situ* hybridization (WISH) analysis of *cmyb* expression from 36 hpf to 8 dpf. (B) WISH assay of *runx1* in mutants at 36 hpf. (C) WISH analysis of key hematopoietic markers and Sudan black analysis. All experiments were independently replicated at least three times. MU: Rnf111 mutants with deletion of 4 base pair nucleotides; hpf: hours post-fertilization; dpf: days post-fertilization.

tion of Rnf111 in the regulation of HSPC development, four vectors - Rnf111 WT, Rnf111 SIM MU, Rnf111 RING MU and Rnf111 -4bp MU - were constructed and the rescue effects of corresponding mRNA on HSPC defects were compared in Rnf111-deficient embryos (Figure 3A). The amino acids of the three SUMO-interacting motifs (SIM) were all mutated to alanine in the Rnf111 SIM mutant (Rnf111 SIM MU), which abolishes its binding to SUMO-modified substrates.[21] Four cysteine residues within the RING domain of the active center of the UbL activity were mutated in the Rnf111 RING mutant (Rnf111 RING MU), which enabled it to still bind to but not to degrade the substrates.[15] The result showed that Rnf111 WT effectively rescued the deficient HSPC in Rnf111 mutants, while the mRNA, whose sequence was consistent with the sequence of the Rnf111 -4bp mutant line (-4bp MU), failed to do so (Figure 3B), demonstrating that the phenotype observed in Rnf111 mutants was indeed Rnf111 dependent. Interestingly, while Rnf111 SIM MU could rescue the defect of HSPC, Rnf111 RING MU lost its rescue effect (Figure 3B). The same rescue assays were carried out in *rnf111* morphants, and similar results were obtained (*Online Supplementary Figure S4A-C*). All these results suggest that Rnf111 regulates HSPC development depending on its UbL function rather than STUbL activity.

Decreased proliferation capacity of hematopoietic stem and progenitor cells was observed in *rnf111* morphants

TGF-β signaling, which was the main pathway that Rnf111 regulated,[12-15] can affect HSPC development by regulating proliferation.[35,36] Therefore, Brdu incorporation assays were carried out to detect the proliferation ability of HSPC in Tg (*runx1*: EGFP) and Tg (*cmyb*: EGFP) *rnf111* morphants. The results showed that the number of $Brdu^+/GFP^+$ cells in both transgenic lines decreased sharply in the caudal hematopoietic tissue of *rnf111* morphants (Figure 4A, B, *Online Supplementary Figure S4D*), indicating that the proliferation capacity of HSPC was severely impaired, which may be related to impaired TGF-β signaling.

The decrease of p-Smad2/3 protein leads to hematopoietic stem and progenitor cell defects in Rnf111-deficient embryos

In line with our finding that Rnf111 regulated the development of HSPC depending on its UbL function, it was reported previously that this function was also required for Rnf111 to regulate the TGF-β signaling pathway. Rnf111 degrades Smad7, an inhibitory protein of the TGF-β signaling pathway, in a SUMO-independent manner and promotes the phosphorylation of receptor Smad (Smad2 and Smad3). It was also reported that TGF-β at low doses (picogram levels) stimulated colony formation from $CD34^+$ cells.[36] Considering the reduced

