Interferon regulatory factor 2 binding protein 2b regulates neutrophil *versus* macrophage fate during zebrafish definitive myelopoiesis

Luxiang Wang,[1*] Shuo Gao,[1*] Haihong Wang,[1*] Chang Xue,[1*] Xiaohui Liu,[1] Hao Yuan,[1] Zixuan Wang,[1] Saijuan Chen,[1] Zhu Chen,[1] Hugues de Thé,[1,2] Yiyue Zhang,[3] Wenqing Zhang,[3] Jun Zhu[1,2] and Jun Zhou[1]

[1]CNRS-LIA Hematology and Cancer, Sino-French Research Center for Life Sciences and Genomics, State Key Laboratory of Medical Genomics, Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Université de Paris 7/INSERM/CNRS UMR 944/7212, Equipe Labellisée No. 11 Ligue Nationale Contre le Cancer, Hôpital St. Louis, Paris, France and [3]Division of Cell, Developmental and Integrative Biology, School of Medicine, South China University of Technology, Guangzhou, China

**LW, SG, HW and CX contributed equally to this work.*

 doi:10.3324/haematol.2019.217596

Received: January 25, 2019.

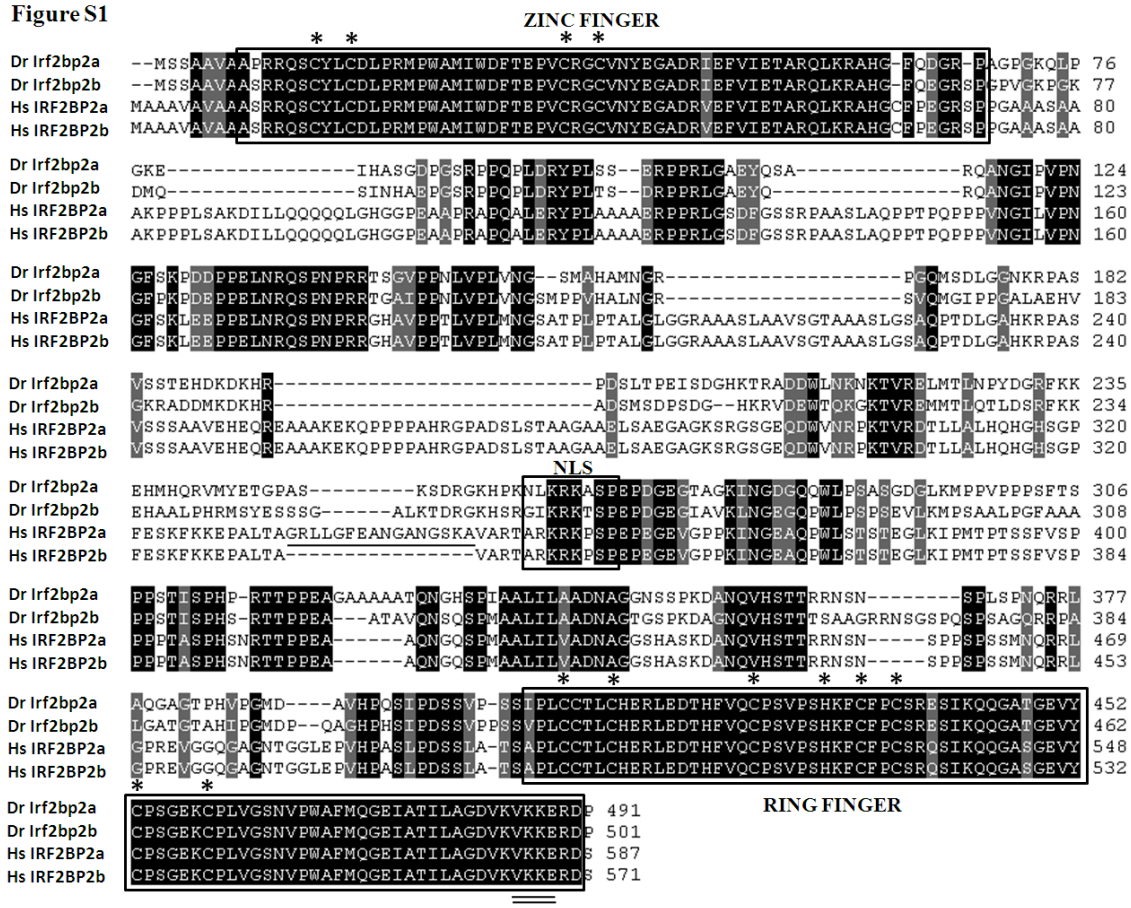
Accepted: May 20, 2019.

Pre-published: May 23, 2019.

Correspondence: *JUN ZHOU* - zj10802@rjh.com.cn

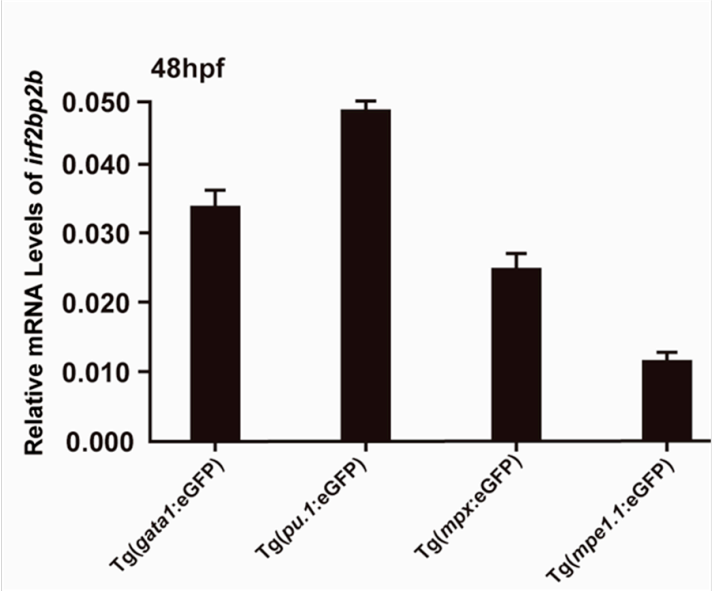
JUN ZHU - zhuj1966@yahoo.com or jun.zhu@paris7.jussieu.fr

