The Znf711-Phf8 complex functions as a transcriptional rheostat essential for neutrophil development

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Abstract

Neutrophil differentiation is governed by a precise transcriptional and epigenetic program. Here, we identify the zinc finger protein 711 (Znf711) and its partner, the histone demethylase PHD finger protein 8 (Phf8), as essential regulators of terminal granulopoiesis. Contrary to their established role as a transcriptional activator-co-activator pair, we found that the Znf711-Phf8 complex operates through a repressive mechanism. Znf711 promotes neutrophil maturation in a DNA-binding-independent manner by sequestering Phf8. Upon loss of Znf711, Phf8 is recruited by the growth factor independent 1 transcription repressor (Gfi1aa) to the promoter of the master regulator *c/ebpα*, where SUMOylated Phf8 acts as a corepressor to inhibit its transcription. Furthermore, we delineate a positive feedback loop wherein C/ebpα directly activates *znf711* expression, ensuring a high level of *c/ebpα* at the onset of differentiation. Our findings define the Znf711-Phf8 complex as a critical transcriptional rheostat in neutrophil development.

Introduction

Neutrophils are a vital component of the immune system, acting as the first responders to infections and playing a critical role in combating invading pathogens.[1] As the most abundant type of short-lived white blood cells, they require continuous daily replenishment from hematopoietic stem cells (HSC). Within the classical hierarchy of hematopoiesis, HSC sequentially differentiate into common myeloid progenitors (CMP) and granulocyte-monocyte progenitors (GMP), which ultimately give rise to the neutrophil and macrophage lineages. This process culminates in terminal differentiation to achieve full neutrophil maturation.[2]

The steady-state production of mature neutrophils is tightly regulated by a variety of transcription factors and co-factors.[3,4] Pioneering studies have demonstrated that key transcription factors such as the C/EBP and ETS family proteins act in succession to drive neutrophil development.[5] Concurrently, various epigenetic regulators including histone methyltransferases, histone demethylases (KDM), and histone deacetylases (HDAC) collaborate with lineage-specific transcription factors throughout the process of neutrophil differentiation.[6] Mutations or abnormal expression of these critical regulators are closely associated with pathologies such as neutropenia or leukemia.[7] Despite extensive research in this area, the full spectrum of regulators involved in neutrophil development and their precise mechanisms remain to be fully elucidated.

The human *ZNF711* gene encodes a C2H2-type transcription factor characterized by a Zfx/Zfy transcription activation region at the N-terminus and 13 consecutive zinc finger motifs at the C-terminus.[8] Multiple loss-of-function mutations within this gene have been identified in several families with X-linked intellectual disability.[9] Mechanistic studies in neuronal cells have demonstrated that ZNF711 functions as a transcriptional activator by recruiting the histone demethylase PHF8 to activate *KDM5C* expression in neuronal cells.[10,11] Thus, a tight functional link between ZNF711 and PHF8 has been established in the context of neurodevelopmental disorders.[10,11]

In addition to its expression in the brain, *ZNF711* is also widely expressed in myeloid lineages.[12] Notably, *ZNF711* is

highly expressed at the early stages (myeloblast and promyelocyte) of terminal granulopoiesis but significantly declines as neutrophils undergo differentiation (the BloodSpot database) (*Online Supplementary Figure S1A*). Consistent with a potential role in leukemogenesis, lowered expression of *ZNF711* has been observed in most acute myeloid leukemia (AML) subtypes (the BloodSpot database) (*Online Supplementary Figure S1A*). Additionally, the BeatAML database[13] includes five cases harboring *ZNF711* mutations: four carry missense mutations of unknown significance, and one carries a truncating mutation (ZNF711$^{E316/*}$), which deletes part of the transcription activation region, the nuclear localization signal (NLS), and all zinc finger motifs, rendering it a loss-of-function mutation (*Online Supplementary Figure S1B*). The expression profile, coupled with the AML-associated mutation, strongly suggests potential roles for *ZNF711* in both normal myelopoiesis and its malignant counterpart. However, its precise function in hematopoiesis remains to be elucidated.

PHF8 contains a PHD finger that mediates binding to specific nuclear protein partners and chromatin, as well as a Jumonji C (JmjC) domain exhibiting histone demethylase catalytic activity.[14,15] Thus, PHF8 is generally identified as a transcriptional co-activator which removes repressive histone marks to activate gene expression.[14-6] Notably, amplification of *PHF8* has been frequently observed in AML patients (The Cancer Genome Atlas database). Furthermore, PHF8 is a proven regulator of the cell-intrinsic immune response in AML, as its transduction impedes leukemic transformation and suppresses clonogenic growth while facilitating apoptosis of AML cells.[17] Conversely, in chronic myeloid leukemia (CML), PHF8 inhibits differentiation of CML cells and promotes their proliferation by activating transcription of the *BCR-ABL1* fusion gene.[18] These contradictory findings suggest that the role of PHF8 in leukemogenesis is context-dependent and intricate. Similar to *ZNF711*, the potential functions of *PHF8* in normal myelopoiesis also remain unclear.

In this study, we generated two knockout zebrafish lines to demonstrate that both *znf711* and *phf8* are essential for neutrophil development. Mechanistically, we found that Znf711 and Phf8 do not function synergistically as a canonical combination of transcriptional activator and co-activator during this process. Instead, Znf711 acts in a DNA-binding-independent manner by impeding its partner Phf8 from inhibiting the expression of *c/ebpα*, the master regulator driving granulopoiesis. Concurrently, rather than functioning solely as a canonical histone demethylase that activates transcription, Phf8 serves as an important co-repressor of the transcription factor Gfi1aa to regulate neutrophil maturation. Moreover, we demonstrate that SUMOylation is required for Phf8 to engage in transcriptional repression. Finally, we identified a positive feedback loop between *c/ebpα* and *znf711*, which ensures a high level of *c/ebpα* expression at the early stages of terminal granulopoiesis to promote cell differentiation. Overall, our findings reveal that Znf711 and Phf8 are two novel and critical regulators in terminal granulopoiesis that operate through a fine-tuned transcriptional regulatory circuit centered on *c/ebpα*.

Methods

Zebrafish strains

Zebrafish strains including Tubingen, Tg(*mpx*:GFP), *znf711, phf8*, and *gfi1aa* homozygous mutants, were raised under standard conditions (28.5°C in system water). All animal work was approved by the Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Procedures and methods

Detailed information about generation of *znf711* and *phf8* knockout lines using CRISPR/Cas9 system, morpholinos, whole-mount *in situ* hybridization (WISH), Sudan Black staining, flurescence-activated cell sorting (FACS) analysis and cell collection, RNA sequencing (RNA-seq) and real-time quatitative polymerase chain reaction (RT-qPCR), plasmid construction, dual-luciferase reporter assay, co-immunoprecipitation (co-IP) and western blot assay, chromatin immunoprecipitation qPCR (ChIP-qPCR), cell lines and treatment are provided in the *Online Supplementary Appendix.*

Statistical analysis

Data were analyzed by GraphPad Prism 9.0 software using two tailed Student's *t* test for comparisons between two groups and one-way analysis of variance (ANOVA) among multiple groups. Differences were considered significant at $P<0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Data availability

RNA-seq dataset generated in this study is available in the Gene Expression Omnibus (GEO) database (GSE295370).

Results

Zebrafish *znf711* is required for neutrophil development across the lifespan

The zebrafish (*Danio rerio*) has emerged as a powerful model for hematopoietic research over the past two decades.[19] Given the high evolutionary conservation between zebrafish Znf711 and its human ortholog (*Online Supplementary Figure S2*), we sought to define its role in hematopoiesis. We generated a *znf711* knockout zebrafish line using the CRISPR/Cas9, resulting in a truncated protein that lacked the Zfx/Zfy transactivation domain, the nuclear localization signal (NLS), and all zinc finger motifs (*Online Supplementary Figure S3A, B*). RT-qPCR data confirmed the significant

reduction of *znf711* transcripts in the homozygous mutants (*Online Supplementary Figure S3C*).

Zebrafish hematopoiesis occurs in two waves, primitive and definitive, at distinct anatomical sites.[20,21] Primitive neutrophils and monocytes/macrophages originate from the rostral blood island (RBI), while the intermediate cell mass (ICM) gives rise to primitive erythrocytes and some neutrophils.[22,23] During definitive hematopoiesis, HSC emerge from the ventral wall of the dorsal aorta (VDA), migrate to the caudal hematopoietic tissue (CHT), and finally colonize the kidney marrow (KM) in adults.[22,23] To dissect the function of *znf711* in hematopoiesis, we performed whole-mount *in situ* hybridization (WISH) with lineage-specific markers on mutant embryos and larvae. At 22 hours post-fertilization (hpf), the development of primitive monocytes/macrophages and erythrocytes was unperturbed in *znf711* mutants (*Online Supplementary Figure S4*). In sharp contrast, expression of neutrophil markers including *c/ebp1* (the zebrafish ortholog of human *C/EBPε*), lysozyme (*lyz*), and myeloperoxidase (*mpx*), was significantly impaired in primitive neutrophils derived from both the RBI and ICM (Figure 1A-D', N).