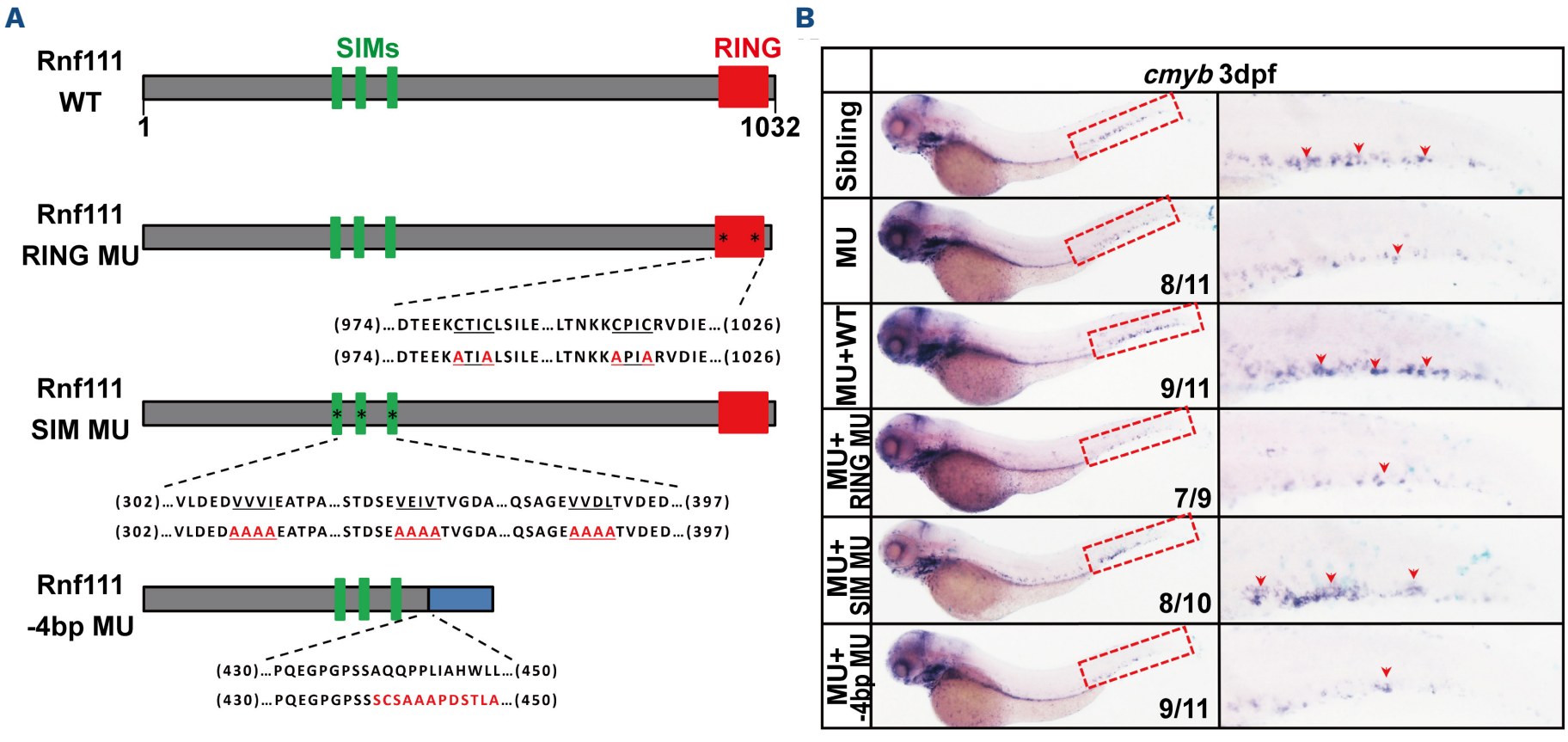

Figure 3. Schematic representation and rescue assay of the four Rnf111 constructs. (A) Schematic diagram of the Rnf111 wild-type, Rnf111 RING mutant, Rnf111 SIM mutant and -4 base pair (bp) mutant proteins. (B) Whole-mount *in situ* hybridization analysis of the rescue efficiency of Rnf111 wild-type, Rnf111 RING mutant, Rnf111 SIM mutant and Rnf111 -4 bp mutant RNA. Red arrows indicate *cmyb*-positive hematopoietic stem and progenitor cells. All experiments were independently replicated at least three times. WT: wild-type; 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 motifs; -4bp MU: Rnf111 -4 bp mutant with deletion of 4 bp nucleotides; dpf: days post-fertilization; MU: Rnf111 mutants with deletion of 4 bp nucleotides.

proliferation capacity of HSPC observed in *rnf111* morphants, we speculated that Rnf111 may regulate the development of HSPC by affecting the phosphorylation of Smad2/3. We, therefore, performed anti-p-Smad2/3 and EGFP double immunostaining assays in Tg (*cmyb*: EGFP) embryos. The results showed a basal level of Smad2/3 phosphorylation in HSPC of wild-type embryos (Figure 5A, A', A''), while Smad2/3 phosphorylation was significantly reduced in HSPC of *rnf111* morphants (Figure 5B, B', B'', C). Immunohistochemistry and western blot analysis of whole embryos demonstrated the same downward trend of p-Smad2/3 (Figure 5D-F) with no obvious changes in general Smad2 and Smad3 protein levels. Meanwhile, the protein level of Smad7 was upregulated (Figure 5G), which may be responsible for the reduction of p-Smad2/3. Then, to further investigate whether the observed Rnf111 ablation causing the HSPC defective phenotype was due to the decrease of p-Smad2/3, definitive endoderm 2 inducer (IDE2), a drug that specifically activates TGF-β signaling and downstream Smad2 phosphorylation with no effect on hemogenic endothelium and HSPC emergence (*Online Supplementary Figure S5A, B*), was used to treat Rnf111 mutants. As expected, IDE2 effectively rescued *cmyb* expression (Figure 5H). We also carried out the IDE2 rescue assay in *rnf111* morphants, obtaining the same result (*Online Supplementary Figure S5C, D*). All these results demonstrated that the reduction of p-Smad2/3 was the key cause of the HSPC defect in Rnf111-deficient embryos.

The Gcsf signaling pathway was the downstream target of Rnf111-Smad2/3

As an important regulator of classical TGF-β signaling pathway, Rnf111 promotes TGF-β signaling by degrading the repressor proteins and facilitates the phosphorylation of receptor-Smad2/3. It has been reported that TGF-β can regulate the amount of granulocyte-macrophage colony-stimulating factor receptors (GM-CSFR), interleukin-3 receptors (IL-3R), and granulocyte colony-stimulating factor receptors (GCSFR) in mouse hematopoietic progenitor cell lines without affecting the receptor affinity.[37] Gcsf signaling is required for HSPC emergence and expansion in zebrafish.[38]

