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PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

c-Myb acts in parallel and cooperatively with Cebp1 to regulate neutrophil maturation in zebrafish

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Key Points

- c-Myb is essential for neutrophil terminal differentiation by targeting granule gene expression.
- c-Myb and Cebp1 act cooperatively to regulate neutrophil maturation in zebrafish.

Neutrophils are the key effectors for generating innate immunity in response to pathogenic infection and tissue injury in vertebrates. Dysregulation of neutrophil development and function is known to associate with various human disorders. Yet, the genetic network that orchestrates lineage commitment, differentiation, and maturation of neutrophils remains incompletely defined. Here, we present an in vivo study to delineate the genetic program underlying neutrophil development during zebrafish embryonic myelopoiesis. We show that loss of c-Myb function has no effect on macrophages but severely impairs neutrophil terminal differentiation, resulting in the accumulation of neutrophils with unsegmented nuclei and scant granule. This neutrophilic defect, which resembles the neutrophil-specific granule deficiency (SGD) caused by the mutations in CCAAT/enhancer-binding protein ε (*C/EBP*ε) in humans, is attributed, at least in part, to the downregulation of the granule protein transcription. Likewise, genetic inactivation of Cebp1, the zebrafish functional homolog of mammalian C/EBPε, also leads to a similar SGD-like phenotype in zebrafish. Genetic epistasis and biochemical analysis further reveals that c-Myb and Cebp1 act in parallel and cooperatively to control neutrophil differentiation by directly regulating granule protein gene transcription. Our study indicates that c-MYB is an intrinsic master regulator for neutrophil terminal differentiation and a potential target in SGD patients. (*Blood.* 2016;128(3):415-426)

Introduction

Neutrophils are the most abundant white blood cells in the human body and are essential for removing pathogens such as bacteria and fungi to prevent human infection. On the other hand, hyperactivation of neutrophils can also cause chronic inflammatory damage to tissues.[1-4] Because the numbers and activities of neutrophils are tightly controlled, dysregulation of neutrophil development and function can lead to the onset and progression of a variety of human disorders including neutropenia, neutrophil-specific granule deficiency (SGD), chronic granulomatous disease, and leukemia.[1-4]

The development of neutrophils has been best characterized in mice.[5] In adult mice, the earliest myeloid-restricted precursors derived from hematopoietic stem/progenitor cells are granulocyte-macrophage progenitors (GMPs). The multipotent granulocyte-macrophage progenitors subsequently differentiate into neutrophil-monocyte progenitors, which further commit to the earliest neutrophilic progenitors termed myeloblasts/promyelocytes. The myeloblasts then differentiate into myelocytes which finally undergo terminal differentiation to give rise to mature neutrophils. Genetic and biochemical studies in mice and cell lines have indicated that neutrophil development is mainly governed by cell type–specific transcription factors including the Ets transcription factor PU.1, Runt domain protein RUNX1, and the CCAAT/enhancer-binding protein α (C/EBPα); C/EBPβ and C/EBPε are the best-known transcription factors involved in the lineage commitment and subsequent differentiation and maturation of neutrophils.[1-4]

A hallmark of mature neutrophils is the presence of granules in their cytoplasm.[6,7] There are 3 types of granules: primary, secondary, and tertiary granules, which share common features in structure, but substantial differences in protein contents. The primary granules are the first granules to appear and they are peroxidase positive. As neutrophil precursors undergo further differentiation, the profile of granule proteins synthesized in the differentiating cells changes, which leads to the formation of peroxidase-negative secondary and tertiary granules. The secondary are identified by a high content of lactoferrin, whereas the tertiary are enriched in gelatinase. Although the biological significance of the formation of various granule subtypes remains