This neutrophil-specific defect persisted into the definitive hematopoiesis stage, with no apparent impact on other lineages (*Online Supplementary Figure S4*). From 36 hpf to 5 days post-fertilization (dpf), we observed a marked reduction in the expression of *c/ebp1*, *lyz*, and *mpx* (Figure 1E-K', N). The impairment in neutrophil development was further confirmed by Sudan Black (SB) staining (Figure 1L, L', N) and by a pronounced reduction of GFP$^+$ cells in *znf711*$^{-/-}$//Tg(*mpx:eGFP*) larvae at 48 hpf (Figure 1M, M', N). The specificity of this phenotype was confirmed by the following experiments: knockdown of *znf711* with specific morpholino antisense oligonucleotides (MO) in wild-type embryos recapitulated the neutrophil deficiency, and this defect was fully rescued by reintroducing either zebrafish *znf711* or human *ZNF711* mRNA (Figure 1O, P). The functional complementation by human *ZNF711* indicates that its role in granulopoiesis is evolutionarily conserved.

To determine whether this requirement extends into adulthood, we analyzed whole kidney marrow (WKM) from 1-year-old zebrafish. FACS revealed a significant decrease in both the percentage of GFP$^+$ cells within the myeloid gate (87.4% *vs*. 39.0%) and the GFP fluorescence intensity in *znf711*$^{-/-}$//Tg(*mpx:eGFP*) mutants compared to controls (Figure 2A, B). Consistently, May-Grünwald Giemsa staining showed a substantial reduction in the proportion of mature neutrophils (27.4% *vs*. 8.8%) in the mutants (Figure 2C, D). In contrast, the percentages of cells in the erythrocyte and lymphocyte gates were comparable between wild-type (WT) and mutant WKM (*Online Supplementary Figure S5*).

In conclusion, our findings demonstrate that Znf711 is a neutrophil-specific regulator whose function is required throughout the lifespan of zebrafish.

c/ebpα downregulation mediates the neutrophil developmental defect in *znf711* mutants

To gain insight into the mechanism underlying the neutrophil defect in *znf711* mutants, we performed RNA-seq on GFP$^+$ cells from WT Tg(*mpx*:eGFP) and *znf711*$^{-/-}$//Tg(*mpx*:eGFP) larvae at 48 hpf (*Online Supplementary Appendix*). A heatmap of differentially expressed genes (DEG) clearly illustrates the transcriptional alterations in *znf711*-deficient neutrophils (Figure 3A). We found a significant decrease in the expression of *c/ebpα*, a master transcription factor governing neutrophil differentiation,[24,25] in the mutant cells. Accordingly, the expression of key C/EBPα target genes,[26,27] including *c/ebp1*, *csf3r* (the zebrafish ortholog of human granulocyte colony-stimulating factor receptor, *GCSFR*), as well as the neutrophil-specific markers *mpx* and *lyz*, was markedly reduced. RT-qPCR analysis independently confirmed these findings (Figure 3B).

We hypothesized that the downregulation of *c/ebpα* is the major cause of neutrophil impairment in *znf711* mutants. To test this, we performed a genetic rescue experiment by expressing *c/ebpα* specifically in the neutrophil lineage. To avoid potential confounding effects from misexpression in earlier progenitors, we injected a TOL2(*mpx:c/ebpα*) plasmid into *znf711*-deficient embryos. Neutrophil-specific restoration of *c/ebpα* substantially rescued the neutrophil defects (Figure 3C, D). Conversely, overexpression of *znf711* in WT embryos led to a significant expansion of the *mpx*$^+$ population, which should be caused by *c/ebpα* upregulation (Figure 3C, D, E). Critically, this expansion was abolished when *znf711* mRNA was co-injected with a TOL2(*mpx:c/ebpα-bZIP*) plasmid, which expresses a dominant-negative C/ebpα mutant lacking transactivation capacity[28] (Figure 3C, D).

Altogether, these results demonstrate that the deficiency of *znf711* impairs neutrophil development by reducing *c/ebpα* expression.

Znf711 antagonizes Phf8-mediated repression of *c/ebpα* to promote neutrophil development

To delineate how Znf711 regulates neutrophil differentiation, we began by mapping its functional domains. Strikingly, *in vivo* rescue assays revealed that the mutant lacking the entire DNA-binding domain (Znf711 ΔDBD) restored neutrophil development as effectively as the WT protein. By contrast, the mutant lacking the Zfx/Zfy domain (Znf711 ΔZfx/Zfy) was completely non-functional (Figure 4A, B). These results indicate that Znf711 functions in a DNA-binding-independent manner, challenging its canonical role as a transcription factor.

The involvement of the Zfx/Zfy domain in mediating interactions between Znf711 and its partners[29] led us to hypothesize that the loss of Znf711 may release certain interactant, which subsequently inhibits *c/ebpα* expression. We focused on PHF8, a known ZNF711-interacting partner in neurons that typically functions as a transcriptional co-activator.[16,30]

Intriguingly, integrated ChIP-seq and RNA-seq data from WT and *PHF8*-depleted HeLa cells suggest that PHF8 can also repress transcription,[31] coupled with the finding that PHF8 binds the *C/EBPα* promoter in human myeloid cells (the Cistrome database)[32] (*Online Supplementary Figure S6*). We therefore proposed a model wherein the absence