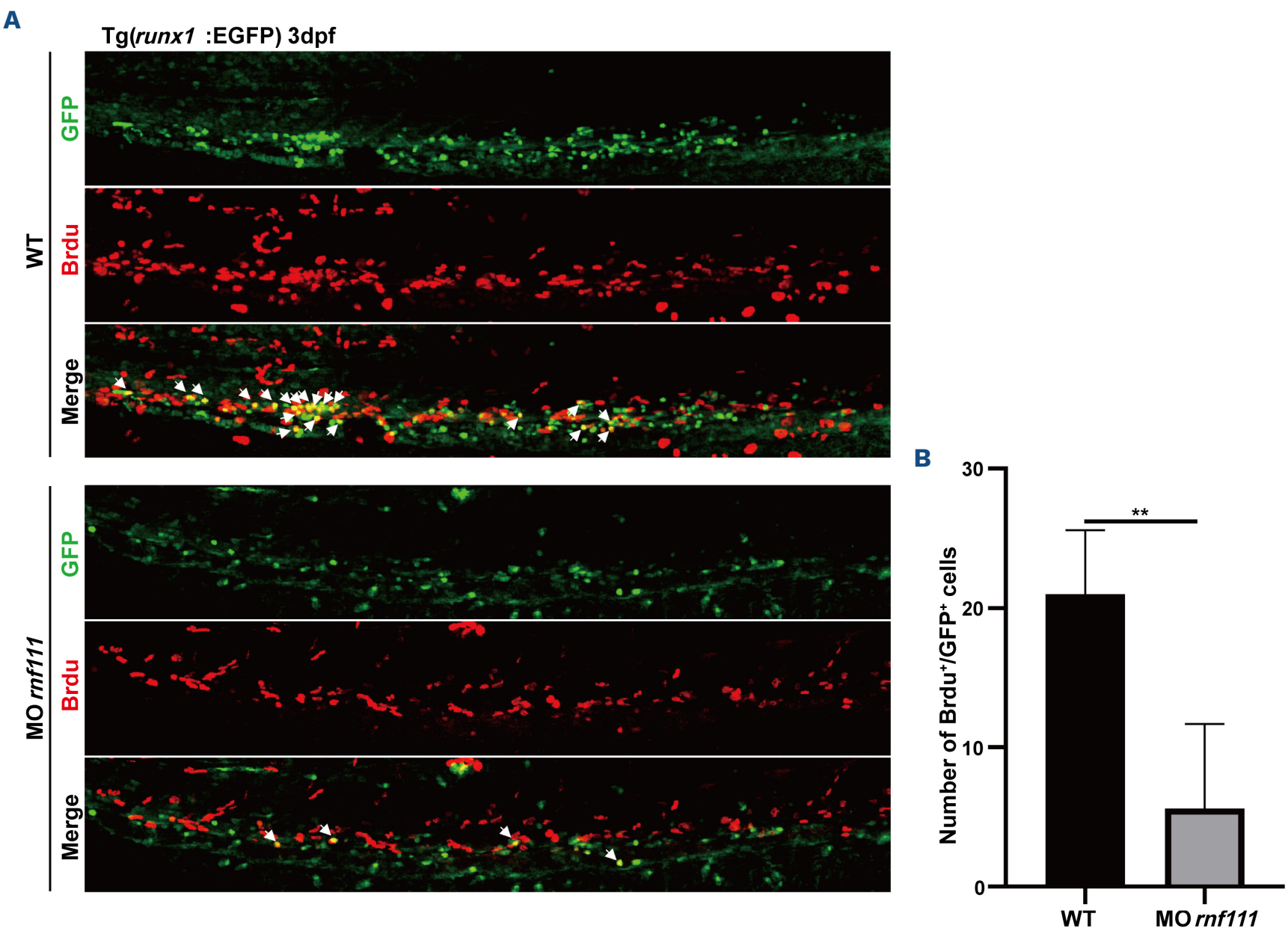

Figure 4. Bromodeoxyuridine incorporation assay. (A) Double immunostaining of *runx1*-EGFP with green fluorescent protein (GFP) and anti-bromodeoxyuridine (Brdu) in the caudal hematopoietic tissue of the transgenic Tg (*runx1*-EGFP) line at 3 dpf. White arrows indicate Brdu and *runx1*-EGFP double-positive cells. (B) Statistics of Brdu and *runx1*-GFP double-positive cells. The data are presented as mean ± standard deviation. with $^{**}P<0.01$. dpf: days post-fertilization; WT: wild-type; MO *rnf111*: *rnf111* morphants.

Based on our results, we hypothesized that Rnf111 regulates phosphorylation of Smad2/3 to promote Gcsf signaling in HSPC development. To test this hypothesis, quantitative RT-PCR was performed to detect the expression of *gcsfr*, *gcsfa* and *gcsfb* in 3 dpf embryos. All these three genes exhibited decreased expression in Rnf111 mutants and *rnf111* morphants (Figure 6A, *Online Supplementary Figure S6A*). The WISH results further confirmed that *gcsfr* expression was reduced (Figure 6B). However, the expression of *gcsfr* in Rnf111 mutants (36 hpf) and the aorta-gonad-mesonephros region of *rnf111* morphants (31 hpf) was unchanged (*Online Supplementary Figure S6B, C*). Then, *cmyb*-GPF positive HSPC at 3 dpf were sorted to detect *gcsfr* expression with quantitative RT-PCR assay. The result showed that *gcsfr* expression was reduced in HSPC (Figure 6C).

Then the -2.5k promotor sequence of *gcsfr* was cloned into the PGL3-basic vector and luciferase activity assay was performed. The results indicated that IDE2, Rnf111 WT and Rnf111 SIM MU could activate the luciferase expression, while Rnf111 RING MU failed (Figure 6D). These results were in line with those of the *in vivo* rescue assay (Figure 3). Then, GFP and Smad2-GFP RNA were injected in zebrafish embryos and chromatin immunoprecipitation PCR (ChIP-PCR) analysis was carried out to detect the binding ability of Smad2 to endogenous *gcsfr* promotor. The results showed that the promoter region of *gcsfr* could be specifically co-immunoprecipitated with Smad2-GFP (Figure 6E).

In order to further confirm the pivotal role of Gcsf signaling in Rnf111 regulation of HSPC development, the RNA rescue assay of both ligand (Gcsfb) and receptor (Gcsfr) was implemented. As expected, both *gcsfb* and *gcsfr* RNA rescued the developmental defects of HSPC in both Rnf111 mutants and *rnf111* morphants (Figure 6F, *Online Supplementary Figure S6D, F*). Moreover, when the expression of Gcsfr was knocked down by *gcsfr* morpholinos, the rescue effect of IDE2 was abolished (*Online Supplementary Figure S6E, F*), suggesting that Gcsfr was a pivotal target of Smad2.