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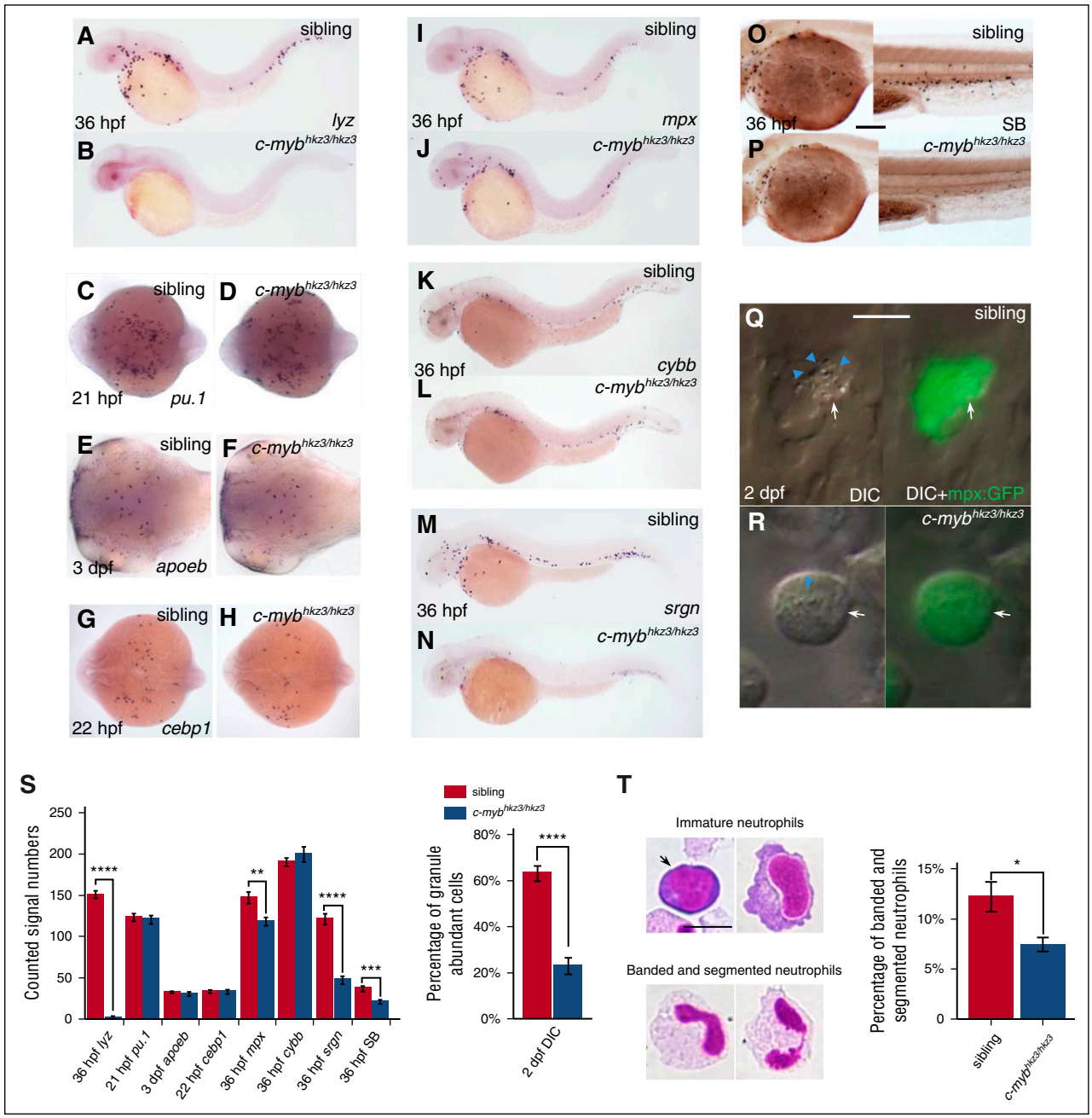