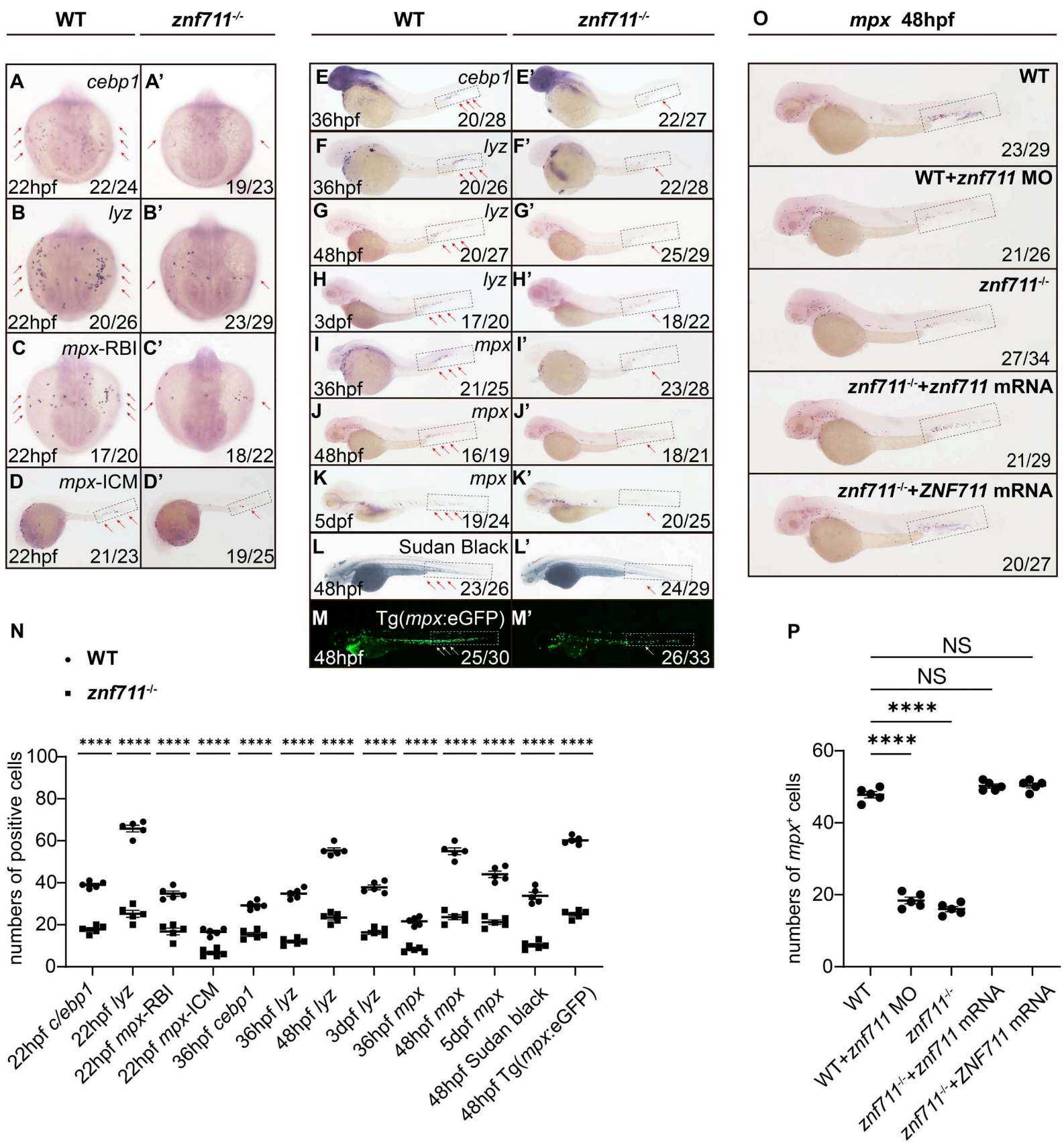


Figure 1. *znf711* deficiency specifically impairs neutrophil development during embryogenesis. (A-K') Whole-mount *in situ* hybridization (WISH) analyses of neutrophil markers *c/ebp1*, *lyz, mpx* from 22 hours post fertilization (hpf) to 5 days post fertilization (dpf) in wild-type (WT) and *znf711*-deficient zebrafish. Grey boxes and red arrows indicate the main positions of positive cells for each marker in rostral blood island (RBI), intermediate cell mass (ICM), ventral wall of the dorsal aorta (VDA), and caudal hematopoietic tissue (CHT) regions. N/N: the number of embryos/larvae showing representative phenotype/total number of embryos/larvae examined. (L, L') Sudan Black staining in WT and *znf711*-deficient larvae at 48 hpf. (M, M') GFP fluorescence in WT Tg(*mpx*:eGFP) and *znf711*$^{-/-}$//Tg(*mpx*:eGFP) larvae at 48 hpf. (N) Statistical results for A-M' (Student's *t* test, N=5, 20-30 embryos/larvae were used for each probe in each experiment. Each dot represents the mean value obtained from all counts within the same group). (O) WISH assays of *mpx* in WT embryos injected with *znf711* morpholino antisense oligonucleotides (MO), and *znf711*$^{-/-}$ mutant embryos rescued by WT zebrafish *znf711* or human *ZNF711* mRNA at 48 hpf. (P) Statistical results for (O). The statistical significance was calculated by using one-way analysis of variance (ANOVA). N=5, 25-35 larvae were used for each experiment. Each dot represents the mean value of 1 experiment. Error bars represent mean ± standard error of the mean; NS: not statistically significant; ****P<0.0001.

of Znf711 releases Phf8, which in turn represses *c/ebpα* to block neutrophil maturation.

To validate this hypothesis, we first examined whether Phf8 interacts with Znf711 in myeloid cells. co-IP assays in 32Dcl3 cells (a murine myeloid progenitor cell line) confirmed this interaction using endogenous antibodies (Figure 4C). Further co-IP assays in HEK293T cells demonstrated that the interaction with Phf8 is mediated by the Zfx/Zfy domain of Znf711 (Figure 4D).

Second, we evaluated whether Phf8 inhibits the *c/ebpα* promoter using dual-luciferase reporter assays. Overexpression of *phf8* significantly repressed the promoter, while depletion of endogenous *PHF8* using small hairpin (shRNA) activated it (Figure 4E, lanes 1-3). The role of PHF8 as a histone demethylase depends on its H3K4me3-binding PHD finger motif and catalytic JmjC domain.[14-16] Notably, the ΔPhd finger and ΔJmjC mutants of Phf8 repressed the promoter similarly to WT Phf8, indicating that Phf8 does not function as a canonical histone demethylase here (Figure 4E, lanes 4 and 5). Moreover, the Znf711 ΔDBD mutant activated the promoter comparably to WT Znf711 (Figure 4E, lanes 6 and 7), implying this activation is indirect. Indeed, Phf8's repression was markedly impaired in the presence of either WT or Znf711 ΔDBD mutant (Figure 4E, lanes 8 and 9).

Third, we investigated Phf8 binding to the *c/ebpα* promoter using *in vivo* ChIP-qPCR. Due to the technical challenge of performing chromatin immunoprecipitation on the limited number of GFP⁺ cells that can be practically isolated from zebrafish larvae, we conducted these assays in whole larvae expressing HA-Phf8. Even within this heterogeneous cellular context, we observed specific enrichment of the endogenous *c/ebpα* promoter. This enrichment was significantly greater

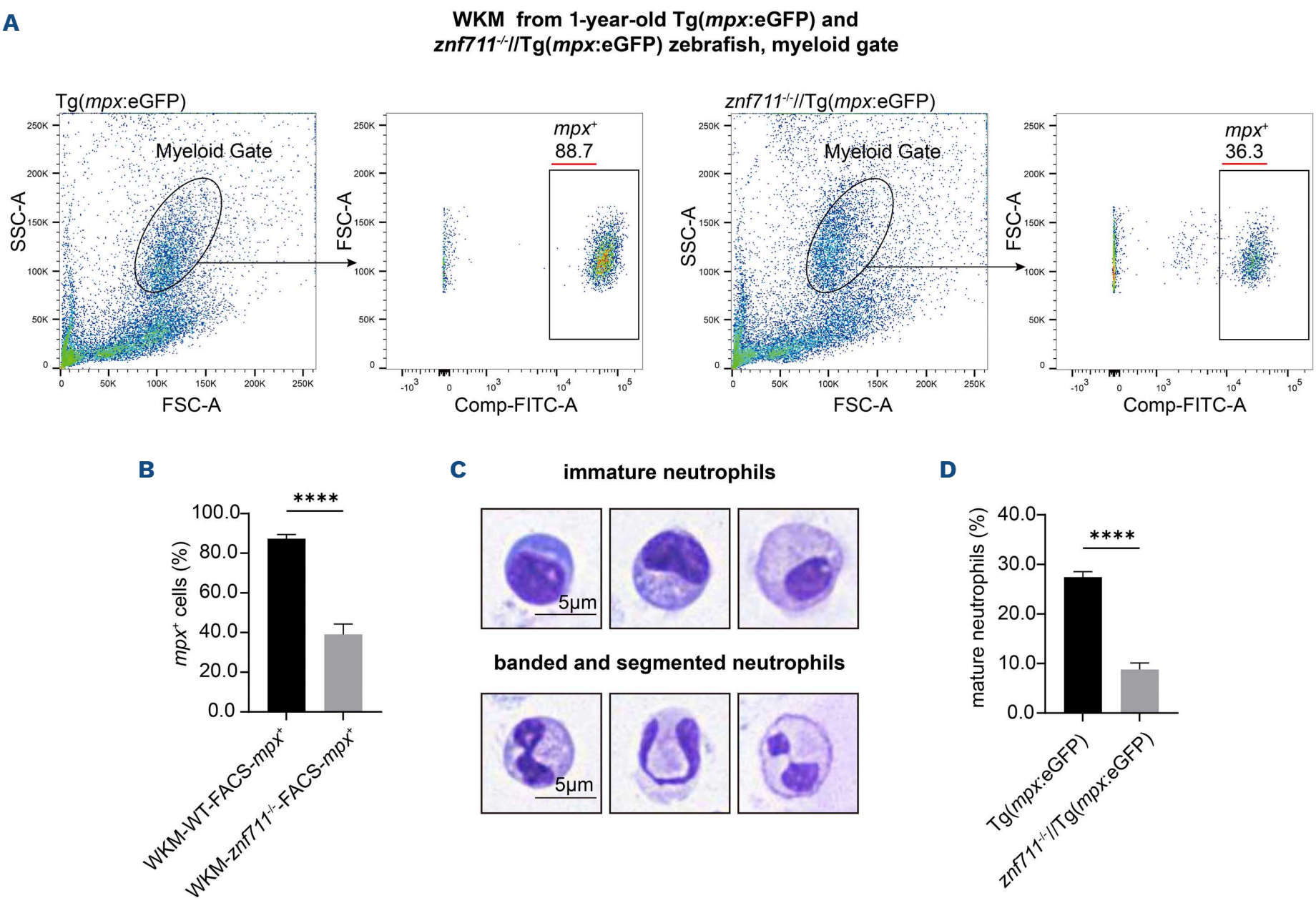


Figure 2. Neutrophil impairment persists in adult *znf711*-deficient zebrafish. (A) Fluorescence-activated cell sorting (FACS) analysis of GFP-positive cells within the myeloid gate of whole kidney marrow (WKM) from 1-year-old wild-type (WT) Tg(*mpx*:eGFP) and *znf711*$^{-/-}$//Tg(*mpx*:eGFP) zebrafish. Light scatter and fluorescence profiles of WT and mutant groups in 1 representative experiment were shown. FSC: forward light scatter, SSC: side light scatter. (B) Statistical results for (A). The statistical significance was calculated by using Student's *t* test, N=5, each time 2 WKM were used in the WT and mutant groups, respectively. (C) May-Grünwald Giemsa staining of *mpx*⁺ neutrophils isolated from the myeloid gate of WT siblings and *znf711*$^{-/-}$//Tg(*mpx*:eGFP) mutants. Scale bar, 5 μm. (D) 300 cytospin-collected *mpx*⁺ cells were counted on slides. Immature and mature neutrophils were distinguished and quantitated by morphology, and the proportion of mature neutrophils was compared between WT and mutant groups (Student's *t* test, N=5). Error bars represent mean ± standard error of the mean; ****P<0.0001.

in *znf711*$^{-/-}$ zebrafish (Figure 4F). This result provides direct *in vivo* evidence that Znf711 loss leads to increased occupancy of Phf8 on the *c/ebpα* promoter, consistent with our model that Znf711 sequesters Phf8 from chromatin.