WENQING ZHANG - mczhangwq@scut.edu.cn



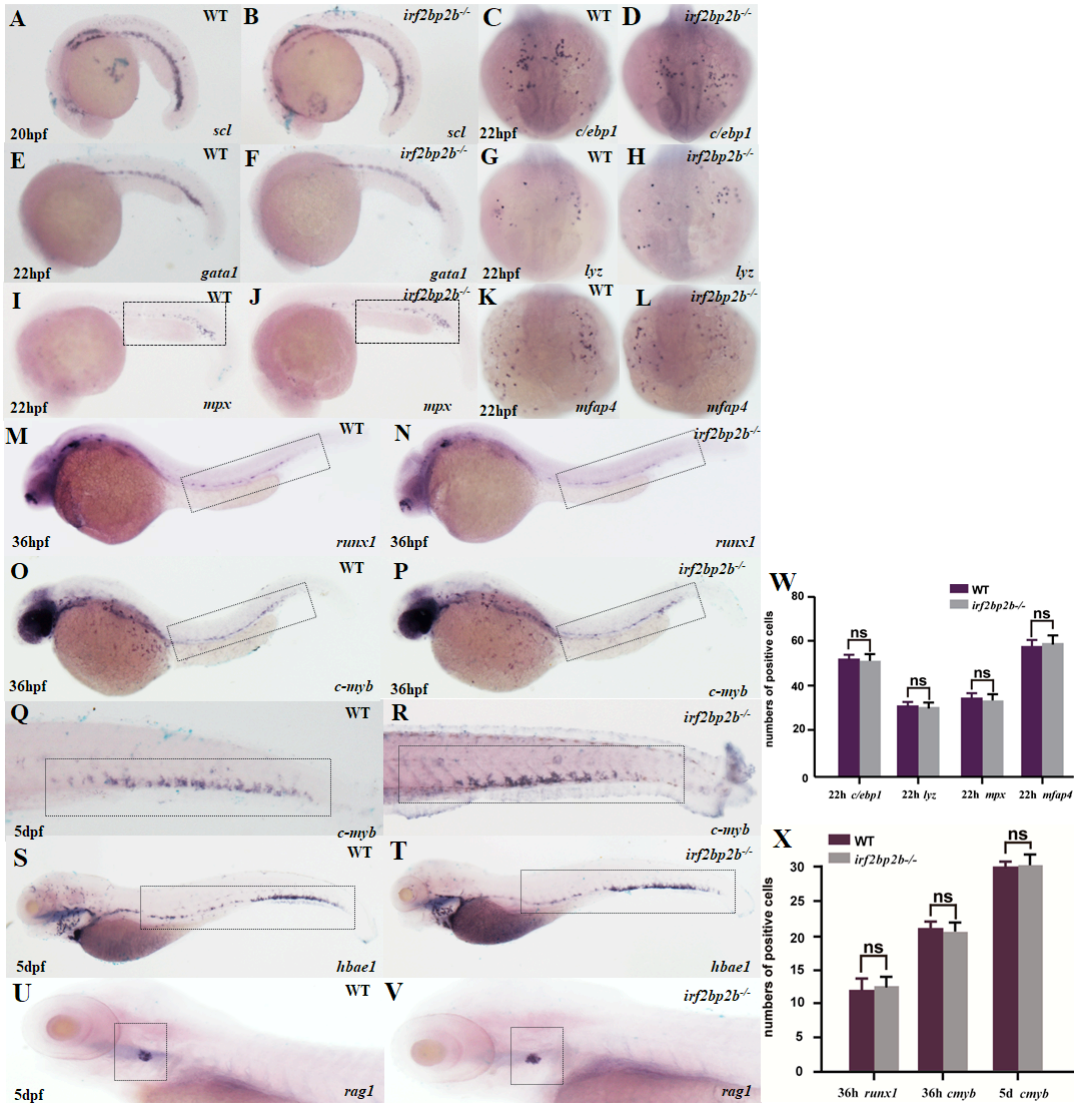
Supplemental Figure 1. Alignment of protein sequences of IRF2BP2 family members. Alignment of zebrafish (Dr) Irf2bp2a and Irf2bp2b, human (Hs) IRF2BP2a and IRF2BP2b protein sequences. Functional domains including the C4-type zinc finger and the C3HC4-type ring finger are boxed, and the critical cysteine (C) and histidine (H) residues are denoted by asterisks. The conserved nuclear localization signal (NLS) in the intermediate domain is also boxed. The extra 16 amino acids of the human IRF2BP2a isoform compared to IRF2BP2b are underlined. The consensus SUMOylation site VKKE located at the C-terminus is double underlined.