Figure 1. Neutrophil differentiation is severely impaired in *c-myb*$^{hkz3/hkz3}$ mutants. (A-B) Loss of *lyz* expression in *c-myb*$^{hkz3/hkz3}$ mutants. WISH of *lyz* in 36-hpf siblings (A) and *c-myb*$^{hkz3/hkz3}$ mutants (B). (C-H) Myeloid progenitor, macrophage lineage, and neutrophil progenitor markers were not affected in *c-myb*$^{hkz3/hkz3}$ mutants as evidenced by unaltered expression of *pu.1* (C-D), *apoeb* (E-F), and *cebp1* (G-H). (I-J) *mpx*$^{+}$ cells were slightly decreased in *c-myb*$^{hkz3/hkz3}$ mutants. WISH of *mpx* expression in 36-hpf siblings (I) and *c-myb*$^{hkz3/hkz3}$ mutants (J). (K-L) Unaltered *cybb* expression in *c-myb*$^{hkz3/hkz3}$ mutants. WISH of *cybb* expression in 36-hpf siblings (K) and *c-myb*$^{hkz3/hkz3}$ mutants (L). (M-N) Decreased *srgn* expression in *c-myb*$^{hkz3/hkz3}$ mutants. WISH of *srgn* expression in 36-hpf siblings (M) and *c-myb*$^{hkz3/hkz3}$ mutants (N). (O-P) Decreased SB^{+} cells in *c-myb*$^{hkz3/hkz3}$ mutants. SB staining in 36-hpf siblings (O) and *c-myb*$^{hkz3/hkz3}$ mutants (P). (Q-R) In vivo imaging of 2-dpf *Tg(mpx:GFP)* embryos by VE DIC microscopy. Left panel, Bright-field DIC image; right panel, an overlay of bright-field DIC and fluorescent image. Blue arrowheads identify granules contained by neutrophils (white arrows) expressing GFP. (S) Quantifications of WISH, SB staining, and DIC analysis for panels A to R (Student *t* test, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$; WISH and SB, n ≥ 20; DIC, n ≥ 60). (T) May-Grünwald-Giemsa staining of neutrophils in 3-dpf embryos (left) and neutrophils were quantitated by morphology (right) (Student *t* test, $^{*}P < .05$, n ≥ 500). Scale bars, 200 μm (O-P) and 5 μm (Q, R, T).

incompletely understood, they appear to display different accessibility for exocytosis[8] and inflammatory responses.[9-11] The heterogeneity of neutrophil granules can be partially explained by targeting-by-timing hypothesis, meaning that targeting of protein into a certain subset of granules is determined by the time window of their synthesis.[12,13] Yet, the molecular basis controlling granule protein expression remains poorly defined. To date, C/EBPε is the best characterized transcription factor known to be involved in the granule protein expression. Targeted deletion of *C/EBPε* in mice results in the production of defective neutrophils with immature nuclei and absent or low levels of several secondary granule protein.[14,15] Likewise, mutations of *C/EBPε* in humans cause SGD, in which neutrophils display bilobed nuclei and lack secondary and tertiary granules.[16] However, some SGD patients contain intact *C/EBPε* alleles and express normal levels of C/EBPε