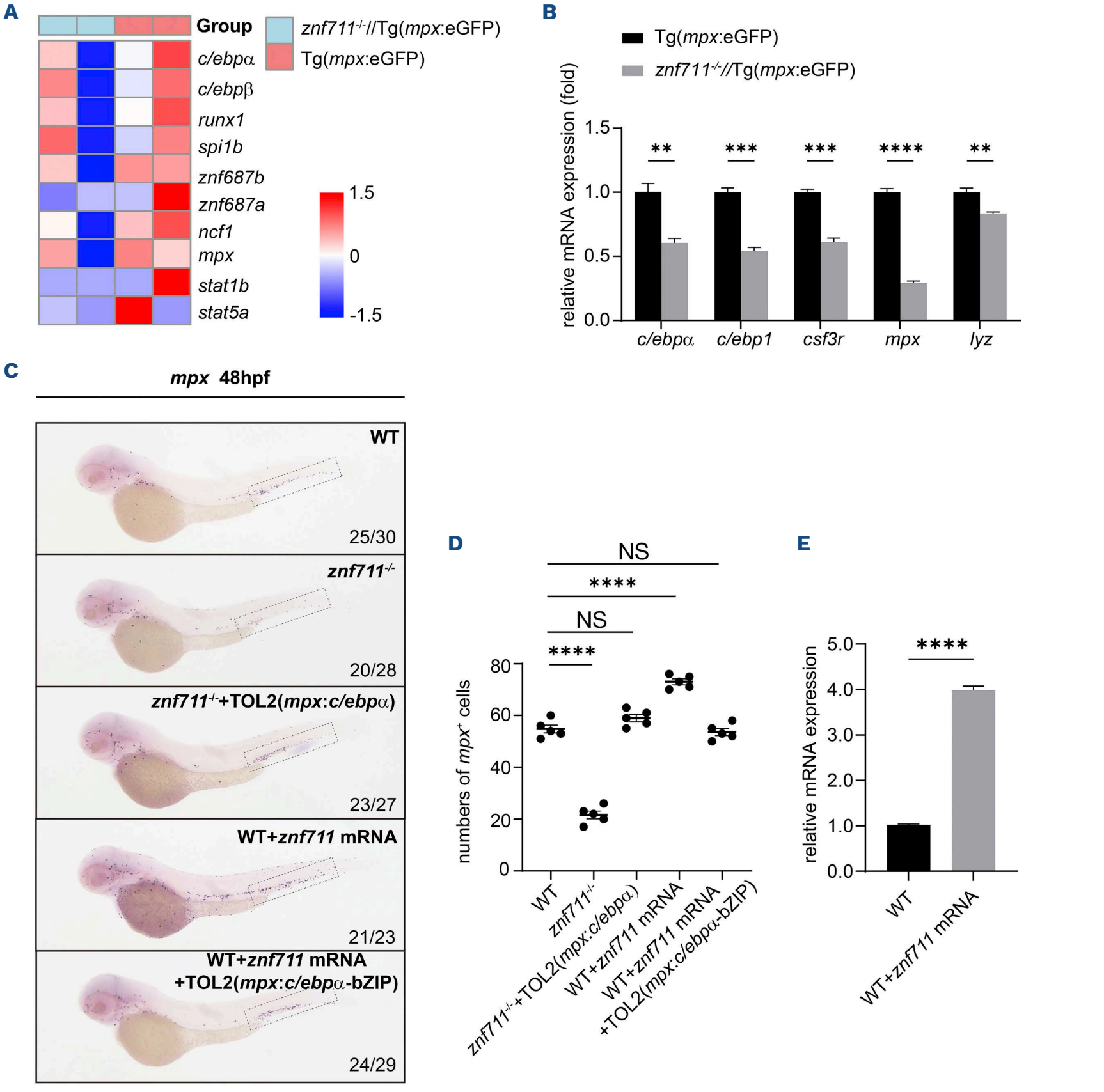


Figure 3. *c/ebpα* downregulation mediates the neutrophil developmental defect in *znf711* mutants. (A) Heatmap displays the expression patterns of key differentially expressed genes in *znf711-deficient* neutrophils *versus* wild-type (WT) cells. (B) Expression of *c/ebpα, c/ebp1, csf3r, mpx*, and *lyz* in GFP-positive cells enriched from Tg(*mpx*:eGFP) and *znf711* morpholino antisense oligonucleotides (MO) injected Tg(*mpx*:eGFP) larvae at 48 hours post fertilization (hpf). To determine the relative expression rate, data were normalized to the expression level of WT groups (which were set to 1.0) after normalized to the internal control of β-*actin* (Student's *t* test, N=3). (C) Rescue assays with the TOL2(*mpx:c/ebpα*) plasmid in *znf711*$^{-/-}$ mutants at 48 hpf. Overexpression of *znf711* mRNA in WT embryos in the absence and presence of the TOL2(*mpx:c/ebpα*-BZIP) plasmid. *Mpx* probe was used in whole-mount *in situ* hybridization (WISH). (D) Statistic result for (C). The statistical significance was calculated by using one-way analysis of variance (ANOVA). The asterisk indicates a statistical difference. (N=5, 23-30 larvae were used for each experiment). Each dot represents the mean value of 1 experiment. (E) Real-time quantitative polymerase chain reaction (RT-qPCR) analyses of *c/ebpα* transcripts in *mpx*$^{+}$ cells sorted from WT Tg(*mpx*:eGFP) and WT zebrafish injected with *znf711* mRNA at 48 hours post fertilization (hpf). Data were normalized to the expression level of WT groups (which were set to 1.0) after normalized to the internal control of β-*actin* (Student's *t* test, N=3). Error bars represent mean ± standard error of the mean; NS: not statistically significant; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

