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

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ABSTRACT

A proper choice of neutrophil-macrophage progenitor cell fate is essential for the generation of adequate myeloid subpopulations during embryonic development and in adulthood. The network governing neutrophil-macrophage progenitor cell fate has several key determinants, such as myeloid master regulators CCAAT enhancer binding protein alpha (C/EBPα) and spleen focus forming virus proviral integration oncogene (PU.1). Nevertheless, more regulators remain to be identified and characterized. To ensure balanced commitment of neutrophil-macrophage progenitors toward each lineage, the interplay among these determinants is not only synergistic, but also antagonistic. Depletion of interferon regulatory factor 2 binding protein 2b (Irf2bp2b), a well-known negative transcription regulator, results in a bias in neutrophil-macrophage progenitor cell fate in favor of macrophages at the expense of neutrophils during the stage of definitive myelopoiesis in zebrafish embryos. Mechanistic studies indicate that Irf2bp2b acts as a downstream target of C/EBPα, repressing PU.1 expression, and that SUMOylation confers the repressive function of Irf2bp2b. Thus, Irf2bp2b is a novel determinant in the choice of fate of neutrophil-macrophage progenitor cells.

Introduction

Hematopoiesis is the process by which uncommitted hematopoietic stem cells proliferate and differentiate into all mature blood cell types.[1] The stepwise development of multipotent hematopoietic stem cells undergoes sequential lineage potential limitations toward oligopotent and unipotent progenitor cells, eventually restricting their output.[2] The molecular network governing every stage of hematopoiesis involves an interplay between multiple lineage-specific transcription factors/cofactors and epigenetic modifiers.[3] Any tiny disturbance of these factors could bias the lineage-restricted cell fate toward an alternate fate.[4]

Neutrophil-macrophage progenitors (NMP) generate neutrophil-macrophage lineage cells, mainly neutrophils, monocytes, and macrophages. The gene regulatory network governing NMP cell fate is composed of primary determinants, CCAAT enhancer binding protein alpha (C/EBPα) and spleen focus forming virus proviral integration oncogene (PU.1), along with secondary determinants Gfi and Egr/Nab.[5,6] Neutrophil cell fate specification requires C/EBPα, whereas macrophage cell fate specification depends on PU.1.[7,8] The relative levels of C/EBPα and PU.1 determine the choice of NMP cell fate. A low C/EBPα:PU.1 ratio shifts the balance toward macrophage differentiation, whereas a high ratio directs granulocyte differentiation.[6] To keep myeloid lineage fidelity, the interplay among

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the determinants is important not only in initiating the differentiation toward one lineage, but also in inhibiting that of the other lineage. Gfi1 and Egr/Nab, the downstream transcription factors of C/EBPα and PU.1, function as mutually antagonistic repressors to inhibit lineage-specific genes in mice.[5,9] It has also been reported that the suppression of *irf8*, a downstream gene of Pu.1, leads to a depletion of macrophages and an expansion of neutrophils during zebrafish primitive myelopoiesis.[10] *Irf8* knockout mice even develop a chronic myeloid leukemia-like disease.[11,12] Mechanistically, interferon regulatory factor 8 (IRF8) impedes the ability of C/EBPα to stimulate neutrophil differentiation by preventing its binding to chromatin.[12] In addition to the transcription factors involved in the C/EBPα and PU.1 network, Runx1 was shown to repress *pu.1* in a Pu.1-Runx1 negative feedback loop and determine macrophage *versus* neutrophil fate.[13]

Interferon regulatory factor 2 binding protein (IRF2BP)2 is a member of the IRF2BP family that was initially identified as an interferon regulatory factor 2 (IRF2)-dependent corepressor in inhibiting the expression of interferon-responsive genes.[14] The IRF2BP family is highly conserved during evolution, and is structurally characterized by an N-terminal zinc finger motif which mediates homo- or hetero-dimerization/multimerization between different IRF2BP2 family members, and a C-terminal ring finger motif that interacts with its partners.[15] IRF2BP2 is described as a corepressor in most published works.[14,16,17] The significance of IRF2BP2 in hematopoiesis was first revealed by genetic studies in I*rf2bp2*-deficient mice. IRF2BP2, with its binding partner ETO2, and the NCOR1/SMRT corepressor complex, participates in erythroid differentiation.[16] As a ubiquitously distributed nuclear protein, IRF2BP2 plays multiple roles in various types of hematopoietic cells. For example, IRF2BP2 exerts a repressive effect on target genes of nuclear factor of activated T cells (NFAT), which is another partner of IRF2BP2.[17] IRF2BP2 has also been shown to restrain naïve CD4 T-cell activation by inhibiting proliferation and CD25 expression.[18] Moreover, *Irf2bp2*-deficient macrophages were inflammatory in mice.[19] In recent years, four patients with acute promyelocytic leukemia carrying a novel fusion IRF2BP2-RARα have been reported. Nevertheless, the potential role of IRF2BP2 in leukemogenesis is still unclear.[20-23]

In this study, we provide *in vivo* evidence demonstrating that a deficiency of *irf2bp2b* triggers biased NMP cell fate choice, favoring macrophage development during zebrafish definitive myelopoiesis, which adds Irf2bp2b to the repertoire of factors regulating NMP cell fate decision. Mechanistic studies indicate that Irf2bp2b, which is under the control of C/ebpα, inhibits *pu.1* expression. We further reveal that SUMOylation is indispensable for the transcriptional repression of Irf2bp2b.

Methods

Maintenance and generation of mutant zebrafish

Zebrafish were raised, bred, and staged according to standard protocols.[24] For the generation of crisp9-mediated *irf2bp2b* knockout zebrafish, guide RNA targeting exon 1 of *irf2bp2b* was designed using an online tool, ZiFiT Targeter software.

Plasmid construction

The zebrafish *irf2bp2b* gene and its serial mutants were cloned into PCS2^{+} vector. The upstream sequences of zebrafish *pu.1* and *irf2bp2b* genes were cloned into PGL3 promoter vector (Promega).

Whole-mount in situ hybridization

Digoxigenin-labeled RNA probes were transcribed with T7, T3 or SP6 polymerase (Ambion, Life Technologies, USA). Whole-mount *in situ* hybridization (WISH) was performed as described previously.[25]

Semi-quantitative reverse transcriptase polymerase chain reaction

The RNA preparation, cDNA synthesis, and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) were performed as described in the *Online Supplementary Methods*.

Retroviral transduction

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

Statistical analysis

The statistical significance of a difference between two means was evaluated by the unpaired Student *t*-test. For multiple comparisons, one-way analysis of variance was performed, followed by a least significant difference post-hoc test for multiple comparisons. Differences were considered statistically significant at $P<0.05$.

Ethics

The animal protocol described above was reviewed and approved by the Animal Ethical and Welfare Committee, Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China).

Results

Deficiency of zebrafish *irf2bp2b* causes a reduction of the neutrophil population and a simultaneous expansion of the macrophage population during definitive myelopoiesis

The *IRF2BP* gene family includes three members, *IRF2BP1*, *IRF2BP2* and *IRF2BPL*, which are highly conserved throughout evolution.[15] All the family members bear a nearly identical N-terminal C4-type zinc finger motif and a C-terminal C3HC4-type ring finger motif, whereas the intermediate domain between the zinc finger and the ring finger motifs shows relatively low similarity at the protein level.[15] There are two paralog genes of *irf2bp2* named *irf2bp2a* and *irf2bp2b* in zebrafish, whereas a unique *IRF2BP2* gene exists in the human genome, which generates two isoforms also named *IRF2BP2a* and *IRF2BP2b* due to alternative splicing. Human IRF2BP2a has a 16 amino acid-long additional sequence in its intermediate domain compared with IRF2BP2b. This additional sequence in human IRF2BP2a is not conserved in zebrafish Irf2bp2a/2b (*Online Supplementary Figure S1*). Phylogenetic analysis showed that the two paralogs and human *IRF2BP2* arose from a common ancestor, suggesting that

functional divergence occurred early in vertebrate evolution.[26]

The zebrafish is an excellent model organism for the study of hematopoiesis.[27] Like mammalian hematopoiesis, zebrafish hematopoiesis also consists of primitive and definitive waves which emerge sequentially in distinct anatomical sites.

Human *IRF2BP2* mRNA is distributed in dozens of tissues, with the most prominent expression being found in bone marrow (*https://www.ncbi.nlm.nih.gov/gene/359948)*. Zebrafish *irf2bp2b* is also ubiquitously expressed in developing embryos. *irf2bp2b* transcript was detected in the green fluorescent protein (GFP)-positive cells enriched from Tg(*gata1*:eGFP), Tg(*pu.1:*eGFP), Tg(*mpx*:eGFP), and Tg(*mpeg1.1*:eGFP) embryos (*Online Supplementary Figure S2*). To evaluate the effects of *irf2bp2b* on hematopoietic differentiation and lineage commitment, a mutant line was generated using the CRISPR/Cas9 system targeting the first exon of the *irf2bp2b* gene and introducing a 26 nt deletion which results in a truncated protein by frameshifting (Figure 1A, B). Moreover, the mutant *irf2bp2b* gene was cloned into an HA-tagged expressing vector and transfected into HEK293T cells. As expected, a short protein was detected by western blot analysis. Meanwhile, immunofluorescence analysis showed that this Irf2bp2b mutant protein lost its nuclear localization due to loss of the nuclear localization signal[28] (Figure 1C, D).