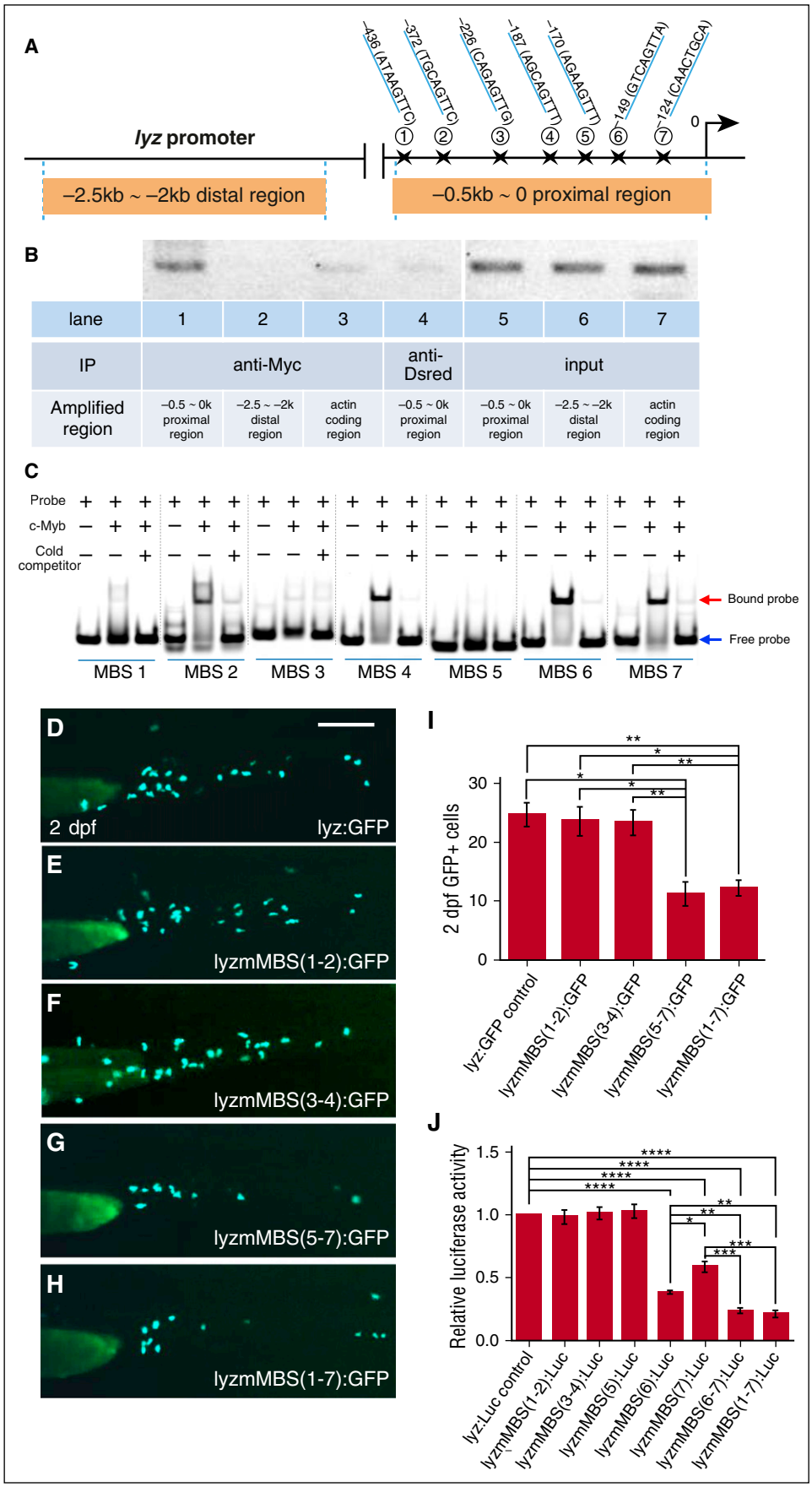


Figure 2. *lyz* is a direct downstream target of c-Myb. (A) Schematic diagram of the 2.5-kb *lyz* promoter region. The transcription initiation site is designated as 0. Seven putative c-Myb consensus sites are identified within −0.5~0 kb proximal region using promo 3.0 online software and their positions (marked by stars) relative to the transcription initiation site are shown. (B) ChIP shows that c-Myb binds to the −0.5-kb proximal promoter region of the *lyz* promoter. Lysates from the embryos injected with the Myc-tagged *c-myb* mRNA were precipitated with anti-Myc (lanes 1-3) and anti-Dsred (lane 4, negative control) antibodies. The precipitates were then subjected to semiquantitative PCR analysis of the enrichment of the −0.5~0-kb proximal region (lanes 1 and 4), the −2.5- ~ −2-kb distal region (lane 2), and the actin-coding region (lane 3). Lanes 5-7 are input DNA control. (C) Gel shift image shows that 7 FAM-labeled probes containing putative MBSs were incubated with purified His-c-Myb N-terminal (amino acids 33-202) of the zebrafish c-Myb protein, encompassing the highly conserved DNA-binding domain. Excess unlabeled probes compete with the FAM-labeled probes. Equal amounts of proteins were used, specifically bound to the FAM-labeled probes. (D-I) Transient GFP reporter assay. Embryos were injected at the 1-cell stage with pTol-*lyzProm*oter-GFP reporter constructs driven by an intact 2.4-kb *lyz* promoter: pTol-*lyz*:GFP (D) or *lyz* promoter with subsets of the mutated c-MBSs (mMBS): pTol-*lyz*mMBS(1-2):GFP (E), pTol-*lyz*mMBS(3-4):GFP (F), pTol-*lyz*mMBS(5-7):GFP (G), and pTol-*lyz*mMBS(1-7):GFP (H). The promoter activity was then measured by calculating the numbers of GFP$^+$ cells at 2 dpf. Representative images (D-H) and GFP$^+$ quantifications (I) of 2-dpf injected embryos (ANOVA, Dunnett T3, n ≥ 50). Scale bars represent 200 μm. (J) Luciferase reporter assay. Bars showed the relative luciferase activity of *lyz* promoter with different subsets of MBS mutation compared with unmutated control. The statistical significance was calculated using 1-way ANOVA followed by Dunnett T3 correction. An asterisk indicates a statistical difference (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, triplicated).