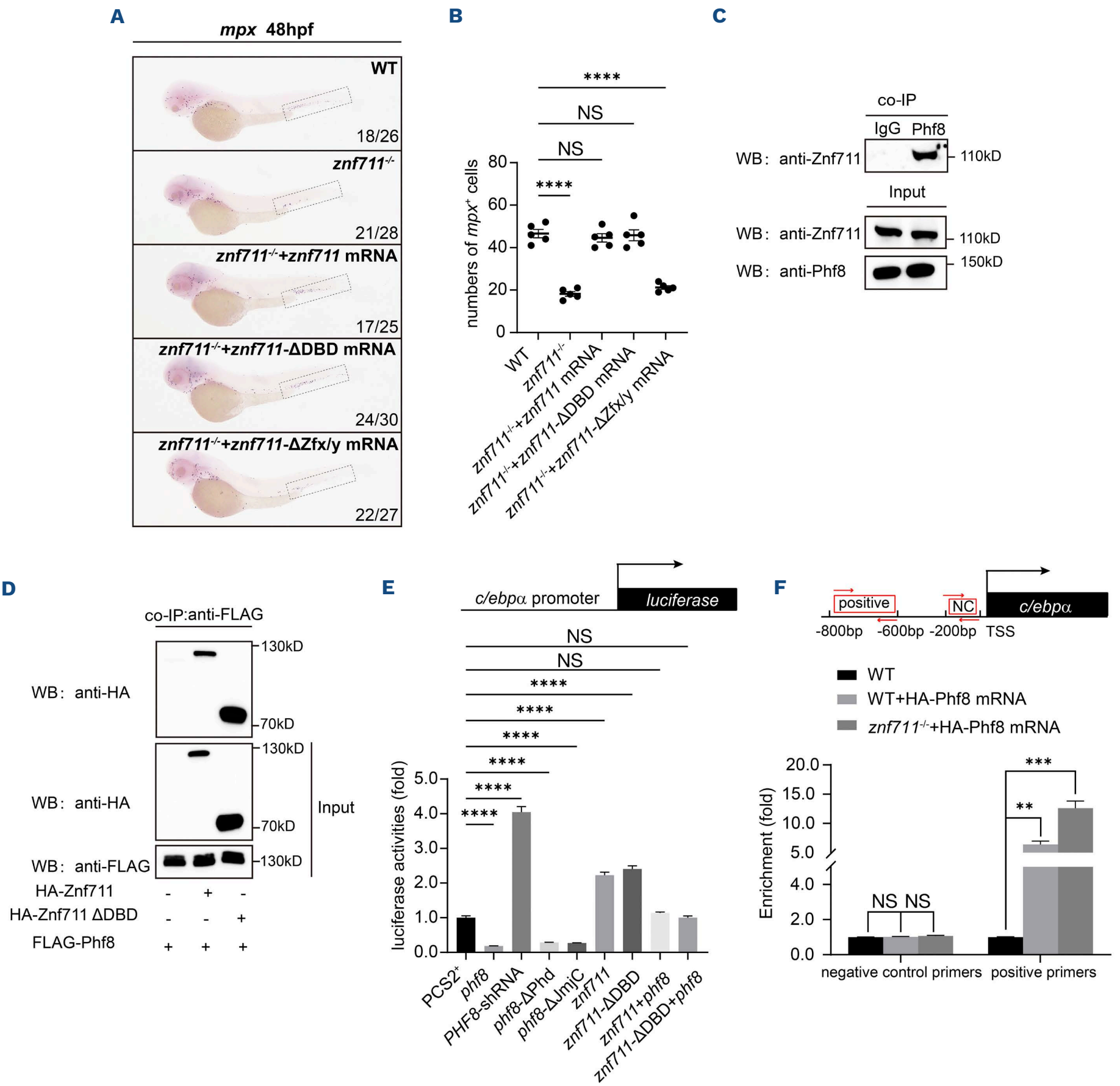


Figure 4. Znf711 antagonizes Phf8-mediated repression of *c/ebp*α to promote neutrophil development. (A) Whole-mount *in situ* hybridization (WISH) assays of *mpx* were conducted in wild-type (WT), *znf711*$^{-/-}$ mutants, and *znf711*$^{-/-}$ mutants injected with WT Znf711, Znf711 ΔZfx/Zfy, and Znf711 DNA binding domain (ΔDBD) mutant mRNA at 48 hours post fertilization (hpf). (B) Statistical analysis for (A). The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=5, each experiment used 25-30 larvae. Each dot represents the mean value of 1 experiment). (C) Co-immunoprecipitation (co-IP) assays indicated endogenous Znf711 could be immunoprecipitated with an anti-Phf8 antibody in the 32Dcl3 cell line. (D) HA-tagged WT Znf711 and Znf711 ΔDBD mutant proteins could both be pulled down by FLAG-tagged Phf8 in HEK293T cells. (E) Dual-luciferase reporter assays. Bars showed the relative luciferase activity on the zebrafish *c/ebp*α promoter (-600 bp ~ -960 bp). Luciferase activities with WT *phf8*, short hairpin RNA (shRNA) targeting *PHF8*, *phf8* ΔPhd finger mutant, *phf8* ΔJmjC mutant, WT *znf711*, *znf711* ΔDBD mutant, WT *znf711* and *phf8* co-expression, *znf711* ΔDBD mutant and *phf8* co-expression, were detected and normalized to empty vector pCS2^{+} which was set to 1.0 (Student's *t* test, N=3). (F) Chromatin immunoprecipitation quantitative polymerase chain reaction analyses of *c/ebp*α promoter in WT or *znf711*$^{-/-}$ zebrafish larvae expressing HA-Phf8 by using an anti-HA antibody. Positive: the location of the positive primers. NC: the location of the negative control primers. The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=3). Error bars represent mean ± standard error of the mean; NS: not statistically significant; ***P*<0.01; ****P*<0.001; *****P*<0.0001. WB: western blotting.

Finally, knockdown of *phf8* in *znf711*-deficient embryos restored *mpx*$^{+}$ cells to normal levels (Figure 5A, B). Additionally, we also generated a *phf8* knockout line (*Online Supplementary Figure S7*), which exhibited expanded *mpx*$^{+}$ cells (Figure 5C, D) and a profound (~18-fold) increase in *c/ebpα* expression within these cells (*Online Supplementary Figure S8A*). Notably, *znf711* MO failed to reduce neutrophils in *phf8*$^{-/-}$ zebrafish (Figure 5C, D), indicating that *znf711* is epistatic to *phf8*.

In summary, our findings indicate that neither Znf711 nor Phf8 functions as a canonical transcriptional activator or co-activator. Instead, Znf711 promotes neutrophil development by sequestering Phf8, thereby preventing Phf8-mediated repression of *c/ebpα*.

Gfi1aa recruits Phf8 to the *c/ebpα* promoter to exert transcriptional repression

Previous studies indicate that PHF8 is recruited to the promoters of target genes through interactions with specific transcription factors.[33] Given the DNA-binding-independent role of Znf711 in neutrophil development, we investigated how Phf8 is recruited to the *c/ebpα* promoter.

We focused on Gfi1aa (the zebrafish ortholog of GFI1) for three reasons: (i) it is a well-characterized transcriptional repressor expressed in early neutrophils[34]; (ii) it directly cooperates with Lsd1 to repress *c/ebpα* expression in zebrafish neutrophils;[35] and (iii) similar neutrophil expansion occurs in *gfi1aa, lsd1*,[35] and *phf8*-deficient zebrafish, respectively. We therefore hypothesized that Gfi1aa recruits Phf8 to the

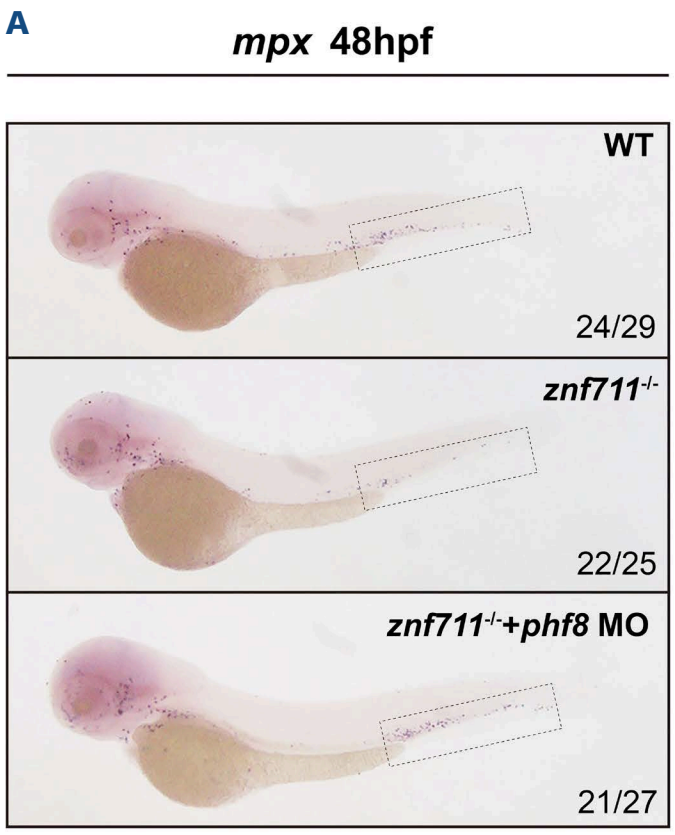


Figure 5. *znf711* is epistatic to *phf8* in neutrophils. (A) Whole-mount *in situ* hybridization (WISH) assays of *mpx* in wild-type (WT) and *znf711*$^{-/-}$ mutants injected with *phf8* morpholino antisense oligonucleotides (MO) at 48 hours post fertilization (hpf). (B) Statistic results for (A). The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=5, 25-30 larvae were used for each experiment). (C) WISH assays of *mpx* in WT, *phf8*$^{-/-}$ mutants, and *phf8*$^{-/-}$ mutants injected with *znf711* MO at 48 hpf. (D) Statistic results for (I). The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=5, 20-30 larvae were used for each experiment). Each dot represents the mean value of 1 experiment. Error bars represent mean ± standard error of the mean; NS: not statistically significant; ****P<0.0001.

c/ebpα promoter to exert repression.

To test this, we first confirmed the Gfi1aa-Phf8 interaction by co-IP experiments in both HEK293T (Figure 6A) and 32Dcl3 myeloid cells (Figure 6B). Next, dual-luciferase assays in HEK293T cells showed that overexpression of *gfi1aa* or *phf8* repressed the *c/ebpα* promoter, while their depletion activated it (Figure 6C, lanes 1-6). Critically, the repressive function of each was abolished upon depletion of the other (Figure 6C, lanes 7-8), demonstrating their mutual dependence.