A series of hematopoietic-related markers was detected by WISH analysis during the stage of primitive hematopoiesis in *irf2bp2b*-defecient embryos. The primitive macrophages and neutrophils derived from the rostral blood island, as well as the erythrocytes and neutrophils originating from the intermediate cell mass remained unchanged (*Online Supplementary Figure S3A-L, W*).

Definitive pluripotent hematopoietic stem cells arise from the ventral wall of the dorsal aorta, the zebrafish equivalent of the aorta/gonad/mesonephros of mammals, then migrate through the caudal hematopoietic tissue to the thymus and kidney marrow. WISH analyses revealed that the expression of the hematopoietic stem/progenitor

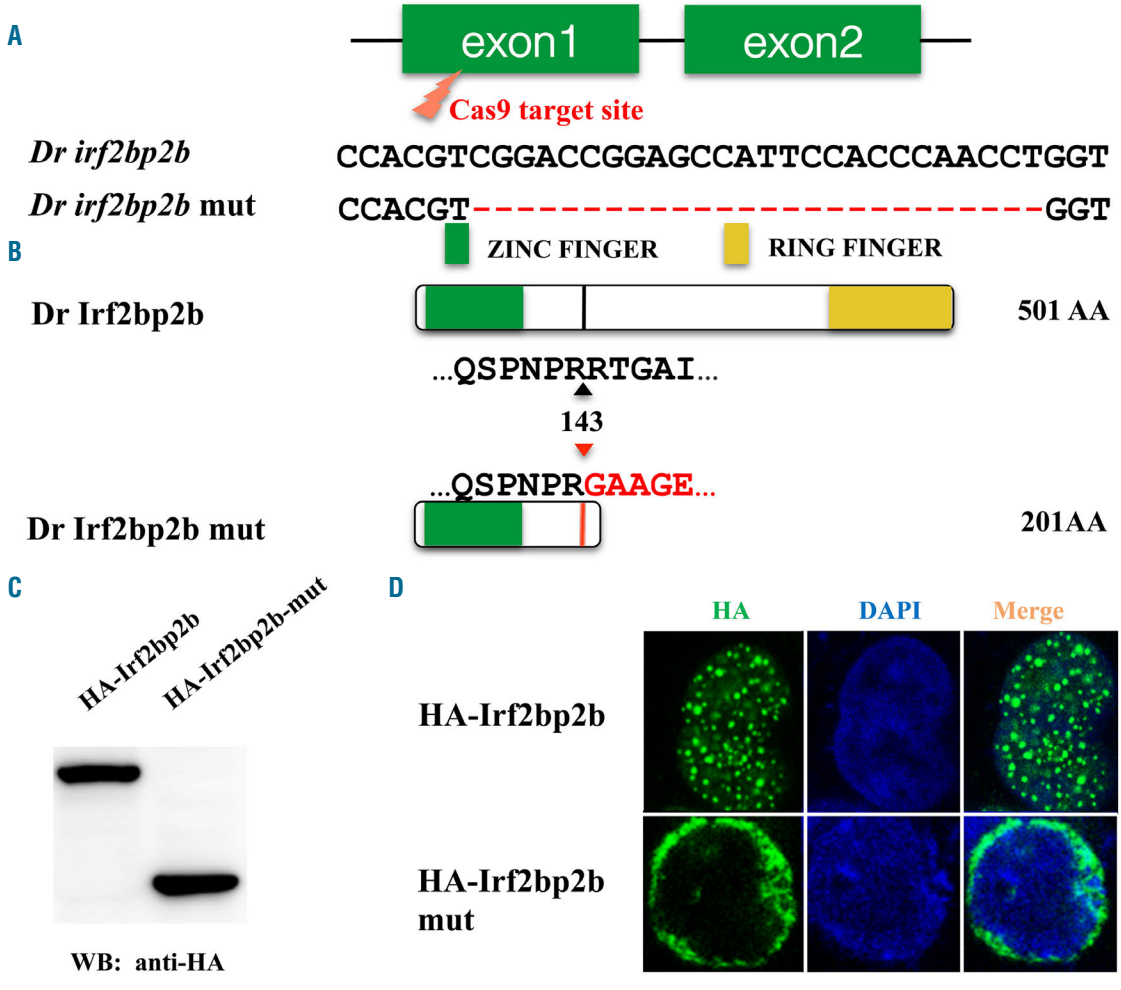

Figure 1. The establishment of a zebrafish *irf2bp2b* knockout line. (A) Schematic representation of the Cas9 target site in the first exon of zebrafish *irf2bp2b*. The deleted nucleotides in the mutant gene are marked by hyphens. (B) Schematic representation of wildtype (501 amino acids) and mutant Irf2bp2b proteins (201 amino acids). The site where the frameshift was introduced is marked by triangles. (C) Western blot analysis of HA-tagged wildtype and mutant Irf2bp2b proteins. (D) Immunofluorescence analysis of wildtype (top panel) and mutant Irf2bp2b (bottom panel) proteins, demonstrating that the truncated protein lost its nuclear localization. AA: amino acids; mut: mutated; WB: western blot; HA: human influenza hemagglutinin; DAPI: 4′,6-diamidino-2-phenylindole.

cell-related markers runx1 and c-myb was relatively unchanged in *irf2bp2b*-deficient embryos (*Online Supplementary Figure S3M-R, X*). The erythroid marker *hbαe1* (*Online Supplementary Figure S3S-T*), and the lymphoid marker *rag1* (*Online Supplementary Figure S3U-V*) were also unaffected.

As for myelopoiesis, a significant decrease in multiple neutrophil markers, including *c/ebp1* (a marker of neutrophil progenitors)[29] and *mpx/lyz* (a marker of mature neutrophils),[30] and a simultaneous increase of monocyte and macrophage markers such as *csf1r* (a monocyte/macrophage marker)[30] and *mfap4/mpeg1.1* (an early embryonic macrophage marker)[31,32] were observed from 36 hours post fertilization (hpf) to 5 days post fertilization (dpf) in *irf2bp2b*-deficient mutants compared to controls (Figure 2A-I). The decreased neutrophil population was further confirmed by Sudan black staining[33] at 3 dpf in the ventral wall of the dorsal aorta (Figure 2J-J', M), as well as in *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) embryos at 5 dpf in caudal hematopoietic tissue (Figure 2K-K', M). Similarly, an expanded macrophage population was found in *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) embryos at 5 dpf (Figure 2L-L', M). Flow cytometry analysis was performed to quantify the numbers of neutrophils and macrophages, and the results showed a 34.9% reduction of eGFP-positive cells in *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) embryos and a 21.4% increase in *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) embryos (Figure 2N-P). The *irf2bp2b*$^{-/-}$ zebrafish were not only viable but also fertile, which made the myelopoiesis study possible in adults. Morphological staining of the 3-month old adult zebrafish kidney marrow further confirmed the expanded macrophages and reduced neutrophils (Figure 3A-C). Meanwhile, FACS analyses were also done with whole kidney marrow from Tg(*mpx:*eGFP) and *irf2bp2b-/-*//Tg(*mpx*:eGFP) lines in 3-month old adults. The myeloid cell populations were analyzed, and many fewer neutrophils were found in *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) zebrafish than in controls (29.7% mpx^{+} *vs.* 84.0% mpx^{+}) (Figure 3D, E).

An opposite phenotype emerged when *irf2bp2b* mRNA was injected into one-cell stage wildtype embryos (Figure 3F-H). It is worth noting that the overall numbers of cells positive for the pan-myeloid marker *l-plastin*[30] (which is a marker of both neutrophils and macrophages), were comparable among *irf2bp2b*-deficient mutants, *irf2bp2b*-overexpressing embryos and wildtype embryos (Figure 3I-L). In addition, embryos injected with a specific *irf2bp2b* morpholino (MO) exactly phenocopied the aberrant myelopoiesis that occurs in *irf2bp2b* knockout embryos (*Online Supplementary Figure S4A-D, I*).

All of the abnormalities in *irf2bp2b*-deficient and morphant embryos could be effectively rescued with the wildtype zebrafish *irf2bp2b* mRNA, confirming the specificity of the phenotype (*Online Supplementary Figure S4E-F, I*). It should be noted that zebrafish *irf2bp2a* mRNA did not rescue the defects of myelopoiesis, indicating that the two paralogs might have distinct roles (*data not shown*). Accordingly, loss of *irf2bp2a* resulted in a quite different phenotype in zebrafish myelopoiesis, which could not be rescued by *irf2bp2b* mRNA, either (experiments ongoing). Moreover, human *IRF2BP2b* mRNA, but not *IRF2BP2a* mRNA, could rescue the biased myelopoiesis in zebrafish *irf2bp2b*-deficient mutants, suggesting that human *IRF2BP2b* is the functional ortholog of zebrafish *irf2bp2b* in this process (*Online Supplementary Figure S4G-H, I* and *data not shown*).