proteins,[17] indicating that SGD in these patients is caused by other unknown genetic lesions.

c-MYB is the cellular homolog of v-MYB oncogene involved in acute lymphocytic leukemia and myeloid leukemia.[18] The physiological role of c-MYB is unveiled by scrutinizing a cohort of *c-MYB* mutant alleles. Phenotype analysis of *c-Myb*–null mice indicates that c-MYB is essential for definitive hematopoiesis.[19] Lineage-specific roles for c-MYB are uncovered by several *c-MYB* conditional and hypomorphic alleles which suggest that c-MYB positively regulates erythroid and lymphoid development but negatively regulates megakaryocyte and adult hematopoietic stem cells (HSCs).[20-25] Interestingly, except for lack of eosinophils in *c-MYB*^{M303V} mice,[24] myeloid lineage defects appear to be subtle in these viable *c-MYB* mutant alleles, perhaps due to inadequate suppression of c-MYB activity and lack of in-depth analysis in these alleles.

Integrating the advantages for both embryological and genetic analysis, zebrafish have emerged as a prominent model for study of vertebrate development and human disorders.[26] In particular, the zebrafish embryonic myelopoiesis arises directly from the rostral blood island (RBI) and produces macrophages and neutrophils,[27-32] allowing for direct assessment of the roles of transcription factors in myeloid development in the absence of their potentially confounding epistatic requirement in the earlier hematopoietic progenitors. Here, we describe a regulatory network showing that during zebrafish embryonic myelopoiesis the neutrophil terminal differentiation is differentially controlled by c-Myb and Cebp1. We further demonstrate that c-Myb and Cebp1 act in parallel and cooperatively to govern neutrophil terminal maturation by impinging on the transcriptional expression of granule proteins.

Methods

Zebrafish husbandry

Zebrafish were raised, bred, and staged according to standard protocols.[33] The following strains were used: AB, *Tg(mpx:GFP)i114*,[34] *Tg(coro1a:EGFP)hkz04t*,[35] *Tg(lyz:Dsred2)nz50tg*,[36] *c-myb*^{hkz3} mutant,[37] and *cebp1*^{smu1} mutant.

Histology

Single-color whole-mount in situ hybridization (WISH) was performed based on standard protocol.[33] Conventional Sudan Black (SB) staining and combined detection of SB staining and protein targets were carried out according to a previous report.[31]