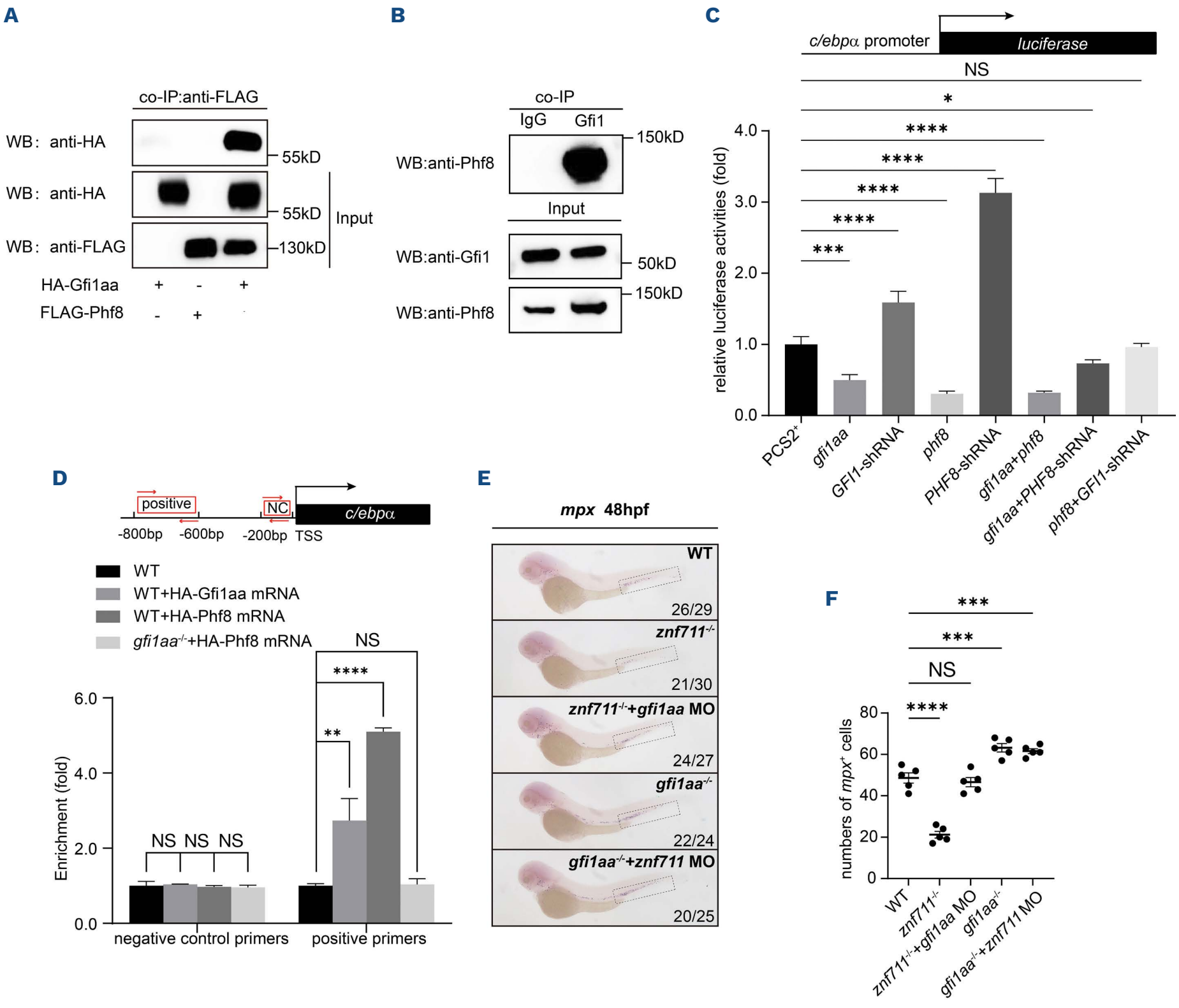


Figure 6. Gfi1aa recruits Phf8 to the *c/ebpα* promoter to exert transcriptional repression. (A) Co-immunoprecipitation (co-IP) assays in HEK293T cells co-expressing FLAG-Phf8 and HA-Gfi1aa. (B) co-IP assays in 32Dcl3 cells where endogenous Phf8 could be immunoprecipitated with an anti-Gfi1 antibody. (C) Dual-luciferase activities on the *c/ebpα* promoter (-600 bp ~ -960 bp) with *gfi1aa* or *phf8* overexpression, *gfi1aa* and *phf8* co-overexpression, small hairpin RNA (shRNA) targeting endogenous *GFI1* or *PHF8*, *gfi1aa* overexpression with *PHF8* depletion, and *phf8* overexpression with *GFI1* depletion, were detected and normalized to empty vector pCS2^{+} group which was set to 1.0 (Student's *t* test, N=3). (D) Chromatin immunoprecipitation quantitative polymerase chain reaction (ChIP-qPCR) analyses of *c/ebpα* promoter in wild-type (WT) zebrafish larvae expressing HA-Gfi1aa or HA-Phf8, as well as in *gfi1aa*$^{-/-}$ zebrafish expressing HA-Phf8 by using an anti-HA antibody. Positive: the location of the positive primers. NC: the location of the negative control primers. The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=3). (E) Whole-mount *in situ* hybridization (WISH) assays of *mpx* in WT, *znf711*$^{-/-}$, *znf711*$^{-/-}$ injected with *gfi1aa* MO, *gfi1aa*$^{-/-}$, and *gfi1aa*$^{-/-}$ zebrafish injected with *znf711* morpholino antisense oligonucleotides (MO) at 48 hours post fertilization (hpf). (F) Statistic results for (E). The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=5, 20-30 larvae were used for each experiment). Each dot represents the mean value of 1 experiment. Error bars represent mean ± standard error of the mean; NS: not statistically significant; *P<0.1; **P<0.01; ***P<0.001; ****P<0.0001. WB: western blotting.

This Gfi1aa-Phf8 axis was further solidified *in vivo*. ChIP-qPCR analyses revealed that Gfi1aa and Phf8 co-occupy the *c/ebpα* promoter (Figure 6D). Importantly, Phf8 binding was drastically reduced in *gfi1aa* mutants (Figure 6D; *Online Supplementary Figure S8B*), indicating that Gfi1aa is essential for recruiting Phf8 to chromatin.

Furthermore, genetic epistasis studies revealed that knockdown of either *phf8* or *gfi1aa* in *znf711* mutants restored neutrophil levels (Figures 5A, B and 6E, F). Conversely, *znf711* knockdown failed to reduce neutrophils in *phf8* or *gfi1aa* mutants (Figures 5C, D and 6E, F), placing *znf711* upstream of both.

Overall, these results establish that Gfi1aa recruits Phf8 to the *c/ebpα* promoter, where it serves as a co-repressor to exert transcriptional repression.

SUMOylation enables Phf8 to function as a transcriptional co-repressor

Having established that Phf8 functions as a Gfi1aa-dependent co-repressor, we sought to define the mechanism underlying its repressive activity. In myeloid cells, GFI1 is known to recruit the LSD1-CoREST repressor complex.[36] We found that PHF8 is dispensable for the integrity of these complexes, as its loss did not affect GFI1-LSD1 or GFI1-RCOR1 interactions (*Online Supplementary Figure S9*), suggesting Phf8 functions through a distinct mechanism. We therefore considered alternative pathways. SUMOylation is a key post-translational modification that is tightly associated with transcriptional silencing through interactions with co-repressor machinery.[37,38] Intriguingly, we detected a ~10 kD adduct on PHF8, consistent with mono-SUMOylation. This finding, coupled with PHF8's repressive role, prompted us to investigate whether SUMOylation imparts repressive activity to PHF8.

We confirmed that PHF8 undergoes SUMOylation in cells (Figure 7A). By mutating candidate lysine residues within SUMO consensus motifs (ΨKXE), we identified K840 as the essential site, as its mutation (K840R) abolished SUMOylation (Figure 7A). The SUMOylation-deficient mutant ($PHF8^{K840R}$) completely failed to repress the *c/ebpα* promoter in dual-luciferase assays (Figure 7B). Crucially, repressive activity was fully restored by fusing this mutant directly to SUMO1 ($PHF8^{K840R}$-SUMO1), underscoring the importance of SUMOylation for Phf8's repressive function (Figure 7B).

These results were further confirmed *in vivo*. Neutrophil-specific expression of the $PHF8^{K840R}$ mutant failed to rescue the expanded mpx^{+} population in *phf8*-deficient zebrafish, whereas the $PHF8^{K840R}$-SUMO1 fusion rescued the phenotype as effectively as WT PHF8 (Figure 7C, D).