Irf2bp2b regulates neutrophil-macrophage progenitor fate by repressing *pu.1* expression

The imbalanced proportion of neutrophil and macrophage populations in *irf2bp2b*-defective mutants can result from either abnormalities in apoptosis or proliferation rate. To distinguish between these possibilities, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and antiphosphohistone H3 (pH3) antibody staining assays were performed to assess the apoptosis and proliferation status of neutrophils and macrophages, respectively. Neither TUNEL nor pH3 assays revealed discernable differences in the percentages of double-positive stained cells (TUNEL^{+}GFP^{+}, pH3^{+}GFP^{+}) in *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpeg1*:eGFP) embryos compared to the percentage in controls, indicating that there is no change in the status of either apoptosis or proliferation of each lineage in *irf2bp2b*-deficient embryos (*Online Supplementary Figure S5*). Moreover, the fact that *l-plastin*-positive cell numbers remained unchanged in both *irf2bp2b*-overexpressing and -deficient embryos suggest that *irf2bp2b* might participate in regulating neutrophil *versus* macrophage commitment.

The relative levels of the master regulators PU.1 and C/EBPα are critical in macrophage *versus* neutrophil cell fate specification.[6] To ensure balanced commitment of NMP, the endogenous levels of PU.1 and C/EBPα must be appropriately tuned to a proper range. Overexpression of PU.1 can bias myeloid output to macrophages, whereas overexpression of C/EBPα has an opposite effect. Thus either *pu.1* upregulation or *c/ebpα* downregulation within NMP could be the cause of biased myelopoiesis toward macrophages in *irf2bp2b* mutants. We tried to examine the expression levels of *pu.1* and *c/ebpα* by WISH analysis. No obvious difference was observed between the wildtype and *irf2bp2b*$^{-/-}$ embryos. However, considering that *Pu.1* is expressed in multiple hematopoietic cell lineages, such as hematopoietic stem cells, common lymphoid progenitors, and common myeloid progenitors,[34] and *C/ebpα* is also widely expressed in hematopoietic stem cells and myeloid cells,[35] changes in their levels of expression within NMP might be difficult to show. Due to the lack of a lineage cell detection cocktail for the zebrafish hematopoietic system, we were unable to isolate the NMP subpopulation by flow cytometry to compare the endogenous expression levels of *pu.1*and *c/ebpα*. RT-qPCR was performed to detect the expression of *c/ebpα* and *pu.1* in wildtype and *irf2bp2b*-deficient whole embryos, and no obvious changes were observed (*Online Supplementary Figure S6*), suggesting that changes occurring in NMP might be masked. To resolve this problem, we used a murine myeloid progenitor cell line 32Dcl3 retrovirally transduced with human *IRF2BP2b*. RT-qPCR analyses revealed that the transcript level of *Pu.1* was downregulated, whereas that of *C/ebpα* was unaffected (Figure 4A). Meanwhile, expression of multiple monocyte differentiation-related genes such as *Mcsfr*, *Mmp1*, *Tlr2*, and *Irf8* was reduced, whereas expression of neutrophil differentiation-related genes, including *Gcsfr*, *Ltf*, *Prtn3*, and *Elane*, was induced (Figure 4A). These observations imply that an alteration of *pu.1* expression, rather than that of *c/ebpα*, might account for the shift in the balance of neutrophil and macrophage populations in *irf2bp2b*-deficient zebrafish embryos. Since IRF2BP2 is a negative transcription regulator, we wondered whether *pu.1* is a direct target of Irf2bp2b, which could be upregulated in *irf2bp2b*-deficient NMP. To test

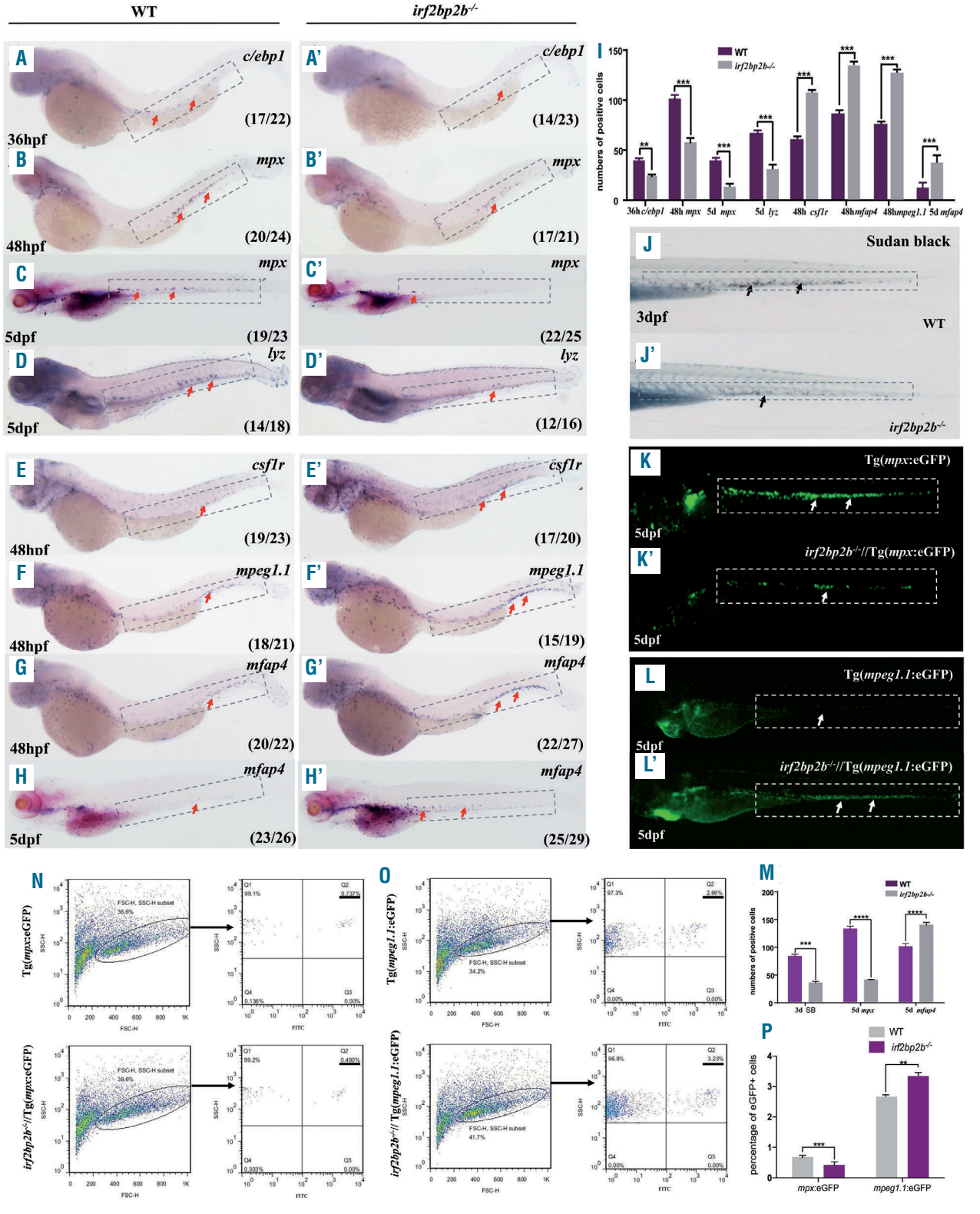

Figure 2. Deficiency of *irf2bp2b* leads to an expanded macrophage population at the expense of the neutrophil population during definitive myelopoiesis. (A-D') Whole-mount *in situ* hybridization (WISH) analyses of neutrophil markers *c/ebp1* (A, A'), *mpx* (B-C'), and *lyz* (D, D') at 36 hours post-fertilization (hpf), 48 hpf, and 5 days post-fertilization (dpf) in wildtype (WT) and *irf2bp2b*-deficient embryos. Gray boxes and red arrows indicate the main position of positive cells for each marker. n/n, number of embryos showing representative phenotype/total number of embryos examined. (E-H') WISH analyses of monocyte/macrophage markers *csf1r* (E, E'), *mpeg1.1* (F, F'), and *mfap4* (G-H') at 48 hpf and 5 dpf. (I) Statistical results for A-H'. Error bars represent the mean ± standard deviation (SD) of at least 15-30 embryos. ***P<0.001 (Student t test). (J, J') Sudan black-positive cells were reduced in *irf2bp2b*-deficient embryos at 3 dpf. (K, K') Green fluorescence protein (GFP)-positive cells were decreased in *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) embryos at 5 dpf. (L, L') GFP-positive cells increased in *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) embryos at 5 dpf. (M) Statistical results for J-L'. ***P<0.001; ****P<0.0001 (Student t test). (N, O) FACS analysis of eGFP-positive cells in wildtype and *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) or *irf2bp2b*$^{-/-}$//Tg(*mpeg1.1*:eGFP) embryos at 2 dpf. (P) Statistical results for N, O. Error bars represent the mean ± SD of three replicates. **P<0.01; ***P<0.001 (Student t test).

this hypothesis, we divided the 8.5 kb zebrafish *pu.1* promoter into four fragments, which were inserted separately into a luciferase reporter vector.[13] The luciferase expression in all of these four constructs was inhibited when co-transfected with *irf2bp2b* in HEK293T cells. The most prominent repression was found within the fragment nearest to the transcription start site (-1.7 kb) (Figure 4B). Next, a series of *in vivo* experiments was performed. The 8.5 kb *pu.1* promoter was cloned into a mCherry reporter vector (*pu.1*:mCherry, Tol2 backbone), which was co-injected with Tol2 transposase mRNA into wildtype zebrafish embryos with or without *irf2bp2b* mRNA. Overexpression of *irf2bp2b* led to significantly reduced expression of mCherry (Figure 4C-D). Moreover, *pu.1* MO was injected into *irf2bp2b*$^{-/-}$ embryos, and effective rescue of aberrant myelopoiesis was obtained (Figure 4E-J, O). These observations suggest that the level of *pu.1* expression might be elevated in NMP in *irf2bp2b* mutants. To further demonstrate that Irf2bp2b regulates zebrafish NMP cell fate choice through repression of *pu.1*, we took advantage of a zebrafish *pu.1*^{G242D} mutant line, in which the level of *pu.1* transcripts is normal but its protein stability is dra-