Figure S2



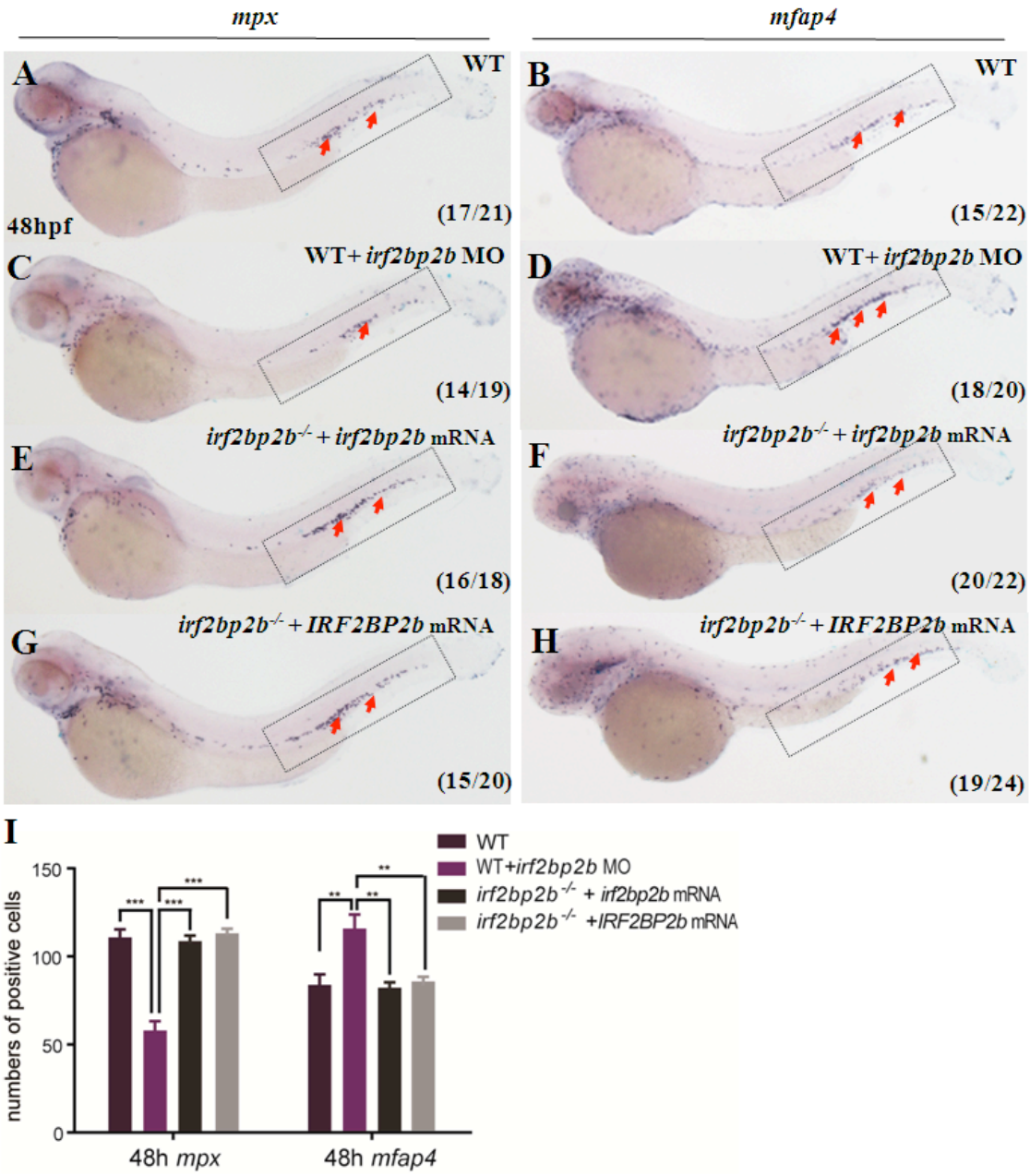
Supplemental Figure 2. Expression of *irf2bp2b* transcript in erythroid and myeloid cells. *Irf2bp2b* transcript was detected by RT-qPCR analysis in the GFP positive cells enriched from Tg(*gata1*:eGFP), Tg(*pu.1*:eGFP), Tg(*mpx*:eGFP), and Tg(*mpeg1.1*:eGFP) embryos at 48hpf, respectively. *β-actin* served as internal control.