Decreased Gcsfr-NO signaling leads to the defect of hematopoietic stem and progenitor cell development in Rnf111-deficient embryos

It was reported that infection-induced Gcsfr-NO signaling can enhance the expansion of HSPC in zebrafish and that Cebpb-Nos2a acts downstream of Gcsf signaling.[39] To verify whether Cebpb is involved in the HSPC defect induced by Rnf111 deficiency, *cebpb* RNA was injected into the Rnf111 mutants. Notably, the defect of HSPC could be effectively rescued (Figure 7A). Since our RT-qPCR results revealed decreased expression of the downstream gene *nos2a* (*inosa*) in both Rnf111 mutants (2 dpf and 3 dpf) and *rnf111* morphants (3 dpf) (Figure 7B, *Online Supplementary Figure S7A*), the cell-permeable DAF-FM DA fluorescent NO probe was used to examine the active NO production.[40] The results showed that NO production was decreased

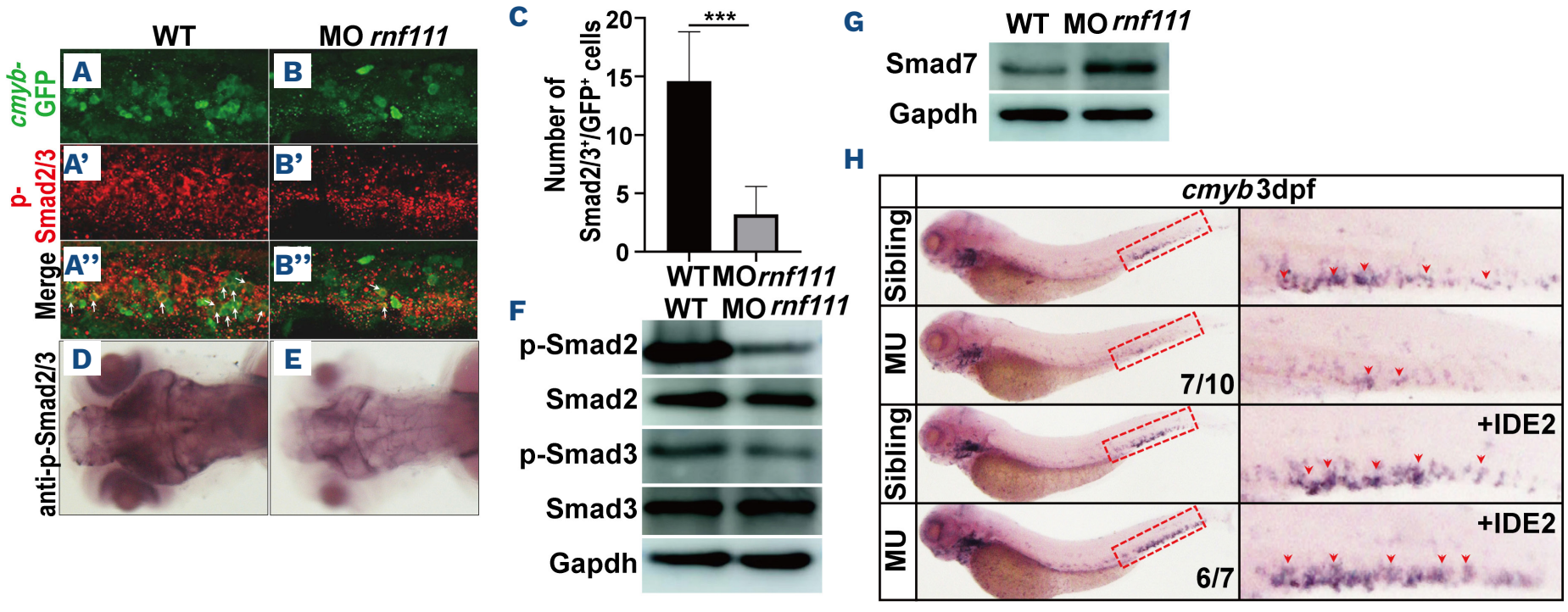

Figure 5. Analysis of p-Smad2/3, Smad7 and IDE2 rescue assay in Rnf111-deficient embryos. (A, A', A'', B, B', B'') Double immunostaining of anti-GFP (A, B) and anti-p-Smad2/3 (A', B') in the caudal hematopoietic tissue of the transgenic Tg (*cmyb*-EGFP) line at 3 dpf. The bottom panel shows merged images (A'', B''). White arrows indicate p-Smad2/3 and *cmyb*-EGFP double-positive cells. (C) Statistics of p-Smad2/3 and *cmyb*-EGFP double-positive cells. The data are presented as mean ± standard deviation with ***P<0.001. (D, E) Immunohistochemistry of p-Smad2/3. (F) Western blot analysis of p-Smad2, Smad2, p-Smad3 and Smad3. (G) Western blot analysis of Smad7. (H) Whole-mount *in situ* hybridization analysis of rescue efficiency of IDE2. Red arrows indicate *cmyb*-positive hematopoietic stem and progenitor cells in the caudal hematopoietic tissue. All experiments were independently replicated at least three times. WT: wild-type; MO *rnf111*: *rnf111* morphants; GFP: green fluorescent protein; MU: Rnf111 mutants with deletion of 4 base pair nucleotides; IDE2: definitive endoderm 2 inducer; EGFP: enhanced green fluorescent protein; dpf: days post-fertilization.