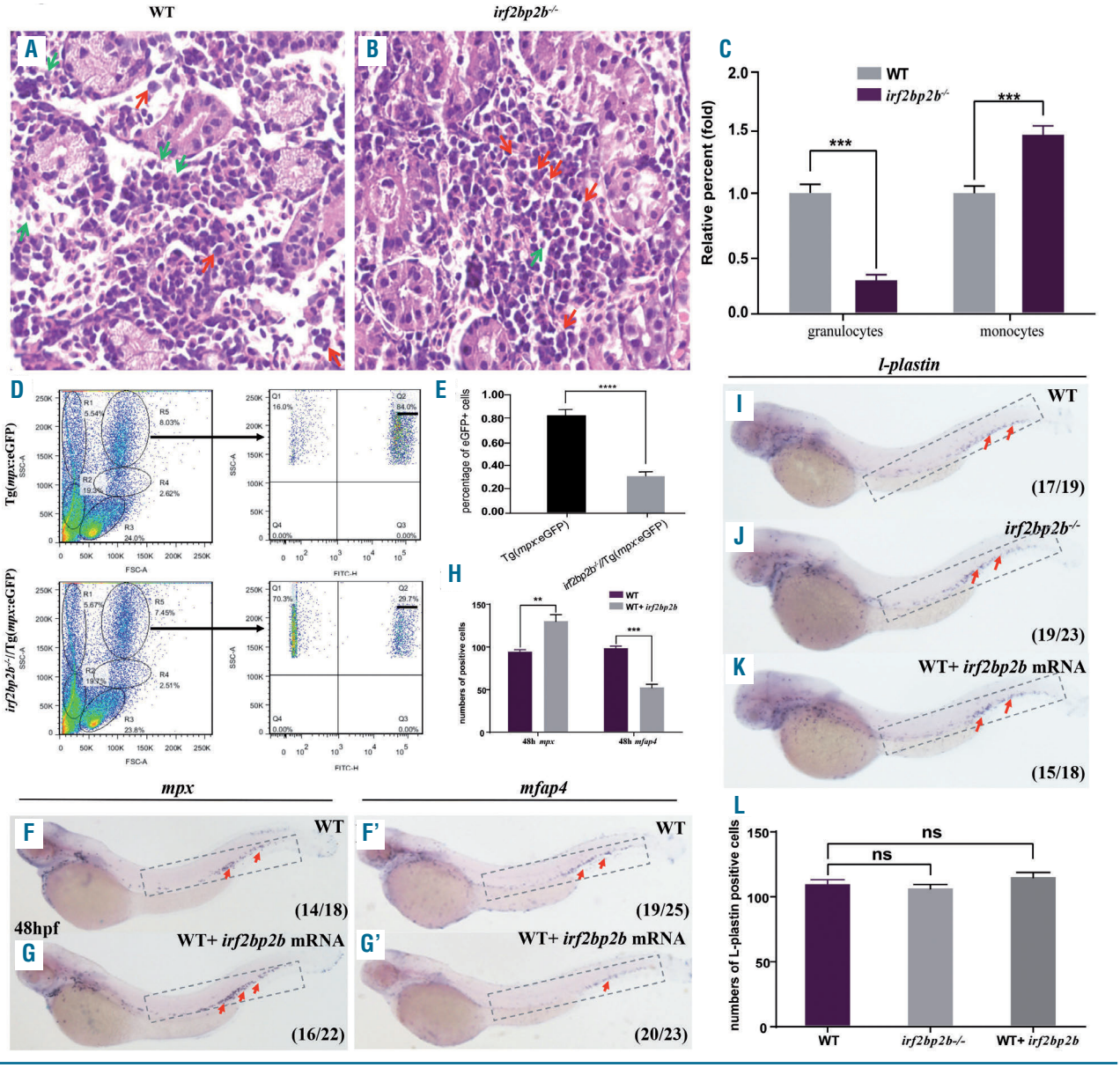

Figure 3. Biased myelopoiesis in *irf2bp2b*-deficient adult zebrafish. (A, B) Hematoxylin & eosin staining for morphological analysis of the kidney marrow collected from 3-month old adult wildtype (1 male and 1 female) and *irf2bp2b*$^{-/-}$ (2 males and 1 female) zebrafish. One representative image of each group is shown. Green and red arrows indicate typical neutrophils and macrophages, respectively. (C) Statistical results for A, B. Error bars represent the mean ± standard deviation (SD) of at least 15 images. ***P<0.001 (Student t test). (D) FACS analysis of whole kidney marrows from Tg(*mpx*:eGFP) and *irf2bp2b*$^{-/-}$//Tg(*mpx*:eGFP) lines in 3-month old adults. The myeloid cells in the R5 gate were analyzed with fluorescence. (E) Statistical results for D. Error bars represent the mean ± SD of three replicates. ****P<0.0001 (Student t test). (F-G') Whole-mount *in situ* hybridization (WISH) using *mpx* and *mfap4* to monitor neutrophil and macrophage development in embryos injected with wildtype or *irf2bp2b* mRNA. (I-K) WISH analyses of the pan-myeloid marker *l-plastin* in embryos injected with wildtype, *irf2bp2b*$^{-/-}$, and *irf2bp2b* mRNA embryos. (H, L) Statistical results for F-G', I-K. Error bars represent the mean ± SD of at least 15-30 embryos. ns: not statistically significant; **P<0.01; ***P<0.001 (Student t test).

matically decreased.[13] In *pu.1*$^{G242D/G242D}$ homozygous embryos, biased myelopoiesis toward neutrophils occurred, as expected. It should be noted that no obvious rescue effect was observed in the *irf2bp2b*$^{-/-}$*pu.1*$^{G242D/G242D}$ double-mutant embryos compared to *pu.1*$^{G242D/G242D}$ embryos, indicating that *pu.1* is indeed downstream of Irf2bp2b in determining NMP cell fate (Figure 4K-O).

Irf2bp2b represses *pu.1* gene transcription by binding directly to its promoter

IRF2BP2 has frequently been described as a co-repressor.[14,16,17] We therefore set out to investigate how Irf2bp2b represses *pu.1* expression. The C-terminal C3HC4-type ring finger motif of IRF2BP2 is responsible for mediating its binding with interacting partners.[14,16,17] The N-terminal C4-type zinc finger motif was believed to enable homo- and hetero-dimerization/multimerization between different IRF2BP2 family members.[15] However, C4 zinc fingers are typically found in DNA-binding domains of transcription factors including GATA1-6 as well as nuclear receptors RAR and RXR.[36,37] The possibility that IRF2BP2 functions as a transcription repressor by directly binding DNA should not, therefore, be excluded.