Figure S3



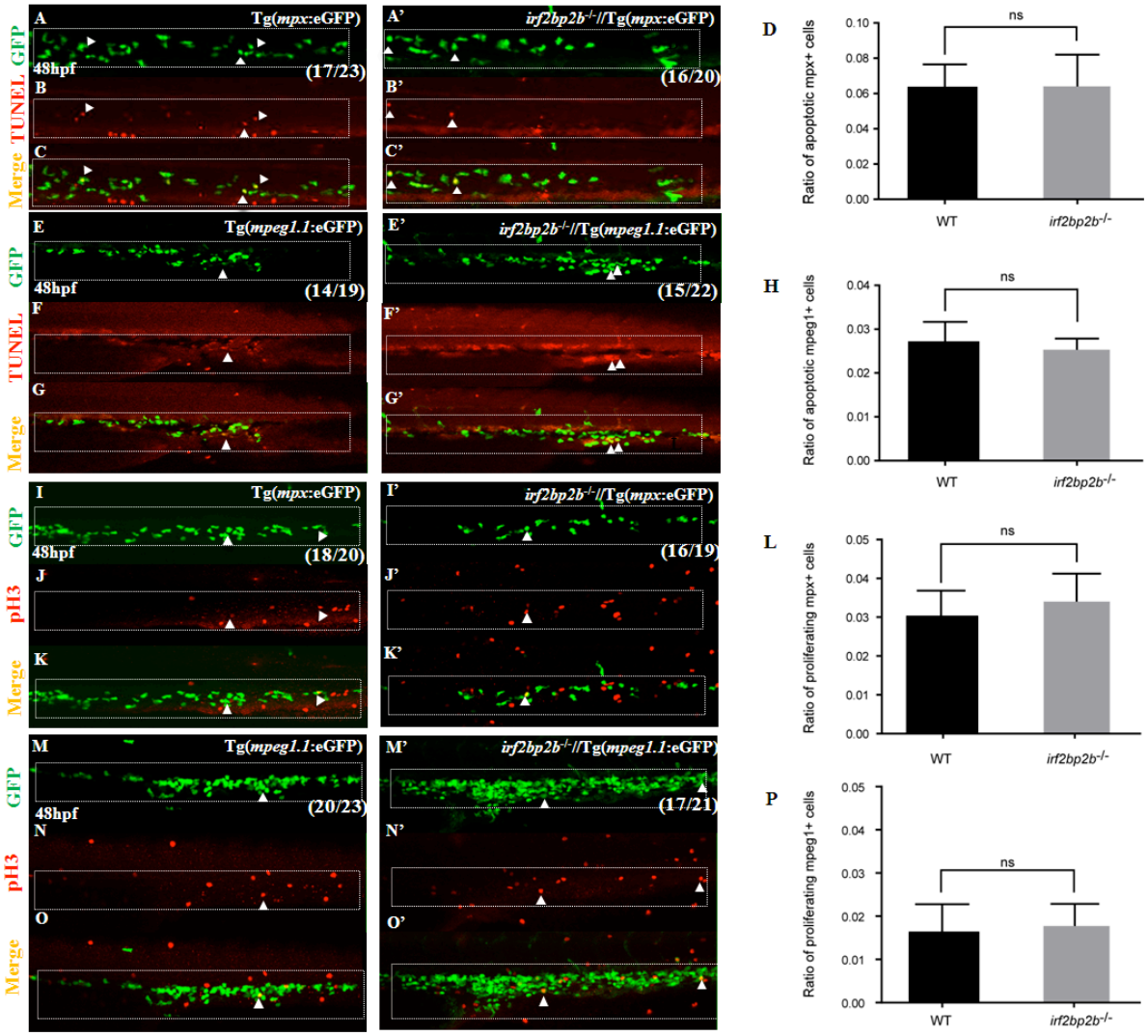
Supplemental Figure 3. Expression of lineage specific markers during primitive and definitive hematopoiesis stages in *irf2bp2b*-deficient embryos. (A-L) WISH analyses of *scl* (A, B) at 20hpf, and *c/ebp1* (C, D), *gata1* (E, F), *lyz* (G, H) *mpx* (I, J), *mfap4* (K, L) at 22hpf. (M, N) WISH analyses of *runx1* at 36hpf, (O-R) WISH analyses of *c-myb* at 36hpf and 5dpf, WISH analyses of *hbae1* (S, T), and *rag1* (U, V) at 5dpf, respectively. (W, X) Statistical results for A-L, M-R. Error bars represent ± SD of at least 15-30 embryos. p values are denoted by asterisks; (ns): no statistical significance.

Figure S4



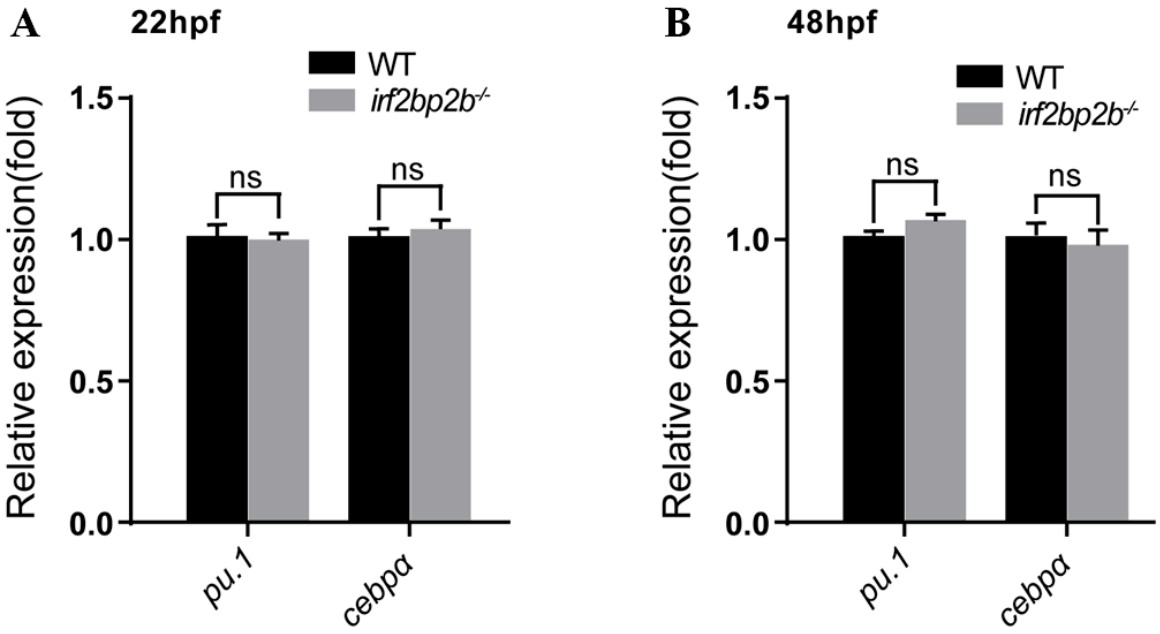
Supplemental Figure 4. Deficiency of *irf2bp2b* leads to an expanded macrophage population at the expense of the neutrophil population during definitive myelopoiesis. (A-D) WISH analyses of *mpx* and *mfap4* in wild type embryos injected with *irf2bp2b* gene specific MO. (E-H) Zebrafish *irf2bp2b* and human *IRF2BP2b* mRNA rescue aberrant myelopoiesis phenotype in *irf2bp2b*$^{-/-}$ mutant embryos. (I) Statistical results for A-H. Error bars represent ± s.e.m of at least 15-30 embryos. p values are denoted by asterisks; (**) P<0.01; (***) P<0.001 (ANOVA followed by LSD post hoc test).