Taken together, these findings demonstrate that instead of functioning as a canonical transcriptional co-activator, Phf8 serves as a co-repressor that collaborates with Gfi1aa to repress *c/ebpα* expression in a SUMOylation-dependent manner.

znf711 is a direct downstream target of C/ebpα

Our previous data placed Znf711 upstream of *c/ebpα* by preventing its repression. To fully delineate this regulatory hierarchy, we examined their expression dynamics during terminal granulopoiesis. Both *C/EBPα* and *ZNF711* peak at the myeloblast/promyelocyte stages and rapidly decline upon differentiation[34] (the BloodSpot database) (Figure 8A), suggesting potential co-regulation. Since Znf711 safeguards *c/ebpα* expression, we hypothesized that C/ebpα might, in turn, directly activate *znf711*, forming a reinforcing circuit. Analysis of the *znf711* promoter revealed multiple putative C/EBPα binding sites (*Online Supplementary Figure S10*). Consistent with direct transcriptional activation, C/ebpα robustly transactivated the *znf711* promoter-luciferase reporter (Figure 8B). Endogenous occupancy of the *znf711* promoter by C/ebpα was confirmed by ChIP-qPCR in zebrafish larvae (Figure 8C). Furthermore, forced *in vivo* expression of *c/ebpα* in the neutrophil lineage significantly upregulated endogenous *znf711* transcripts (Figure 8D).

Thus, we demonstrate that *znf711* is a direct downstream target of C/ebpα. Together with our earlier findings that Znf711 protects *c/ebpα* from repression, this establishes a positive feedback loop. This self-reinforcing circuit ensures a high expression of the master regulator *c/ebpα* at the onset of terminal granulopoiesis, thereby promoting cell differentiation, with subsequent downregulation likely governed by other stage-specific factors.

Discussion

ZNF711 and *PHF8* are linked to XLMR in humans.[9,11] Consistent with this and a prior report in *phf8* morphant zebrafish,[16] our *znf711* and *phf8* homozygous mutants also exhibit delayed brain development and craniofacial abnormalities (*Online Supplementary Figure S11*), confirming that zebrafish recapitulate the conserved neurodevelopmental roles of these genes.

Our study further identifies Znf711, Phf8, and Gfi1aa as components of a coherent transcriptional module that ensures the precise expression of the master regulator *c/ebpα* during terminal granulopoiesis in zebrafish. We demonstrate that the Znf711-Phf8 pair, previously characterized as a transcriptional activator-co-activator complex in other contexts, operates through an unexpected repressive mechanism in neutrophils to fine-tune granulopoiesis.

The dynamic expression of *C/EBPα*, which peaks at the myeloblast and promyelocytes stages and declines upon maturation, is critical for promoting neutrophil development.[34,39] This pattern implies the existence of both permissive and repressive regulatory inputs. While *C/EBPα* can maintain its own expression through auto-regulation,[40] it is also a direct repression target of Gfi1aa.[35] Our work identifies Phf8 as a critical negative regulator of this repressive pathway. In the absence of Znf711, Phf8 is released and recruit-

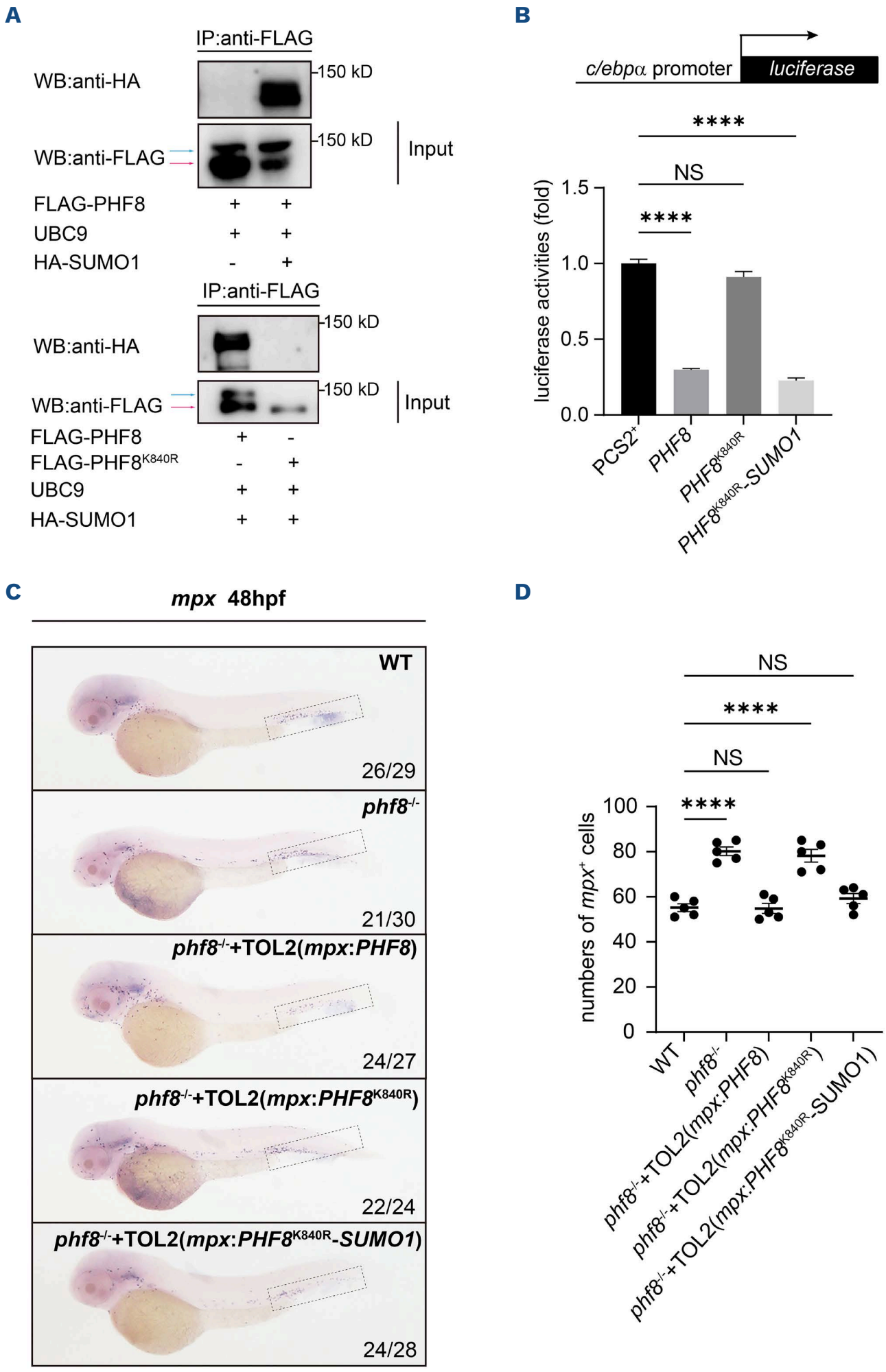


Figure 7. SUMOylation enables Phf8 to function as a transcriptional co-repressor. (A) FLAG-tagged wild-type (WT) PHF8 or PHF8^{K840R} mutant was co-expressed with UBC9 along with HA-SUMO1 in HEK293T cells. PHF8 was immunoprecipitated (IP) with an anti-FLAG antibody, and detected by western blot (WB) with an anti-HA antibody. The red arrow indicates the unmodified form of PHF8, and the blue arrow indicates the SUMOylated form of PHF8. (B) Dual-luciferase reporter assays. Bars showed the relative luciferase activities of WT, PHF8^{K840R}, and PHF8^{K840R}-SUMO1 fusion on the promoter of zebrafish *c/ebpα* (-600 bp ~ -960 bp). Data were normalized to empty vector pCS2^{+} which was set to 1.0 (Student's *t* test, N=3). (C) Whole-mount *in situ* hybridization (WISH) assays of *mpx* in WT, *phf8*$^{-/-}$, and *phf8*$^{-/-}$ zebrafish injected with TOL2(*mpx*:*PHF8*), TOL2(*mpx*:*PHF8*^{K840R}), and TOL2(*mpx*:*PHF8*^{K840R}-*SUMO1*) plasmids. (D) Statistic results for (C). The statistical significance was calculated by using one-way ANOVA. The asterisk indicates a statistical difference (N=5, 20-30 larvae were used for each experiment. Each dot represents the mean value of 1 experiment). Error bars represent mean ± standard error of the mean; NS: not statistically significant; ****P<0.0001.

ed by Gfi1aa to the *c/ebpα* promoter, where SUMOylated Phf8 functions as a potent co-repressor. This mechanism provides a plausible explanation for the loss-of-function *ZNF711* mutations and *PHF8* amplifications observed in AML patients, suggesting that dysregulation of this pathway may contribute to leukemogenesis.