To characterize how Irf2bp2b represses transcription in

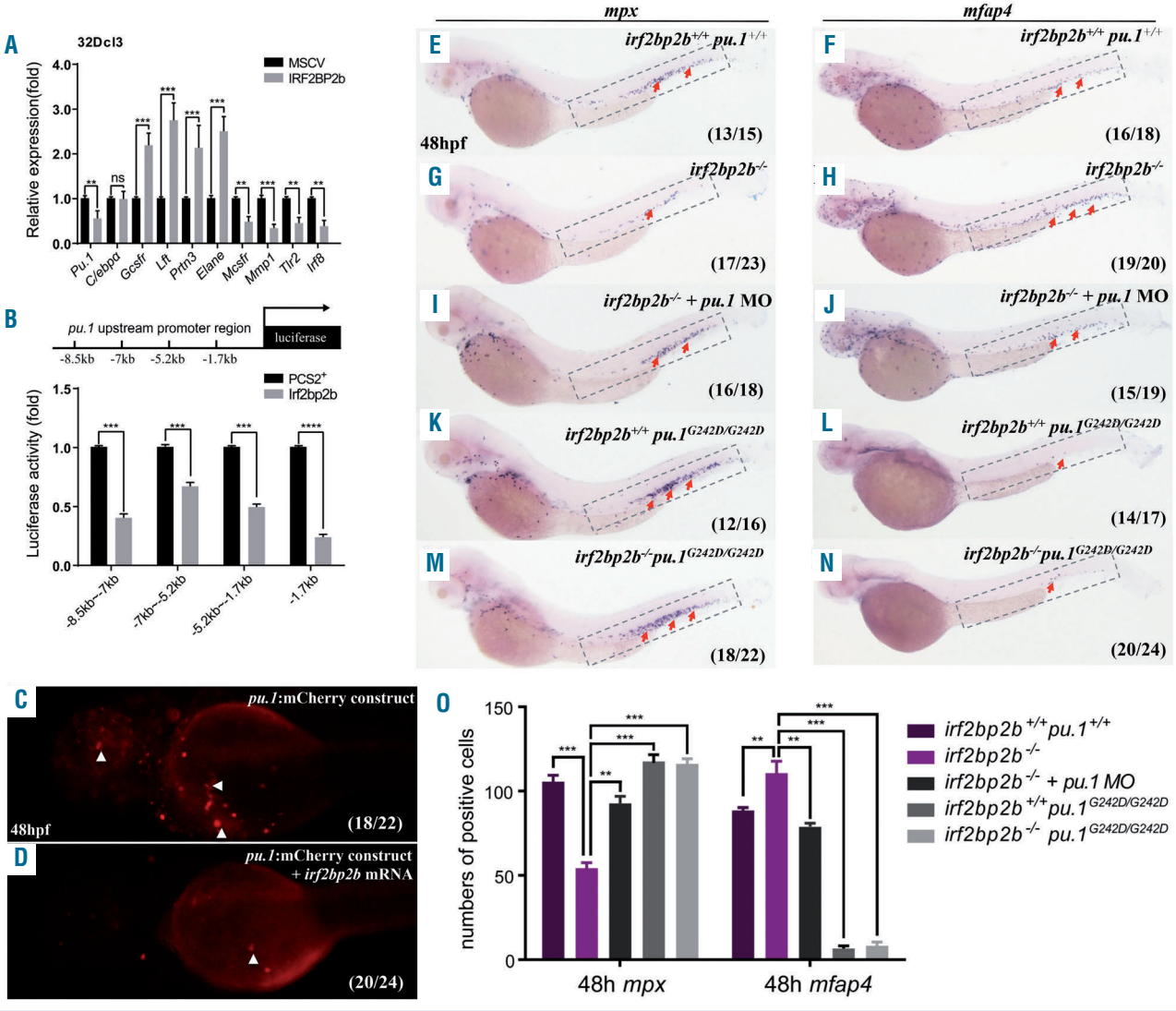

Figure 4. Irf2bp2b dictates neutrophil-macrophage progenitor cell fate through inhibition of pu.1 expression. (A) Quantitative reverse transcriptase polymerase chain reaction analysis of neutrophil and macrophage development-related genes in 32Dcl3 cells constitutively expressing human *IRF2BP2b*. Error bars represent the mean ± standard deviation (SD) of at least three replicates. ns: not statistically significant; ***P*<0.01; ****P*<0.001 (Student *t* test). (B) Schematic diagram of the -8.5kb zebrafish *pu.1* promoter dual luciferase report vector (top panel). Dual luciferase vectors each with a fragment of the zebrafish *pu.1* promoter, as indicated, were co-transfected into HEK293T cells with an *irf2bp2b*-expressing vector or empty vector pCS2^{+}. Luciferase activity with irf2bp2b expression was detected and normalized to empty vector pCS2+ which was set to 1.0 (bottom panel). Error bars represent the mean ± SD of at least three replicates. ****P*<0.001; *****P*<0.0001 (Student *t* test). (C, D) Representative fluorescent images of transient mCherry expression at 48 hours post-fertilization (hpf) of wildtype (WT) and *irf2bp2b*- overexpressing embryos injected with a -8.5 kb *pu.1*:mCherry construct. (E-N) Whole-mount *in situ* hybridization (WISH) assay of *mpx* and *mfap4* in WT embryos, (E, F), *irf2bp2b*$^{-/-}$ mutant embryos (G, H), *irf2bp2b*$^{-/-}$ mutant embryos injected with *pu.1* morpholino (I, J), *pu.1*$^{G242D/G242D}$ mutants (K, L), and *irf2bp2b*$^{-/-}$*pu.1* $^{G242D/G242D}$ double-mutant embryos (M, N). (O) Statistic result for E-N. Error bars represent the mean ± standard error of mean of 15-30 embryos. ***P*<0.01; ****P*<0.001 (analysis of variance followed by the least significant difference *post-hoc* test for multiple comparisons).

the choice of NMP cell fate, a series of point mutations in critical cysteines were introduced into the ring finger motif (C420/423A, named RM hereafter) and the zinc finger motif (C14/17A, named ZM hereafter) of Irf2bp2b, as previously reported[15] (Figure 5A). For the Irf2bp2b RM mutant, interaction with its partners was abolished, while the polymerization and putative DNA-binding capacities of the ZM mutant were both abrogated. A tetramerization motif from human P53 (amino acids 324-355) was fused in-frame with the Irf2bp2b ZM mutant (tet-ZM), restoring the polymerization capacity of this mutant (Figure 5A). Immunofluorescence analysis (anti-HA antibody) of HEK293T cells transfected with the Irf2bp2b mutants described above demonstrated that these mutations did not affect nuclear localization as expected (*Online Supplementary Figure S7*).[28]

The results from *in vivo* rescue assays revealed that only the RM mutant displayed a significant rescue effect similar to wildtype *irf2bp2b*, while the ZM and tet-ZM mutants did not (Figure 5B-L). These data indicate that direct DNA binding would be indispensable for the ability of Irf2bp2b to repress *pu.1* gene expression in NMP cell fate choice.

Correspondingly, the luciferase activity assays showed that only wildtype Irf2bp2b and RM mutant, but not ZM and tet-ZM mutants, exhibited strong repressive effects on luciferase expression with a -1.7 kb zebrafish *pu.1* promoter (Figure 6A). This fragment was further narrowed down to a short 132 bp region (A region) (Figure 6B). To validate that the A region is an Irf2bp2b binding site, *in vivo* chromatin immunoprecipitation polymerase chain reaction (CHIP-PCR) was performed in zebrafish embryos expressing GFP or Irf2bp2b-GFP using an anti-GFP antibody. In this assay, the *pu.1* promoter A region was specifically co-immunoprecipitated with Irf2bp2b-GFP (Figure 6C).

Since positively charged amino acids are important to fit into the negatively charged phosphate backbone of DNA, several arginines (R10/11/36/55/59) within the C4 zinc finger motif were mutated. Luciferase assays showed that only the Irf2bp2b$^{R55/59L}$ double-mutant completely lost the ability to repress luciferase expression from the *pu.1* promoter (Figure 6D). Notably, CHIP-PCR analysis has shown that the Irf2bp2b$^{R55/59L}$ mutant could not co-immunoprecipitate the *pu.1* promoter A region (Figure 6C). As anticipated, this mutant lost the rescue effect in *irf2bp2b*$^{-/-}$ embryos (Figure 6E-J, K). These results indicate that Irf2bp2b represses *pu.1* gene expression by directly binding to its promoter and R55/R59 are two critical amino acids for Irf2bp2b DNA binding.

Taken together, these findings suggest that Irf2bp2b most likely functions as a transcription repressor, rather than a co-repressor, in NMP fate choice during zebrafish myelopoiesis.

The repressive property of Irf2bp2b is dependent on SUMOylation

IRF2BP2 is a co-repressor molecule for its interacting transcription factors.[14,17] In the current study, we demon-

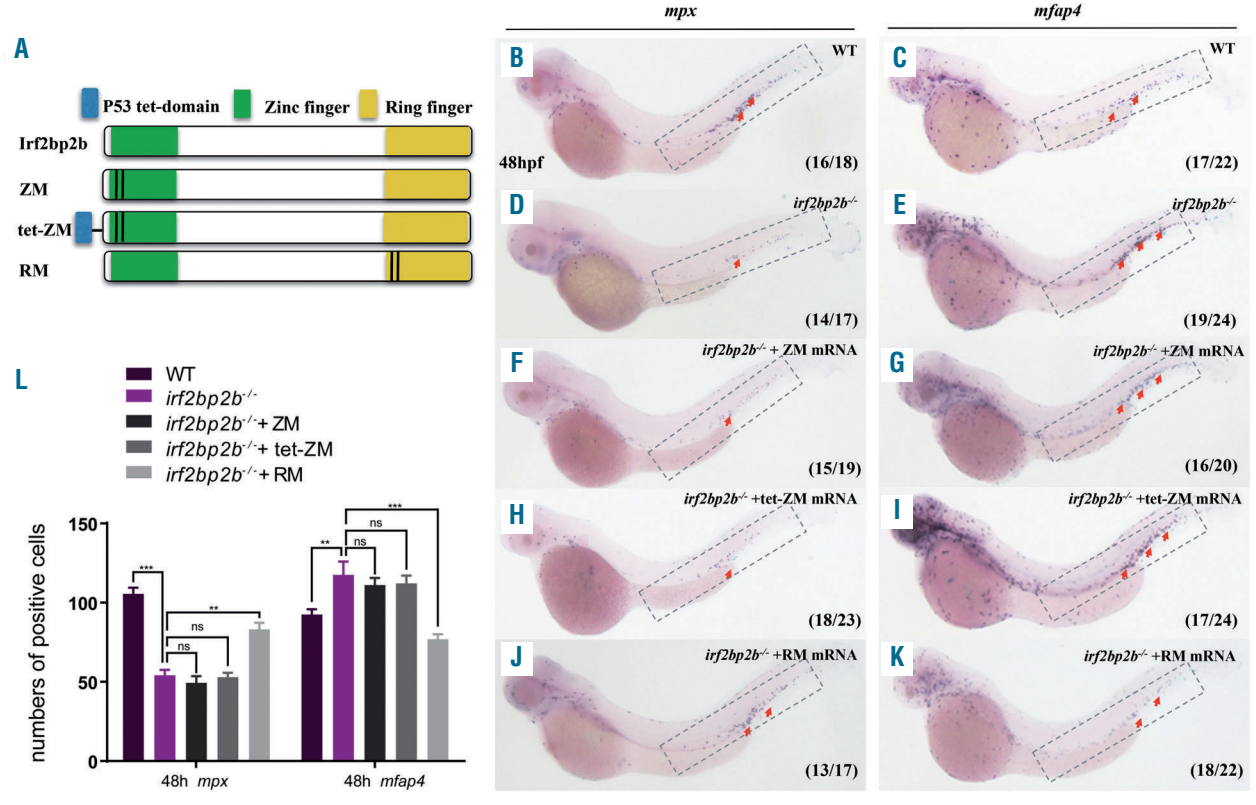

Figure 5. DNA-binding is indispensable for Irf2bp2b in regulating neutrophil-macrophage progenitor cell fate. (A-L) *Irf2bp2b* mRNA rescue assays in *irf2bp2b*$^{-/-}$ embryos. (A) Structure of variant forms of Irf2bp2b, including wildtype (WT), and ZM, tet-ZM, and RM mutants. (B-L) *Mpx* and *mfap4* probes were used in whole-mount *in situ* hybridization (WISH) to examine the rescue effect of injecting *irf2bp2b* ZM (F, G), tet-ZM (H, I), and RM mutant mRNA (J, K). (L) Error bars represent the mean ± standard error of mean of 15-30 embryos. ns: not statistically significant; ***P*<0.01; ****P*<0.001 (analysis of variance followed by the least significant difference post-hoc test for multiple comparisons).