Figure S5



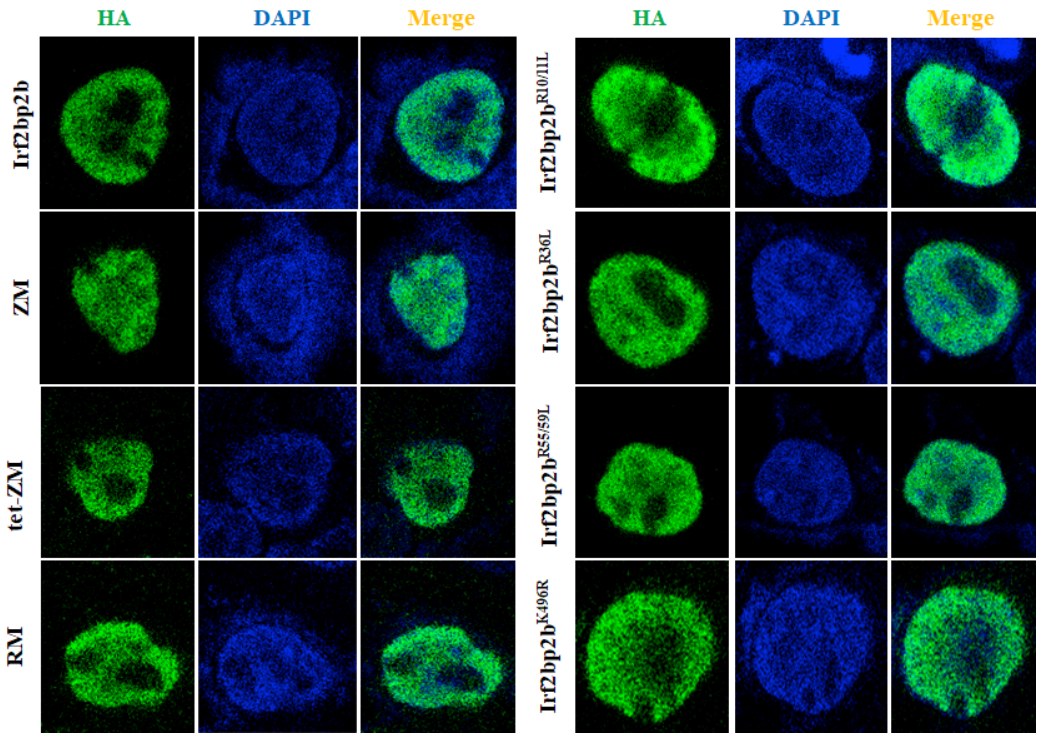
Supplemental Figure 5: **Apoptosis and proliferation of neutrophil and macrophage lineages are normal.** (A-C') Double immunostaining of GFP and TUNEL in CHT at 48hpf of Tg(*mpx*: eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) mutant embryos. (D) Statistic result for C-C'. Error bars represent ± SD of 15-30 embryos. (ns): no statistical significance. (E-G') Double immunostaining of GFP and TUNEL in CHT at 48hpf of Tg(*mpeg1.1*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) mutant embryos. (H) Statistic result for G-G'. Error bars represent ± SD of 15-30 embryos. (I-K') Double immunostaining of GFP and pH3 in CHT at 48hpf of Tg(*mpx*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) mutant embryos. (L) Statistic result for K-K'. Error bars represent ± SD of 15-30 embryos. (M-O') Double immunostaining of GFP and pH3 in CHT at 48hpf of Tg(*mpeg1.1*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) mutant embryos. (P) Error bars represent ± SD of 15-30 embryos.