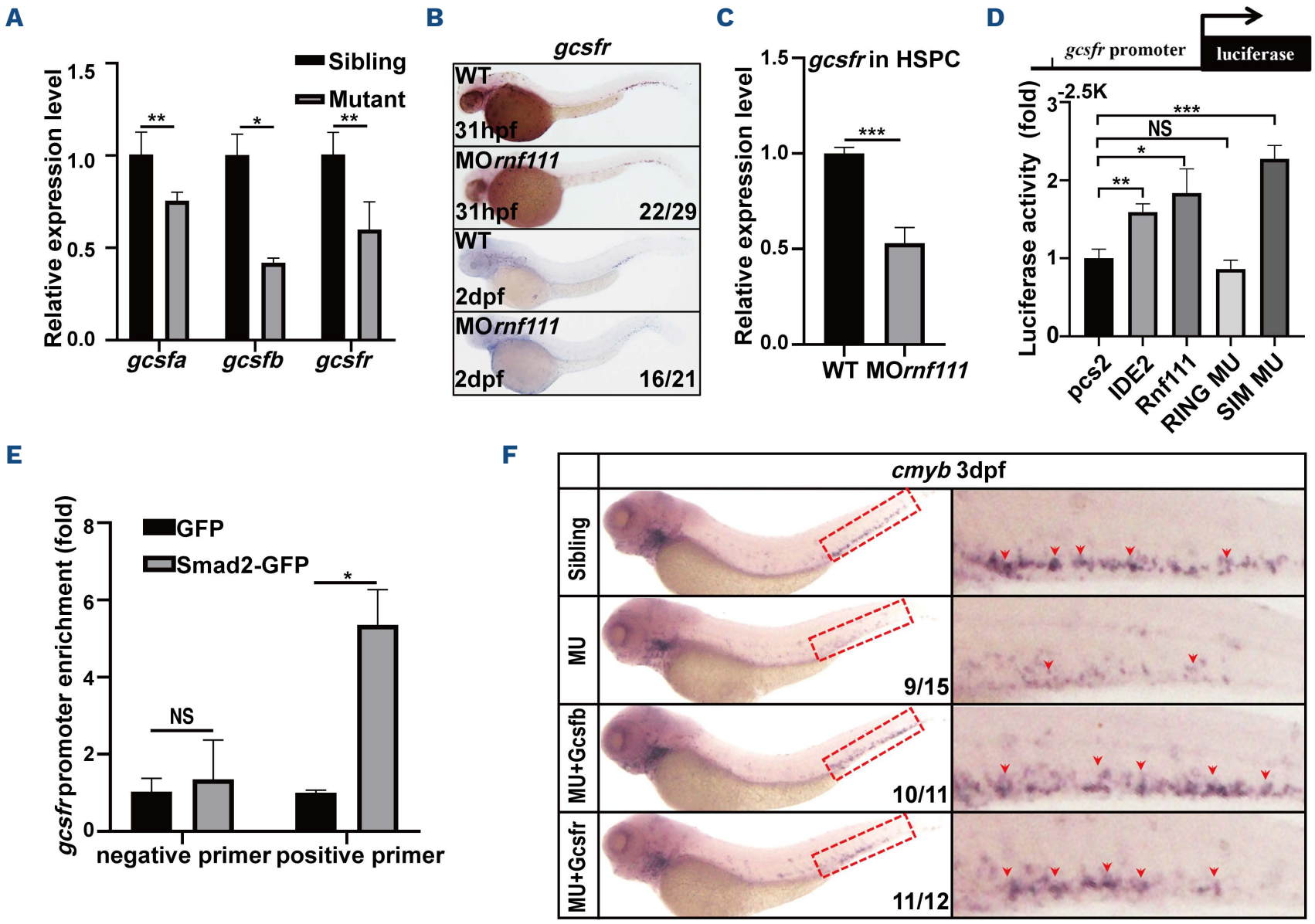

Figure 6. Gcsf signaling was the downstream target of Rnf111-Smad2/3. (A) Real-time quantitative polymerase chain reaction (qPCR) of *gcsfa*, *gcsfb* and *gcsfr* in siblings and mutants at 3 dpf. The data are presented as mean ± standard deviation (SD) with **P*<0.05, ***P*<0.01. (B) Whole-mount *in situ* hybridization analysis of *gcsfr*. (C) Real-time qPCR of *gcsfr* in HSPC of *rnf111* morphants. The data are presented as mean ± SD with ****P*<0.01. (D) Luciferase assay of *gcsfr* promotor in HEK293T cells. The data are presented as mean ± SD with **P*<0.05, ***P*<0.01, ****P*<0.001, NS: no significant difference. (E) Chromatin immunoprecipitation qPCR assay of Smad2-GFP binding to *gcsfr* promotor at 2 dpf. The data are presented as mean ± SD with **P*<0.05, NS: no significant difference. (F) Rescue assay of *gcsfb* and *gcsfr* RNA in Rnf111 mutants. Red arrows indicate *cmyb*-positive hematopoietic stem and progenitor cells in the caudal hematopoietic tissue. All experiments were independently replicated at least three times. WT: wild-type; MO *rnf111*: *rnf111* morphants; HSPC: hematopoietic stem and progenitor cell; IDE2: definitive endoderm 2 inducer; RING MU: Rnf111 RING mutant with the RING domain mutated; SIM MU: Rnf111 SIM mutant with three SUMO-interacting motifs all mutated; GFP: green fluorescent protein; MU: Rnf111 mutants with deletion of 4 base pair nucleotides; hpf: hours post-fertilization; dpf: days post-fertilization.