strated that zebrafish Irf2bp2b inhibits *pu.1* expression. Thus, we investigated the reason underlying the repressive property of IRF2BP2.

Post-translational modification of proteins plays a pivotal role in regulating their function. SUMOylation is an important type of post-translational modification which involves a cascade of dedicated enzymes that facilitate the covalent modification of specific lysine residues on target proteins with monomers or polymers of SUMO (small ubiquitin-like modifier).[38] The SUMOylation of substrate proteins is frequently linked with transcriptional repression.[39] In fact, multiple adducts (the smallest one was about 10 kD larger than the unmodified protein, which was nearly the size of one SUMO molecule) of Irf2bp2b were detected by western blot (Figure 7A). The SUMO-targeted lysine usually lies in the canonical motif ΨkxE.[40] A SUMO consensus motif VKKE (lysine 496) located at the C-terminus of Irf2bp2b was predicted by bioinformatics (*Online Supplementary Figure S1*). The putative lysine was mutated to arginine (Irf2bp2b^{K496R}) to abolish covalent binding with the SUMO molecule. The modified bands of the Irf2bp2b^{K496R} mutant protein disappeared as expected (Figure 7A, B). In addition, an Irf2bp2b^{E498A} mutant was constructed to destroy the conservation of the SUMO consensus motif which still allowed the accessibility of lysine 496 to other modifiers. The modified bands disappeared as Irf2bp2b^{K496R} mutant did (Figure 7C), indicating that Irf2bp2b is a SUMOylated substrate.

In HEK293T cells, GFP-SUMO was co-transfected with HA-tagged wildtype Irf2bp2b or Irf2bp2b^{K496R} mutant. Immunoprecipitation assays showed that GFP-SUMO co-precipitated with HA-tagged wildtype Irf2bp2b, but not with the Irf2bp2b^{K496R} mutant (Figure 7D). This further indicated that Irf2bp2b is indeed SUMOylated in cells.

Luciferase reporter assays with zebrafish *pu.1* promoter were then conducted to assess the repressive capacity of

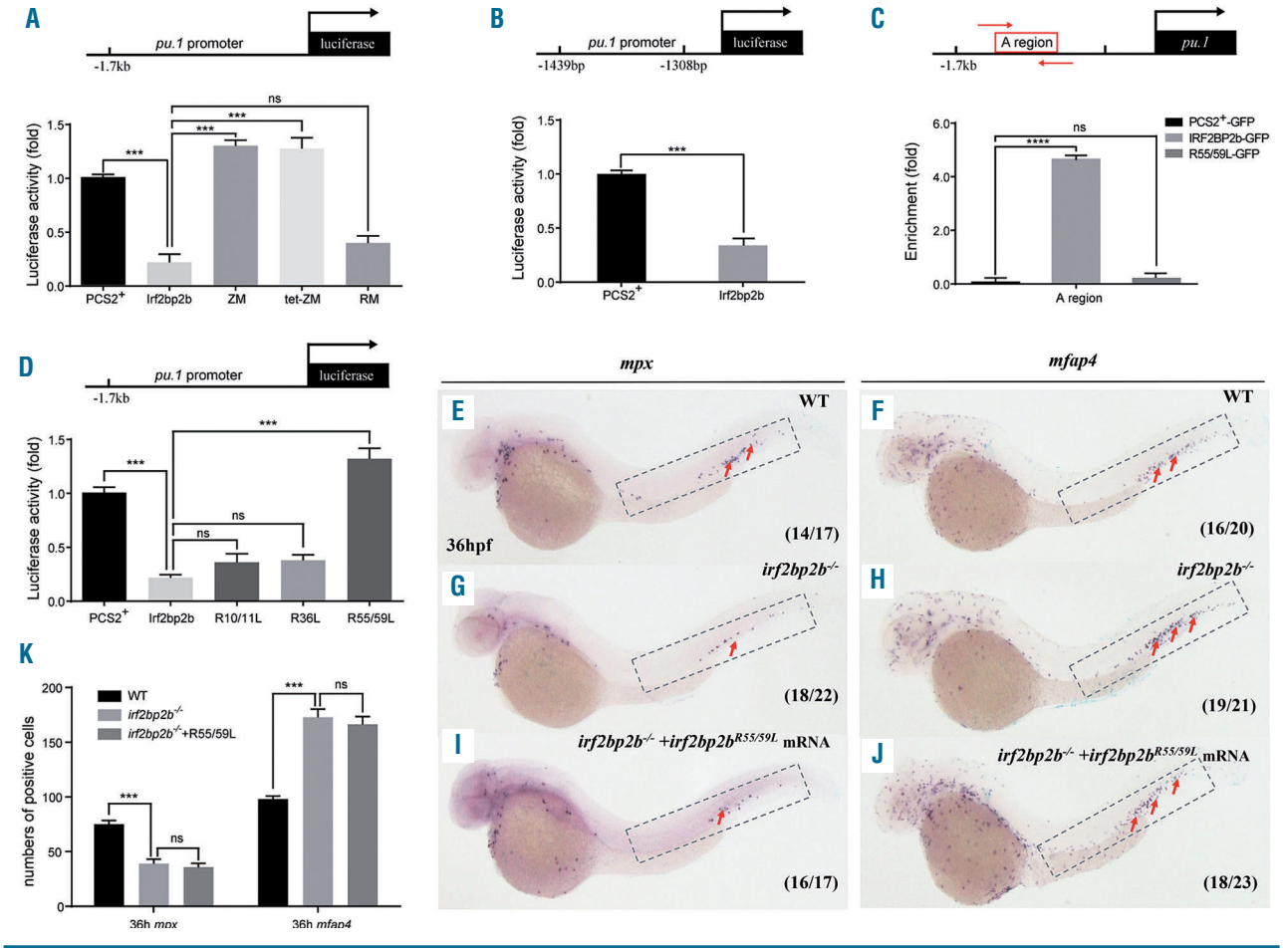

Figure 6. DNA-binding property is indispensable for Irf2bp2b in regulating neutrophil-macrophage progenitor cell fate (continued). (A) Ability of Irf2bp2b mutants to repress the zebrafish *pu.1* promoter (-1.7kb). Error bars represent the mean ± standard error of mean (SEM) of at least three replicates. ***P<0.001 [analysis of variance (ANOVA) followed by the least significant difference (LSD) *post-hoc* test for multiple comparisons]. (B) Irf2bp2b represses luciferase expression from the *pu.1* promoter 132bp A region (from -1308 bp to -1439 bp). Error bars represent the mean ± standard deviation (SD) of at least three replicates. ***P<0.001 (Student *t* test). (C) Chromatin immunoprecipitation polymerase chain reaction analysis of *pu.1* promoter A region in zebrafish embryos expressing green fluorescent protein (GFP), Irf2bp2b-GFP or Irf2bp2b$^{R55/59L-GFP}$ using an anti-GFP antibody. The position of the primers used to amplify the *pu.1* promoter A region are indicated with red arrows. Error bars represent the mean ± SEM of at least three replicates. ****P<0.0001 (ANOVA followed by the LSD *post-hoc* test for multiple comparisons). (D) Luciferase repression assays of Irf2bp2b mutants on zebrafish *pu.1* promoter (-1.7 kb). Error bars represent the mean ± SEM of at least three replicates. ***P<0.001 (ANOVA followed by the LSD *post-hoc* test for multiple comparisons). (E-J) *Irf2bp2b*$^{R55/59L}$ mRNA rescue assays in *irf2bp2b*$^{-/-}$ mutant embryos. *Mpx* and *mfap4* probes were used in whole-mount *in situ* hybridization to examine rescue effects associated with injection of *Irf2bp2b*$^{R55/59L}$ mutant mRNA. (K) Error bars represent the mean ± SEM of at least three replicates. ***P<0.001 (ANOVA followed by the LSD *post-hoc* test for multiple comparisons).