Figure S6



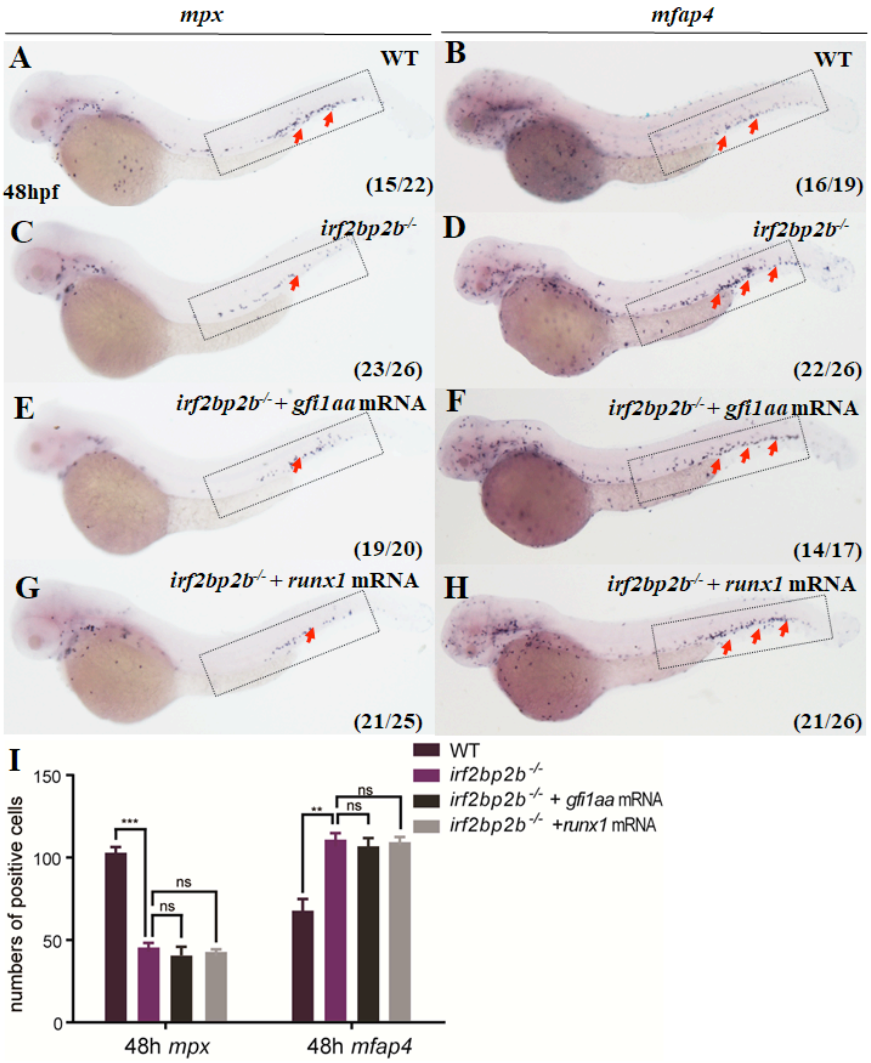
Supplemental Figure 6. **The change of *c/ebpα* and *pu.1* in NMPs might be masked.** RT-qPCR analysis of *c/ebpα* and *pu.1* in wild type and *irf2bp2b*-defecient whole embryos at 22hpf (A) and 48hpf (B). *β-actin* was used as internal control.

Figure S7



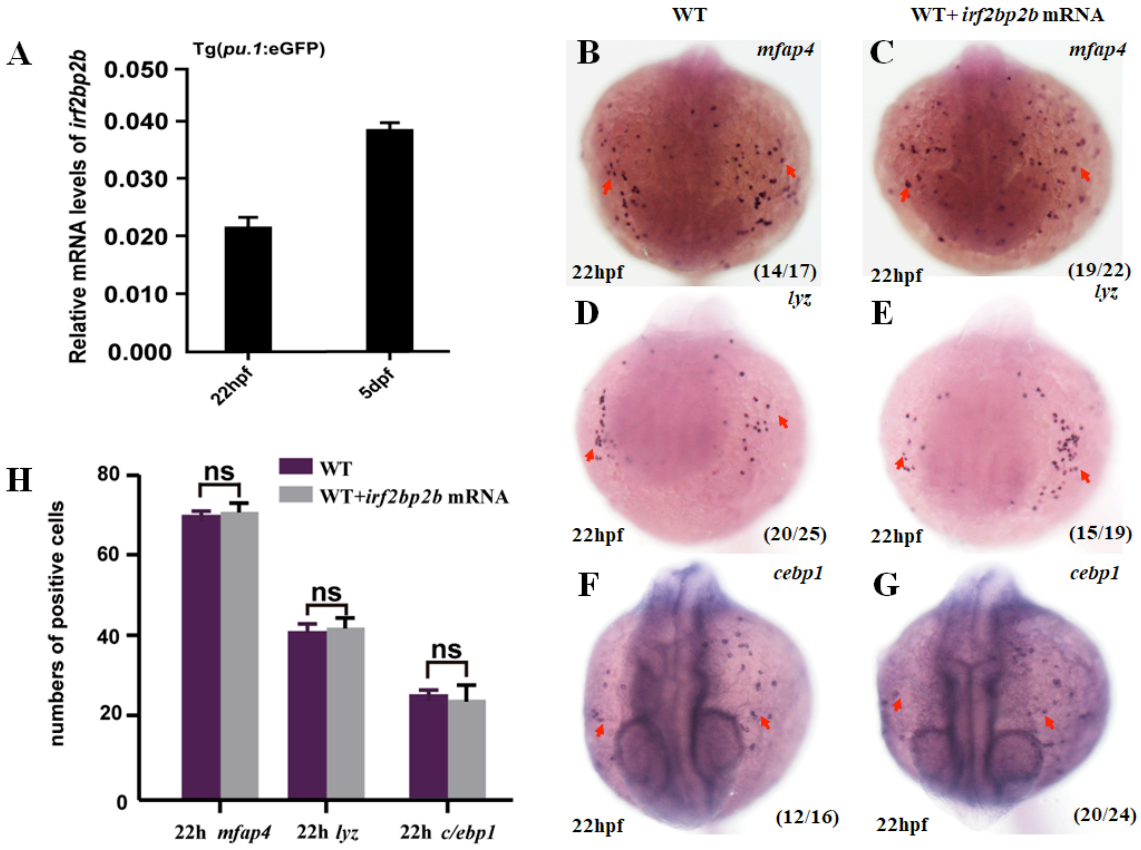
Supplemental Figure 7: Immunofluorescence analysis of HA-tagged wild type and mutant Irf2bp2b proteins. RM, ZM, tet-ZM, Irf2bp2b$^{R10/11L}$, Irf2bp2b^{R36L}, Irf2bp2b$^{R55/59L}$, and Irf2bp2b^{K496R} maintain the nuclear localization.