in *rnf111* morphants at 2 dpf and 3 dpf (Figure 7C, D). It is reported that blood flow-induced Klf2a-NO signaling can regulate NO production.[27] Therefore, we detected the expression of *klf2a* in Rnf111-deficient embryos by WISH assay and quantitative RT-PCR assay. *klf2a* expression was not decreased in Rnf111-deficient embryos compared to control ones (*Online Supplementary Figure S7B, C*), suggesting that Klf2a-NO signaling did not contribute to the defects of HSPC in Rnf111-deficient embryos. In addition, we also employed the rescue assay by treating Rnf111 mutants and *rnf111* morphants with NO agonist, S nitroso N-acetylpenicillamine (SNAP). The WISH results showed that the expression of *cmyb* was partially restored (Figure 7E, *Online Supplementary Figure S7D*). And the rescue effect of SNAP was also proved in Tg(*cmyb*:EGFP) line (*Online Supplementary Figure S7F*). Next, we used the NOS2-specific inhibitor 1400W to block the production of NO and found that the rescue effect of *gcsfr* RNA on HSPC was abolished (*Online Supplementary Figure S7E, F*). Taken together, these data suggest that Rnf111 regulates HSPC expansion, at least in part, through Gcsfr-Cebpb-NO signaling.

Discussion

RNF111 is an UbL that enhances TGF-β signaling by promoting the degradation of repressors (SnoN, Ski and Smad7)

that inhibit the expression of target genes.[13,14] RNF111 also was the second STUbL identified; its targets substrates with SUMO1-capped SUMO2 chains significantly superior to other identical substrates with homogeneous SUMO2 or SUMO1 chains.[21] Rnf111 was responsible for induction of the node through enhancing nodal signaling,[41,42] as well as progression of tissue fibrosis and cancer through regulating TGF-β signaling.[43-45] In this study, we found a novel biological role of Rnf111 in zebrafish hematopoiesis. Both Rnf111 mutants and *rnf111* morphants showed impaired definitive hematopoiesis without defects in the primitive wave, manifested by a decrease of definitive HSPC and downstream lineages. Although we could not exclude the possibility that erythromyeloid progenitors partially contributed to decreased *mpx* expression and Sudan black staining in mutants at 2 dpf, the number of myeloid cells of HSPC origin was indeed reduced, as shown by reduced *mpx* expression and Sudan black staining in Rnf111 mutants and *rnf111* morphants at 2 dpf, 3 dpf, and 4 dpf. The homozygous mutants died within 8 to 10 days, which was consistent with the recessive lethal results in *Rnf111* gene-trap insertion mutation mice.[42] The regulatory role of Rnf111 in HSPC development depends on its UbL function rather than STUbL one. Our mechanism studies unraveled that the decrease of p-Smad2/3 due to stabilization of Smad7 protein caused by Rnf111 deletion was involved in the developmental defect of HSPC and the decrease of downstream Gcsfr/NO signaling results in an attenuated proliferative ability of HSPC (Figure 8). The behavior of HSPC such as self-renewal and quiescence is determined by a huge variety of factors, including external signaling cues present in the microenvironment