Our findings reveal a DNA-binding-independent function for Znf711, expanding the known functional repertoire be-

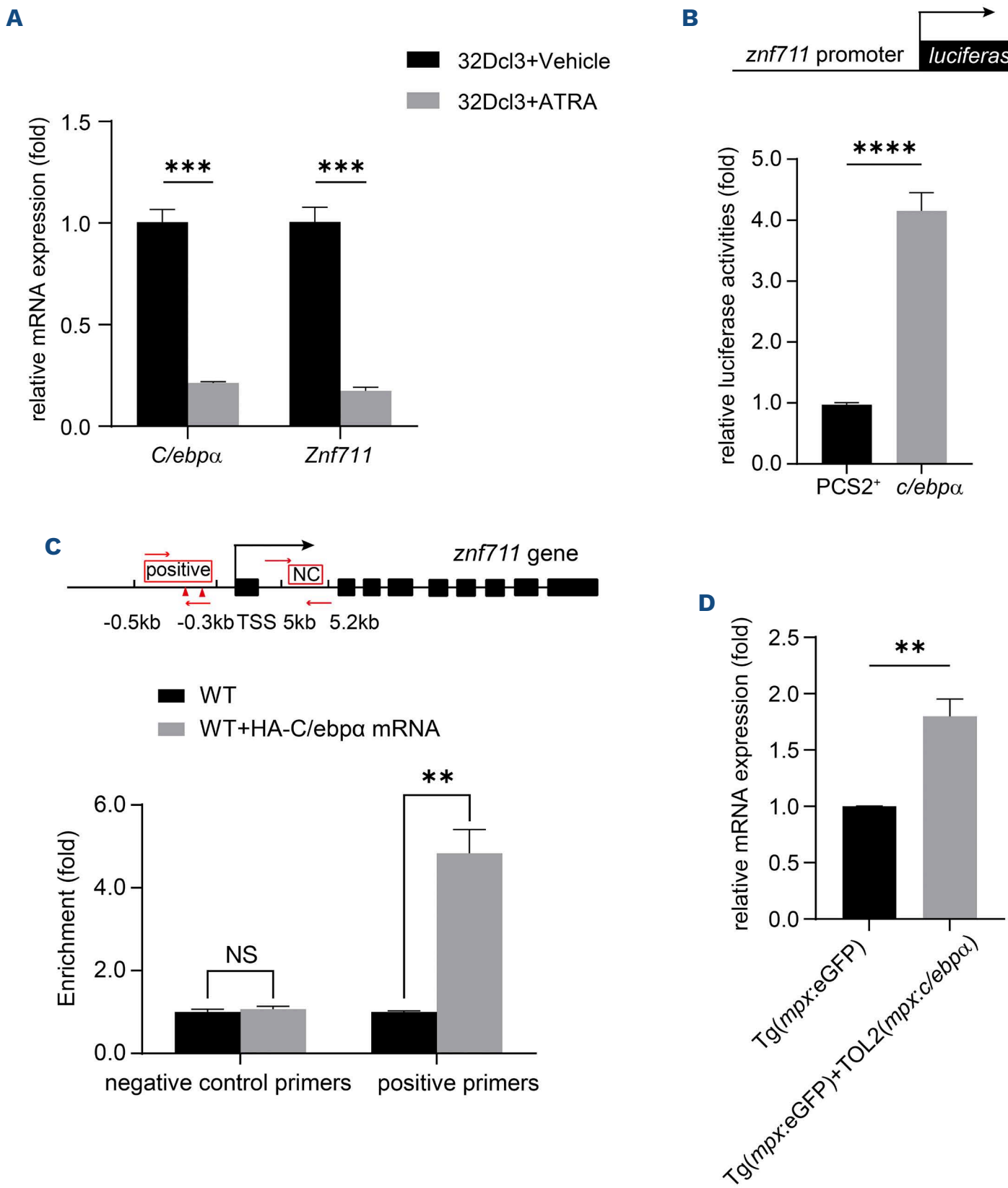


Figure 8. *znf711* is a direct downstream target of C/ebpα. (A) 32Dcl3 cells were induced to differentiate into mature neutrophils using ATRA (1 μM for 72 hours). Real-time quantitative polymerase chain reaction (RT-qPCR) analyses of the expression levels of endogenous *C/ebpα* and *Znf711* before and after ATRA treatment. *GAPDH* serves as internal control, which was set to 1.0 (Student's *t* test, N=3). (B) Dual-luciferase reporter assays. Bars showed the relative luciferase activity on the promoter of zebrafish *znf711* (-2.0 kb). Luciferase activities with C/ebpα were detected and normalized to empty vector pCS2$^+$ which was set to 1.0 (Student's *t* test, N=3). (C, top) Diagram of the *znf711* locus. Two predicted C/EBPα binding sites (JASPAR) within the core promoter region are indicated (triangles). The box denotes the region amplified by chromatin immunoprecipitation quantitative polymerase chain reaction (ChIP-qPCR). (C, bottom) ChIP-qPCR validation of C/ebpα binding to the *znf711* promoter *in vivo*. Enrichment is shown for the region encompassing the predicted sites (positive) *versus* a non-specific control (NC). The statistical significance was calculated by using Student's *t* test. The asterisk indicates a statistical difference (N=3). (D) RT-qPCR analyses of *znf711* in the *mpx*$^+$ cells enriched from wild-type (WT) Tg(*mpx*:eGFP) and Tg(*mpx*:eGFP) zebrafish injected with a TOL2(*mpx:c/ebpα*) plasmid at 48 hours post fertilization (hpf). To determine the relative expression rate, data were normalized to the expression level of WT groups (which were set to 1.0) after normalized to the internal control of β-*actin* (Student's *t* test; N=3). Error bars represent mean ± standard error of the mean; NS: not statistically significant; ***P*<0.01; *****P*<0.0001.

yond its role as a conventional transcriptional activator in neuronal and other tissues.[41] The ability of both the long and short isoforms of human *ZNF711* to rescue neutrophil development in zebrafish mutants (*Online Supplementary Figure S12*) underscores the physiological relevance and evolutionary conservation of this mechanism.

Similarly, we identify a non-canonical, repressive function for Phf8. While widely recognized as a histone demethylase and transcriptional co-activator, Phf8 can also repress transcription in certain contexts. Previous reports have linked PHF8 to repression through mechanisms involving HDAC1-SIN3A recruitment, association with the repressor protein REST/NRSF, or demethylation of the transcription factor YY1.[31,33,42] Our work contributes to this emerging paradigm by demonstrating that in neutrophils, Phf8 is recruited by Gfi1aa and requires SUMOylation, but not its demethylase activity, to repress *c/ebpα*. This suggests that Phf8's repressive function is both context-dependent and mechanistically distinct from its canonical role as an eraser of repressive histone marks.

The dysregulation of PHF8 in hematopoietic malignancies,[43] including its amplification in AML and its ability to activate the oncogenic *BCR-ABL1* fusion gene in CML,[18] underscores its clinical significance. Furthermore, elevated *PHF8* expression is observed in many types of cancers,[42] positioning it as a broad oncogenic driver. Our finding that Phf8 directly represses *c/ebpα*, a gene whose dysfunction is intimately linked to AML, offers a new perspective on its potential oncogenic mechanisms. The Znf711-Phf8-*C/ebpα* regulatory axis we identified in zebrafish may be relevant to human AML, particularly in subtypes with low *ZNF711* expression or *PHF8* amplification. As PHF8 inhibitors are being developed for leukemia and other cancer types,[18,42,44] understanding its critical role in normal neutrophil development will be essential for evaluating potential side effects during therapeutic targeting. Finally, our work expands the repertoire of GFI1 co-repressors. While GFI1 is well-established as a transcriptional repressor in myelopoiesis and is frequently dysregulated in AML, MDS, and SCN,[45,46] its repressive capacity primarily depends on recruitment of the LSD1-CoREST complex.[36] Our data indicate that GFI1 can also recruit Phf8 as an alternative co-repressor. Notably, SUMOylation emerges as a common regulatory theme, as it is essential for the repressive activity of GFI1, PHF8, LSD1, and CoREST,[47-50] suggesting it may be a general mechanism governing the assembly and function of multi-subunit repressive complexes during myeloid development.

In conclusion, our work delineates a novel circuit in neutrophil development, revealing non-canonical functions for Znf711 and Phf8 and expanding our understanding of the GFI1 repressosome. The conservation of this pathway in humans and its potential dysregulation in leukemia underscore its physiological and clinical importance.

Disclosures

No conflicts of interest to disclose.

Contributions

ST and HQ conducted most of the experiments and analyzed data. HW conducted FACS analyses and cell sorting. YC maintained the zebrafish lines. HY, XL, and YW performed RNA sequencing, and conducted analysis of the RNA-sequencing data. HdT provided advice regarding the experiments. JZhu provided advice and analyzed data. JZhou designed the project, analyzed data, and wrote the manuscript.

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Data-sharing statement

RNA-sequencing dataset generated in this study is available in the Gene Expression Omnibus database (GSE295370).

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