Figure S8



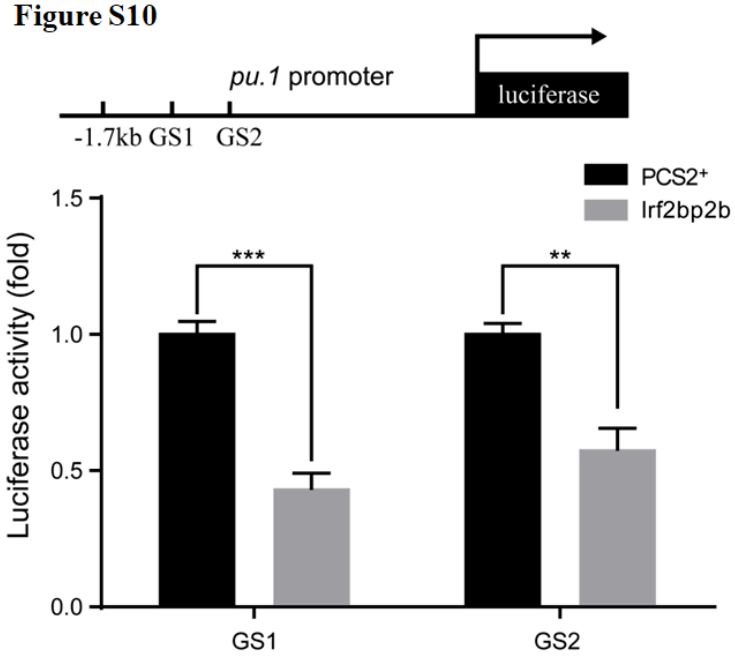
Supplemental Figure 8: *Runx1* and *gfi1aa* overexpressions in *irf2bp2b*-defecient embryos. (A-H) *Mpx* and *mfap4* were probed to monitor neutrophil and macrophage development. (I) Statistical results for A-H. Error bars represent ± s.e.m of at least 15-30 embryos. p values are denoted by asterisks; (**) P<0.01; (***) P<0.001 (ANOVA followed by LSD post hoc test).

Figure S9



Supplemental Figure 9: *Irf2bp2b* does not play a role in RBI-originated macrophage versus neutrophil fate choice. (A) The GFP positive cells were isolated from Tg(*pu.1*:eGFP) embryos at 22hpf (primitive hematopoiesis stage) and 5dpf (definitive hematopoiesis stage), respectively. RT-qPCR analysis (*β-actin* was used as internal control) showed that *irf2bp2b* transcript was detected at both time points, suggesting that *irf2bp2b* is expressed in both primitive and definitive progenitor cells. (B-G) Overexpression of *irf2bp2b* mRNA could not impair RBI-originated myelopoiesis. (H) Statistical results for B-G. Error bars represent ± SD of at least 15-30 embryos. p values are denoted by asterisks; Error bars represent ± SD of 3 replicates. (ns): no statistical significance; (**) P<0.01; (***) P<0.001 (Student's t test).

Figure S10



Supplemental Figure 10: Luciferase results of Irf2bp2b repression on wild type zebrafish *pu.1* promoter A region, and GS1, GS2 mutant constructs. Error bars represent ± SD of at least 3 replications. p values are denoted by asterisks; (**)P<0.01; (****)P<0.001 (Student's t test).

Supplementary Methods

Generation of *irf2bp2b* knockout line

For crisp9 mediated *irf2bp2b* knockout zebrafish generation, guide RNA (gRNA) targeting exon1 of *irf2bp2b* was designed using an online tool ZiFiT Targeter software (http://zifit.partners.org/ZiFiT), which was synthesized by cloning the annealed oligonucleotides into the sgRNA expression vector as previously described(1). The target site was 5'- GGAATGGCTCCGGTCCGACG-3'. The injected F0 founder embryos were raised to adulthood and then outcrossed with wild type zebrafish. F1 embryos carrying potential indel mutations were raised to adulthood. Then PCR amplification and sequencing were carried on genomic DNA isolated from tail clips of F1 fish to identify mutants.

Plasmid construction

Zebrafish *irf2bp2b* was cloned into $PCS2^{+}$ vector. The zebrafish *irf2bp2b* serial mutants were generated with the indicated primers (Table1). As for the luciferase reporter, the -8.5kb upstream frame of zebrafish *pu.1* was devided into four fragments and then inserted into PGL3 promoter vector (Promega) respectively. The zebrafish *irf2bp2b* gene -2.2kb upstream sequence was cloned into PGL3 basic vector (Promega). Primers used were listed in Table1.

In vitro synthesis of antisense RNA probe

Antisense RNA probes were prepared by in vitro transcription according to the standard protocol. The following digoxigenin-labeled antisense probes were used: *c/ebp1*, *mpx*, *lyz*, *mfap4*, *csf1r*, *mpeg1.1*, *l-plastin.*

Whole-mount in situ hybridization (WISH)

Digoxigenin (DIG)-labeled RNA probes were transcribed with T7, T3 or SP6 polymerase (Ambion, Life Technologies, Carlsbad, CA, USA). Whole-mount mRNA in situ hybridization was performed as described previously(2). The probes labeled by DIG (Roche, Basel, Switzerland) were detected using alkaline phosphatase-coupled anti-digoxigenin Fab fragment antibody (Roche, Basel, Switzerland) with BCIP/NBT staining (Vector Laboratories, Burlingame, CA, USA).

MO and mRNA injection

The MO and mRNA were injected at the one-cell stage of the embryos. The MOs were designed and generated by Gene Tools, Philomath, OR, USA. MO sequences were shown in Table 2. Full-length capped mRNA samples were all synthesized from linearized plasmids by using the mMessagemMachine SP6 kit (Life Technologies-Ambion, Austin, TX, USA).

Sudan Black staining

The embryos treated with 4% paraformaldehyde (PFA) overnight at 4℃ were incubated with a Sudan Black (Sigma-Aldrich) solution for about 30 minutes to detect the granules of granulocytes. The detailed method was described previously(3). Staining was then observed under a microscope.