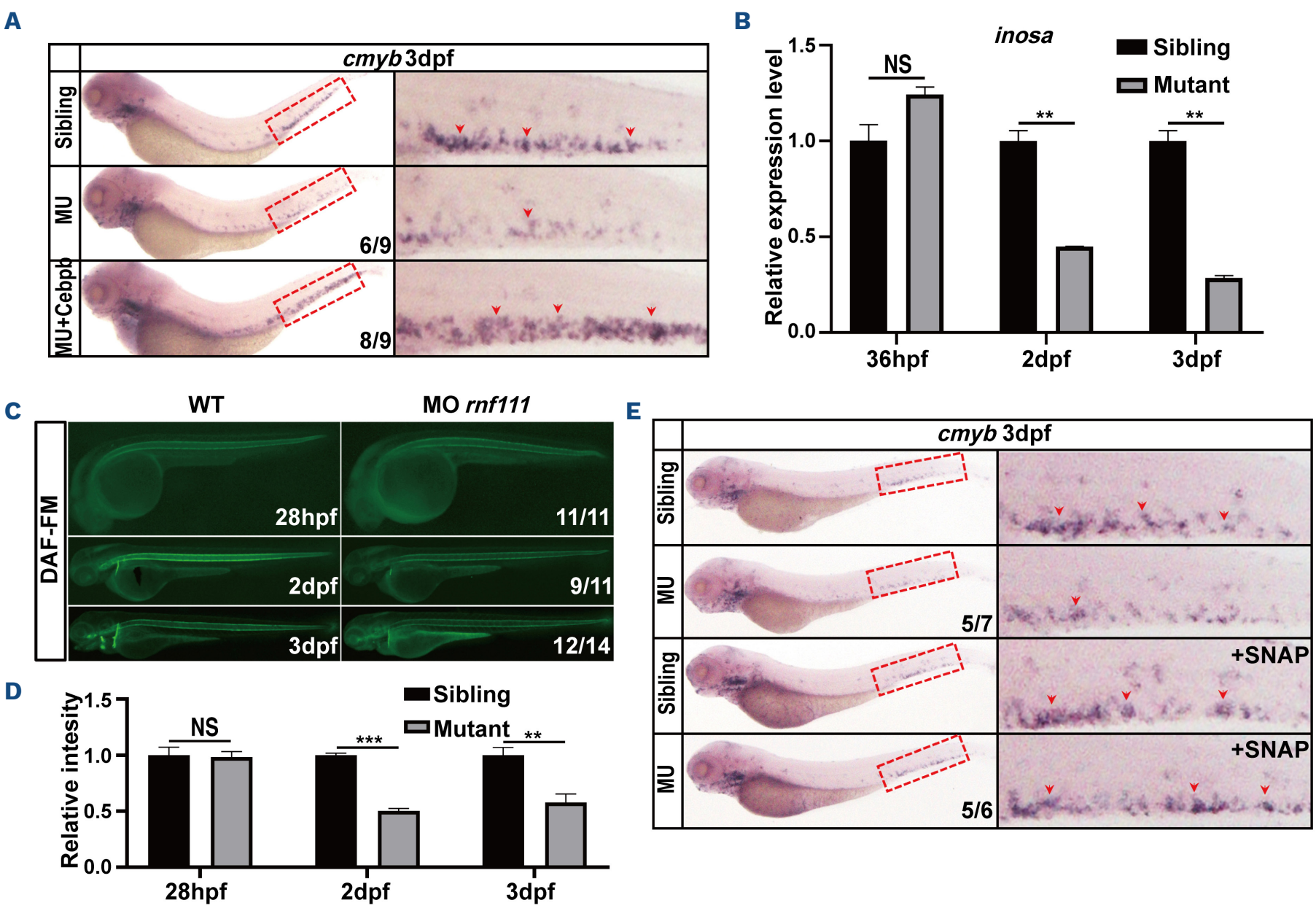

Figure 7. Cebpb-Nos2a acts downstream of Gcsf signaling. (A) Rescue assay of *cebpb* RNA. (B) Real-time quantitative polymerase chain reaction results for *inosa* in Rnf111 mutants compared with wild-type embryos at 36 hpf, 2 dpf and 3 dpf. The data are presented as mean ± standard deviation (SD) with $^{**}P<0.01$, NS: no significant difference. (C) DAF-FM assay showed the decreased production of nitric oxide (NO) morphants at 2 dpf and 3 dpf. (D) Statistics of NO fluorescence intensity. The data are presented as mean ± SD with $^{**}P<0.01$, $^{***}P<0.001$, NS: no significant difference. (E) A rescue effect of the NO agonist S nitroso N-acetyl-penicillamine (SNAP) on *cmyb* expression was observed in Rnf111 mutants. Red arrows indicate *cmyb*-positive hematopoietic stem and progenitor cells in caudal hematopoietic tissue. All experiments were independently replicated at least three times. dpf: days post-fertilization; MU: Rnf111 mutants with deletion of 4 base pair nucleotides; hpf: hours post-fertilization; WT: wild-type; MO *rnf111*: *rnf111* morphants; DAF-FM: 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate.

of bone marrow. In the hematopoietic system, TGF-β signaling controls a wide range of biological processes, from immune system homeostasis to hematopoietic stem cell dormancy and self-renewal.[46] The effect of TGF-β on HSC is bidirectional, with high levels of TGF-β blocking proliferation and low levels of TGF-β promoting proliferation.[36,47] It is also reported that TGF-β at the dose of 0.01 ng/mL can expand normal HSC, and the phosphorylated Smad2/3 may contribute to this proliferative response of HSC under low concentrations of TGF-β.[48] Therefore, during the development of normal HSC, an appropriate concentration of TGF-β is required to maintain the quiescence, self-renewal and differentiation functions. A suitable level of phosphorylation of Smad2/3 in HSC is crucial for the maintenance of their normal function in response to stimulation by low levels of TGF-β. Consistently, our results demonstrated that basal levels of Smad2/3 phosphorylation are present in the HSPC of wild-type embryos (Figure 5 A, A', A'', B, B', B''). However, p-Smad2/3 was downregulated in HSPC of *rnf111* morphants, which may be the main cause leading to the HSPC defect. The Brdu incorporation assay showed the proliferative ability of HSPC in *rnf111* morphants was decreased compared with that in WT embryos (Figure 4), indicating that the proliferative response of HSPC to normal concentrations of TGF-β was impaired in the absence of Rnf111, most likely due to the reduction of downstream effector p-Smad2/3. The rescue assay of IDE2 confirmed this notion. These data suggest that Rnf111 is present to maintain HSPC in response to TGF-β signals at normal concentrations.

It has been reported that TGF-β can regulate the amount of GM-CSFR, IL-3R, and GCSFR in mouse hematopoietic progenitor cell lines without significant changes in receptor affinity.[37] It is also known that Gcsf signaling promotes the expansion of HSPC in zebrafish embryos.[38] Indeed, we did find that the expression of *gcsfr* was downregulated in Rnf111 mutants and morphants at 3 dpf, but unchanged in Rnf111 mutants at 36 hpf and i the aorta-gonad-mesonephros region of *rnf111* morphants at 31 hpf (*Online Supplementary Figure S6B, C*). This may be responsible for the unaffected generation and impaired proliferation of HSPC in Rnf111-deficient embryos. Meanwhile, the HSPC defect could be rescued by *gcsfb* and *gcsfr* RNA. A luciferase reporter assay and ChIP-qPCR further confirmed the direct binding of p-Smad2 on the gcsfr promotor. The rescue effect of IDE2 was blocked by the gcsfr morpholino, further confirming that Gcsfr was the target of Smad2/3.