Irf2bp2b upon its SUMOylation. The results showed that the Irf2bp2b-SUMO fusion, which mimics fully SUMOylated Irf2bp2b, displayed even stronger repression than the wildtype Irf2bp2b, whereas the Irf2bp2b^{K496R} mutant lost the ability to repress transcription (Figure 7B, E). Consistently, Irf2bp2b-SUMO and Irf2bp2b^{K496R} mutants had completely different rescue effects in *irf2bp2b*-deficient mutants (Figure 7F-N).

Overall, these data support the concept that Irf2bp2b is a SUMOylated protein in cells and that SUMOylation is indispensable for its property of repressing transcription.

Irf2bp2b mediates the antagonistic effect of C/ebpα on *pu.1* in neutrophil-macrophage progenitor cell fate

To ensure balanced commitment of NMP toward each lineage, the mutual antagonistic interplay of the master regulators PU.1 and C/EBPα is very important.[5,41] Since Irf2bp2b represses *pu.1* expression in zebrafish NMP cell fate choice, we questioned whether *irf2bp2b* is a C/ebpα target.

Two putative C/ebpα binding sites located at -37 bp (CS1) and -1595 bp (CS2) upstream of the transcription start site were predicted in the zebrafish *irf2bp2b* promoter by bioinformatics analysis. A luciferase reporter vector was constructed with the zebrafish *irf2bp2b* -2.2kb promoter and co-transfected with either a *c/ebpα*-expressing vector or an empty vector. Luciferase expression was significantly enhanced by C/ebpα (Figure 8A). A similar enhancement of expression was also obtained when an mCherry-expressing vector carrying the same *irf2bp2b* promoter (*irf2bp2b:*mCherry, in a Tol2 backbone) was co-injected with *c/ebpα* and Tol2 transposase mRNA into zebrafish embryos (Figure 8B, C). This enhancement was completely abolished when the predicted C/ebpα binding sites were deleted in the *irf2bp2b* promoter (Figure 8D).

Finally, *c/ebpα* mRNA was injected into *irf2bp2b*$^{-/-}$ knock-

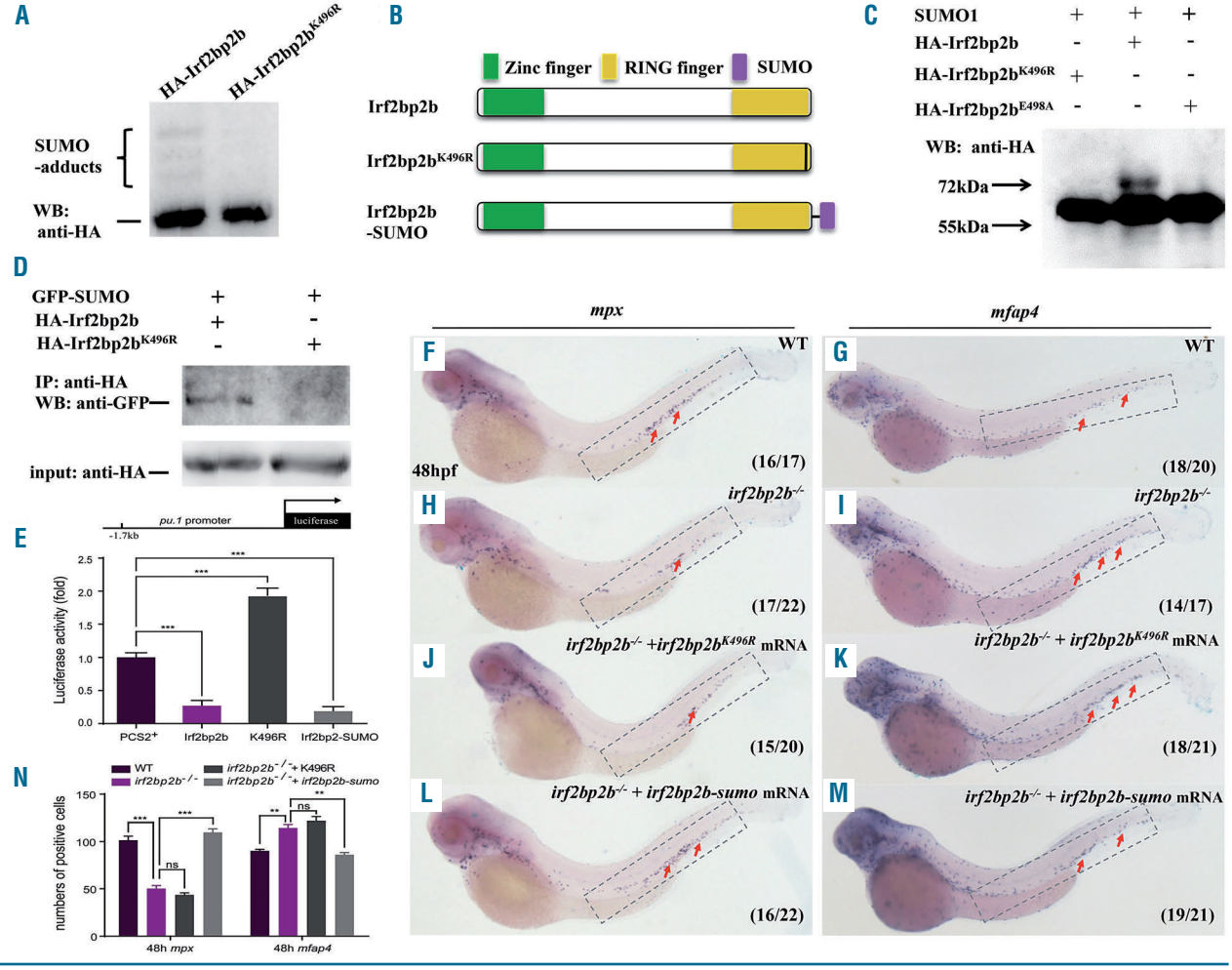

Figure 7. SUMOylation is indispensable for transcription repression of Irf2bp2b. (A) Western blot analysis (anti-HA) of HA-tagged wildtype (WT) and Irf2bp2b^{K496R} mutant proteins expressed in HEK293T cells. (B) The structure of variant forms of Irf2bp2b, including WT, Irf2bp2b^{K496R}, and Irf2bp2b-SUMO mutants. (C) Western blot analysis (anti-HA) of HA-tagged WT, Irf2bp2b^{K496R} and Irf2bp2b^{E498A} mutant proteins expressed in HEK293T cells. (D) HA-tagged WT or Irf2bp2b^{K496R} mutant protein was immunoprecipitated with an anti-HA antibody from HEK293T cells co-expressing GFP-SUMO, and SUMOylated Irf2bp2b protein was detected by western blot with an anti-GFP antibody. (E) Repression of luciferase expression from the zebrafish *pu.1* promoter (-1.7kb) by Irf2bp2b mutants. Error bars represent the mean ± standard error of mean (SEM) of at least three replicates. ***P<0.001 [analysis of variance (ANOVA) followed by the least significant difference (LSD) *post-hoc* test for multiple comparisons]. (F-M) *Irf2bp2b*-SUMO and *irf2bp2b*^{K496R} rescue assays in *irf2bp2b*$^{-/-}$ mutant embryos. *Mpx* and *mfap4* probes were used in whole-mount *in situ* hybridization to examine rescue effects of injections of *irf2bp2b*^{K496R} mutant (J, K) or *irf2bp2b-sumo* (L, M) mRNA. (N) Error bars represent the mean ± SEM of 15-30 embryos. ns: not statistically significant; **P<0.01; ***P<0.001 (ANOVA followed by the LSD *post-hoc* test for multiple comparisons).

out and wildtype embryos. The overexpression of *c/ebpα* mRNA induced biased myelopoiesis toward neutrophils in control embryos (Figure 8E, F, I, J, M), but had no effect on myelopoiesis in *irf2bp2b*$^{-/-}$ embryos (Figure 8G, H, K-M).

Meanwhile, to elucidate whether *gfi1* could also be a secondary determinant of C/ebpα, *gfi1* mRNA was injected into wildtype embryos. Although gfi1 overexpression did give rise to a remarkable expansion of the neutrophil population, the macrophage population was unaffected (*data not shown*). Moreover, this overexpression did not have any rescue effect in irf2bp2b-deficient embryos (*Online Supplementary Figure S8E, F, I*).

In summary, these data indicate that Irf2bp2b plays a pivotal role in mediating the antagonistic function of C/ebpα on *pu.1* transcription regulation, which fine tunes the level of *pu.1* expression in NMP and determines the choice of NMP cell fate in order to maintain a normal neutrophil and macrophage population ratio (Figure 8N).