Cell collection and FACS analysis

Cell collection and FACS analysis were performed essentially as described(4). Wild type Tg(*mpx*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) embryos, as well as wild type Tg(*mpeg1.1*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) embryos were dissociated into single cells using 0.05% trypsin (Sigma) as previously described(5) at 48hfp. These dissociated cells were passed through a 40-μm mesh, centrifuged at 450g, and suspended in 5% FBS/PBS before addition of propidium iodide to a final concentration of 1 μg/ml for exclusion of dead cells. FACS analysis was based on forward and side scatter characteristics, propidium iodide exclusion and GFP fluorescence using a FACS Vantage flow cytometer (Beckton Dickenson). Wild type embryos (without GFP) were used as blank to determine the background values in GPF-controls.

H&E staining of paraffin sections

After being collected and fixed with 4% paraformaldehyde (PFA) overnight at 4 °C, the embryos were dehydrated by progressively higher concentrations of ethanol, substituted with

xylene, embedded in paraffin wax, cut into 3-5μm slices, for hematoxylin and eosin (H&E) staining. Then the samples were imaged under a light microscope (Nikon).

PH3 staining and TUNEL assay

Tg(*mpx*:eGFP) , Tg(*mpeg1.1*:eGFP), irf2bp2b$^{-/-}$//Tg(*mpx*:eGFP), and irf2bp2b$^{-/-}$//Tg(*mpx*:eGFP) embryos were collected at 48hpf and fixed in 4% paraformaldehyde. The fixed embryos were incubated with primary rabbit anti-phospho-histoneH3 (pH3; Upstate Biotechnology) and goat anti-GFP (Abcam) antibodies according to the manufacturer's protocol and subsequently stained with AlexaFluor-647 anti–rabbit and AlexaFluor-488 anti–goat secondary antibodies (Invitrogen). TUNEL assays were performed using the In Situ Cell Death Detection Kit and TMR Red (Roche Diagnostics) according to the manufacturer's recommendations. Images were taken using Olympus FV 1000 confocal microscopy equipped with the FV10-ASW version 3 software.

Retroviral transduction

The IRF2BP2 cDNA was inserted into pMSCV-neo vector. For retroviral transduction, plat-E cells were transiently transfected with retroviral vectors. 32Dcl3 Cells were transduced by spinoculation (1,300 g, 30℃, 90 min) in a retroviral supernatant supplemented with cytokines and 4μg/ml polybrene (Sigma). Transduced cells were selected by G418 treatment (800mg/ml, Sigma).

Quantitative RT-PCR

The quantitativePCR was carried out with SYBR Green Real-time PCR Master Mix (TOYOBO) with ABI 7900HT real-time PCR machine, and analyzed with Prism software. The primers used were listed in Table.

ChIP-PCR

For ChIP analysis, GFP or GFP-Irf2bp2b expressing embryos were harvested at 48hpf for brief fixation. Cross-linked chromatin was immunoprecipitated with anti-GFP antibody

according to the procedure described(6). The resultant immunoprecipitated samples were subjected to semiquantitative PCR using primer pairs (Table1).

Cell culture and Luciferase reporter assay.

HEK293T cells were maintained in DMEM (Life technologies, Grand Island, NY, USA) with 10% Fetal Bovine Serum (Life technologies, Grand Island, NY, USA). Plasmid transfection was carried out with Effectene Transfection Reagent (QIAGEN) according to manufacturer's instruction. For the luciferase reporter assay, cells were harvested 48 hours after transfection and were analyzed using the Dual Luciferase Reporter Assay Kit (Promega, Maddison, WI, USA), according to the manufacturer's protocols.

Western blot and Co-Immunoprecipitation assay

HEK293T cells, which had been transfected with plasmids for 48h, were washed with phosphate-buffered saline (PBS) buffer for 1 minute 3 times. Lysates were prepared using RIPA lysis buffer (Beyotime, Shanghai, China) with proteinase inhibitor (Roche, Basel, Switzerland), after shaking on ice for 30 minutes, the cells were harvested and centrifuged at 15,000 × g for 30 min. Rabbit anti-HA antibody (Santa Cruz) was mixed with the protein-G-agarose beads (30 μl) in the supernatant at 4 °C overnight. The beads were prepared by centrifugation and washed three times with RIPA lysis buffer. Proteins binding to the beads were eluted by adding 30 μl of 2× SDS sample buffer and analyzed by immunoblotting using anti-GFP antibody (Santa Cruz).

References

1. Xiao A, Wang Z, Hu Y, Wu Y, Luo Z, Yang Z, et al. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. Nucleic acids research. 2013 Aug;41(14):e141.
2. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nature protocols. 2008;3(1):59-69.
3. Le Guyader D, Redd MJ, Colucci-Guyon E, Murayama E, Kissa K, Briolat V, et al. Origins and unconventional behavior of neutrophils in developing zebrafish. Blood. 2008 Jan 1;111(1):132-41.
4. Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI. Transplantation and in vivo

imaging of multilineage engraftment in zebrafish bloodless mutants. Nature immunology. 2003 Dec;4(12):1238-46.
5. Yan C, Huo X, Wang S, Feng Y, Gong Z. Stimulation of hepatocarcinogenesis by neutrophils upon induction of oncogenic kras expression in transgenic zebrafish. Journal of hepatology. 2015 Aug;63(2):420-8.
6. Hart DO, Raha T, Lawson ND, Green MR. Initiation of zebrafish haematopoiesis by the TATA-box-binding protein-related factor Trf3. Nature. 2007 Dec 13;450(7172):1082-5.