Further experiments confirmed that the Cebpb/NO pathway was involved in the downstream of Gcsf signaling, evidenced by the rescue effect of *cebpb* RNA and NO donor, and the blocking of the rescue effect of *gcsfr* RNA

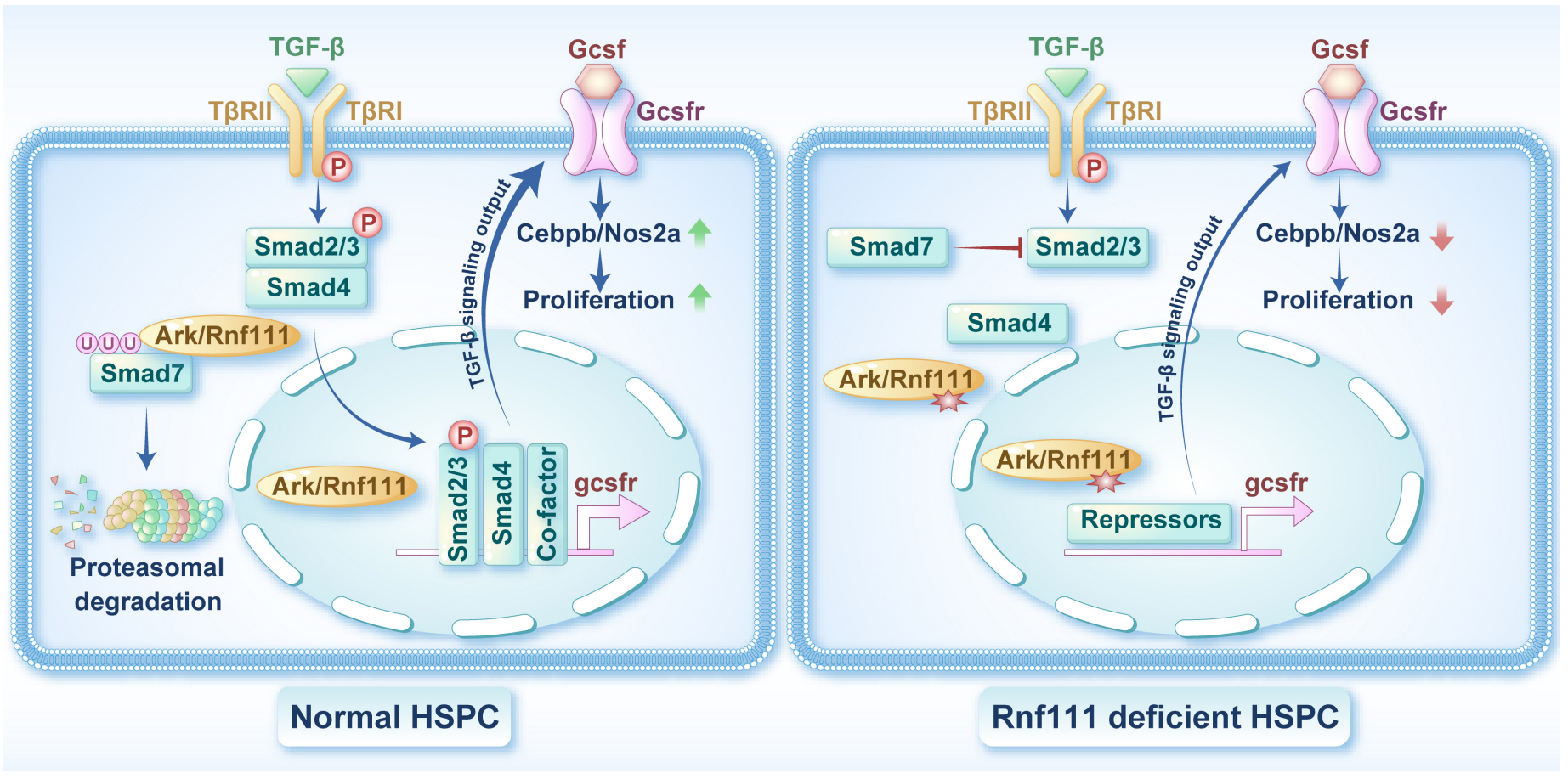

Figure 8. Schema of Rnf111 regulating the development of hematopoietic stem and progenitor cells by maintaining Smad2/3 phosphorylation and activating the Gcsfr/NO signaling pathway. *Left*. At a physiological concentration of transforming growth factor-beta (TGF-β), ARK/Rnf111 maintains Smad2/3 phosphorylation by promoting the degradation of Smad7 and further facilitates activation of the Gcsfr/NO signaling pathway to ensure the proliferative response of HSPC to TGF-β. *Right*. Deletion of ARK/Rnf111 in hematopoietic stem and progenitor cells (HSPC) leads to a weakening of TGF-β signaling output, which is manifested by a decrease in the Gcsfr/NO signal, resulting in an attenuated proliferative response of HSPC to TGF-β.

by a NOS2-specific inhibitor, 1400W. It is worth noting that NO signaling at an earlier developmental stage was not decreased, as shown by the relatively normal DAF-FM signal intensity at 28 hpf in *rnf111* morphants and *nos2a* expression level at 36 hpf in Rnf111 mutants. However, both DAF-FM signal intensity and *nos2a* expression level were significantly reduced at 2 dpf and 3 dpf (Figure 7B-D, *Online Supplementary Figure S7A*), which was consistent with the pattern of HSPC reduction in Rnf111-deficient embryos. The proliferation defect of HSPC caused by decreased NO in Rnf111-deficient embryos was consistent with the reported function of NO on HSPC proliferation at a physiological concentration,[49] which further confirmed the pivotal role of NO in Rnf111 regulation of HSPC development. Taken together, we revealed a fine-tuned regulation of HSPC development by UbL.

Disclosures

No conflicts of interest to disclose.

Contributions

XL and JS performed the experiments and analyzed the data. LW, ZW, ZF, XH, ST and YC assisted with the experiments. HY, HdT and JZ provided suggestions on the experimental design and data presentation. XL and JZ designed the research plan and wrote the paper. All authors read and approved the final manuscript.

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Data-sharing statement

The data involved in this study are available, upon reasonable request, from the corresponding author.

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