Discussion

Although multiple regulators involved in hematopoietic lineage restriction have been characterized, the molecular details of NMP differentiation are still under debate. The relationship between the master regulators PU.1 and C/EBPα in myelopoiesis is complicated, being not only synergistic, but also antagonistic.[41] On the one hand, C/EBPα can stimulate *PU.1* expression by directly binding to its promoter.[42,43] On the other hand, C/EBPα can interact directly with PU.1 and block its function, or inhibit *PU.1* indirectly through activation of the transcription repressor *GFI1*,[44,45] which in turn inhibits PU.1 activity through a

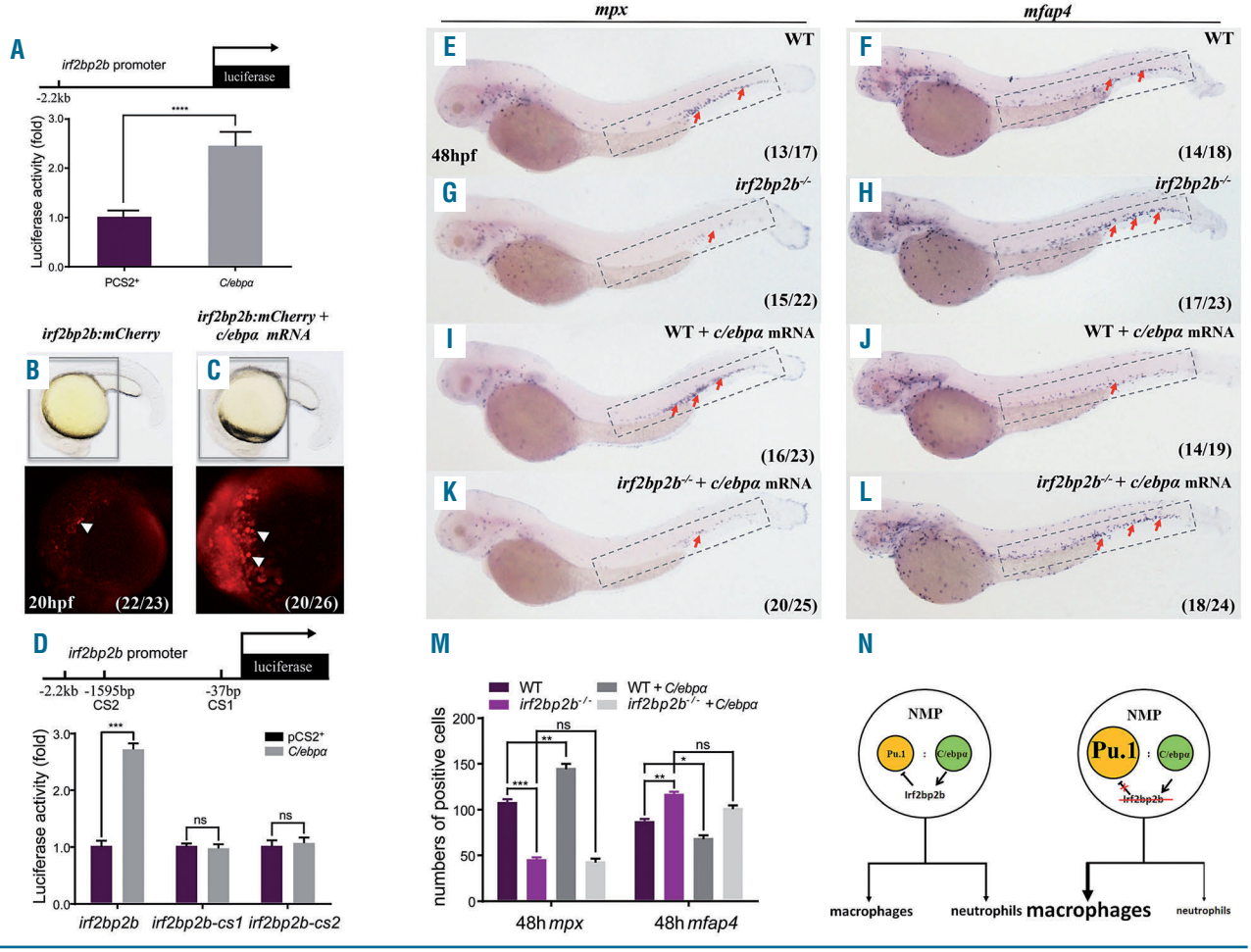

Figure 8. Irf2bp2b mediates the antagonistic effect of C/ebpα on *pu.1*. (A) Schematic diagram of the zebrafish *irf2bp2b* promoter (-2.2 kb) (top panel). C/ebpα activation on the *irf2bp2b* promoter was measured by a luciferase activity assay (bottom panel). Error bars represent the mean ± standard deviation (SD) of at least three replicates. ****P<0.0001 (Student *t* test). (B, C) Representative fluorescent images of transient mCherry expression at 20 hours post-fertilization (hpf) of wildtype (WT) and *c/ebpα*-overexpressing embryos injected with an *irf2bp2b*:mCherry construct (bottom panel). Corresponding bright field images (top panel). (D) Schematic diagram of the zebrafish *irf2bp2b* promoter (-2.2 kb), in which two putative C/ebpα binding sites are predicted (CS1, CS2) (top panel). Luciferase activity assays of C/ebpα activation on the *irf2bp2b* CS1 and CS2 mutant promoters (bottom panel). Data shown are the mean ± SD of at least three independent experiments. Error bars represent the mean ± SD of at least three replicates. ***P<0.001 (Student *t* test). (E-L) *C/ebpα* mRNA overexpression in WT and *irf2bp2b*$^{-/-}$ mutant embryos. *Mpx* and *mfap4* probes were used in whole-mount *in situ* hybridization analysis to investigate any rescue effect. (M) Statistical results for E-L. Error bars represent the mean ± standard error of mean of 15-30 embryos. ns: not statistically significant; *P<0.1; **P<0.01; ***P<0.001 (analysis of variance followed by the least significant difference *post-hoc* test for multiple comparisons). (N) Schematic depiction of the regulation of neutrophil and macrophage fate in WT (left panel) and *irf2bp2b*$^{-/-}$ (right panel) zebrafish.

protein-protein interaction.[46] In the present study, we determined that in the balance between granulocyte and macrophage commitment, zebrafish *irf2bp2b* acts as a direct target of C/ebpα to repress *pu.1* expression. Our data also suggest that during the stage of definitive myelopoiesis in zebrafish, it is the C/ebpα-Irf2bp2b-Pu.1 axis, not the C/ebpα-Gfi1-Pu.1 one, that regulates NMP cell fate. Thus zebrafish Irf2bp2b acts as a novel player in NMP cell fate decision and adds a new layer of complexity to this fine-tuning process.

It should be noted that the primitive macrophages and neutrophils developed normally in *irf2bp2b*-deficient embryos (*Online Supplementary Figure S3C, D, G, H, K, L*). Previously it was reported that a Pu.1-Runx1 negative feedback loop determines the macrophage *versus* neutrophil fate of cells originating in the rostral blood island.[13] Runx1 was shown to inhibit the *pu.1* promoter directly in the study; however, injection of *runx1* mRNA into our *irf2bp2b*-deficient embryos could not rescue the aberrant myelopoiesis (*Online Supplementary Figure S8G, I*). To further elucidate whether *irf2bp2b* regulates primitive myeloid differentiation, we first determined whether *irf2bp2b* is present in primitive *versus* definitive progenitor cells (*Online Supplementary Figure S9A*). We then injected *irf2bp2b* mRNA into one-cell stage wildtype embryos. The biased myelopoiesis could only be observed in the ventral wall of the dorsal aorta at 48 hpf (Figure 3F-H). By contrast, *c/ebp1*, *lyz*, and *mfap4* were all normally expressed in the rostral blood island at 22 hpf (*Online Supplementary Figure S9B-H*). Based on these observations, we believe that even though *irf2bp2b* is expressed in both primitive and definitive myeloid progenitor cells, distinct regulatory mechanisms are implicated in cell fate determination of NMP derived from the ventral wall of the dorsal aorta/caudal hematopoietic tissue and the rostral blood island.

The DNA-binding properties of IRF2BP2 have never been studied. Although C4-type zinc fingers are found in Irf2bp2, GATA, RARα, and RXR, there are still some differences. While a single C-X2-C-X17-C-X2-C type zinc finger exists in Irf2bp2, two consecutive ones are contained in GATA. RARα and RXR have two C-X2-C-X13-C-X2-C type zinc fingers. GATA binds specifically to a consensus sequence.[47] Physiologically the RAR-RXR heterodimer binds to responsive elements that consist of two AGGTCA core motifs.[48] To determine the binding site of Irf2bp2b within the *pu.1* promoter, we first investigated whether it was similar to that of GATA or RAR/RXR. Two putative GATA binding sites (GS1, GS2) were predicted within the 132 bp A region, whereas no RAR/RXR binding sites could be found. However, both GATA site deletion constructs could still be inhibited by Irf2bp2b (*Online Supplementary Figure S10*). Therefore, Irf2bp2b presumably has its own binding site.

The majority of APL patients bear a PML-RARα fusion gene. However, in APL variants RARα is fused with genes other than PML. Recently, four APL cases with a novel fusion, IRF2BP2-RARα, were identified.[20-23] All X-RARα fusion-related APL are characterized by blockage at the promyelocyte stage and inhibition of a large set of differentiation-related genes targeted by co-repressors recruited onto the RARα moiety.[49] It should be noted that the zinc finger motif of IRF2BP2 was intact in all four patients carrying the IRF2BP2-RARα oncoprotein,[20-23] thus two potential DNA-binding domains from each moiety are retained simultaneously in the fusion. Such a phenomenon is very rare in a chimeric fusion protein composed of two transcription factors. This raises a few questions about IRF2BP2-RARα-related APL. Since dimerization is one of the prerequisites for all X-RARα fusions,[50] does the IRF2BP2 moiety serve merely as an interface for dimerization of IRF2BP2-RARα, or does IRF2BP2 make other contributions, such as DNA binding, to the pathogenesis of APL? Does IRF2BP2-RARα arise at the NMP level? If it is expressed in NMP, could IRF2BP2-RARα trigger the biased choice of NMP cell fate favoring granulopoiesis? Further studies are needed to answer these questions.

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