Zebrafish embryo dissociation, FACS, and May-Grünwald-Giemsa staining

Embryo dissociation and fluorescence-activated cell sorter (FACS) were performed as described previously.[38-40] Because *Tg(coro1a:GFP)* specifically labels the myeloid population in zebrafish,[35] *Tg(coro1a:GFP)*$^{+/+}$;*c-myb*$^{hkz3/+}$ were outcrossed with *Tg(lyz:Dsred)*$^{+/+}$;*c-myb*$^{hkz3/+}$ and the resulting *c-myb*$^{hkz3/hkz3}$ mutants and siblings were separated based on green fluorescent protein (GFP) and Dsred: GFP$^+$ single-positive embryos were *c-myb*$^{hkz3/hkz3}$ mutants, whereas GFP$^+$Dsred$^+$ double-positive embryos represented siblings. The *cebp1*$^{smu1/smu1}$ embryos were collected by intercrossing *Tg(coro1a:GFP)*$^{+/+}$; *cebp1*$^{smu1/smu1}$, whereas siblings were collected by intercrossing the same clutch of *Tg(coro1a:GFP)*$^{+/+}$. The GFP$^+$ cells of each group were collected from totally ~5000 embryos using MoFlo XDP (Beckmann) (~1000 embryos once, performed 5 times). Neutrophils were distinguished from macrophages by morphology by May-Grünwald-Giemsa staining with cytospin-collected GFP$^+$ cells on slides.[41]

Transient GFP reporter assay

pTol-lyz:GFP or pTol-lyz:GFP with mutated c-Myb or/and Cebp1 recognition sites were injected into 1-cell stage AB embryos at the dose of 100 pg per embryo. Activity of the GFP reporter was scored by counting the number of GFP$^+$ cells at 2 days postfertilization (dpf).

Luciferase reporter assays

lyz luciferase reporter (150 ng), *c-myb* or/and *cebp1* expression (750 ng), and *Renilla* constructs (5 ng) were cotransfected into 293T cells (per well in 12-well plate) and luciferase activity was measured according to the Dual Luciferase Assay kit (Promega) instructions.

Chromatin immunoprecipitation

Capped 6×Myc-c-myb messenger RNA (mRNA) was injected into 1-cell stage embryos (100 pg per embryo). Six hundred injected embryos were harvested at 8 hours postfertilization (hpf) for brief fixation. Crosslinked chromatin was immunoprecipitated with anti-Myc antibody or anti-Dsred antibody (negative control) according to the procedure described by Hart et al.[42] The immunoprecipitates were subjected to semiquantitative polymerase chain reaction (PCR).

Electrophoretic mobility shift assay

For gel-shift assay, 200 ng of poly(dI-dC), 2 pmol FAM-labeled probes and 3 μg of purified protein were mixed in binding buffer[43] in a total 20-μL reaction and incubated at 4°C for 2 hours. The mixture was then loaded onto a 6% polyacrylamide gel (0.5× Tris/Borate/EDTA) for electrophoresis (160 V at 4°C). For the competition studies, 250-fold molar excess of unlabeled competitor oligonucleotides was added to the binding reactions. Gels were screened by Typhoon Trio scanner (GE Healthcare).

Microscopy and imaging

Video-enhanced (VE) differential interference contrast (DIC) microscopy was done with a 60×/1.00 numerical aperture water-immersion objective mounted on an Olympus BX51 microscope as previously described.[27] VE-DIC images were captured with a DP71 Olympus color camera and recorded. Whole-mount or magnified bright-field images were taken using a Zeiss AxioCam HRc camera mounted on a Zeiss Discovery V20 microscope. Fluorescent images were captured with Olympus FV1000 confocal microscopy.

Statistical analysis

Data were analyzed by SPSS software (version 20) using the Student *t* test for comparisons between 2 groups and 1-way analysis of variance (ANOVA; with Bonferonni or Dunnett T3 posttest adjustment) among multiple groups. Significance was accepted when $P < .05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Suppression of c-Myb function in zebrafish blocks neutrophil maturation

To identify new molecular determinants involved in neutrophil development, we turned our attention to *c-myb*$^{hkz3/hkz3}$zebrafish mutants, which were originally isolated as hematopoiesis-defective mutants lacking lysozyme c (*lyz*), a key component of granule proteins predominantly expressed in neutrophils.[37,44-46] This mutant harbors a splicing mutation that results in the synthesis of a truncated c-Myb protein which lacks the transactivation domain but retains the DNA-binding domain.[37] Our previous study has shown that, similar to the *c-MYB* knockout mice,[19] the *c-myb*$^{hkz3/hkz3}$ mutants fail to develop fetal and adult hematopoiesis due to the impairment of HSCs.[37] However,

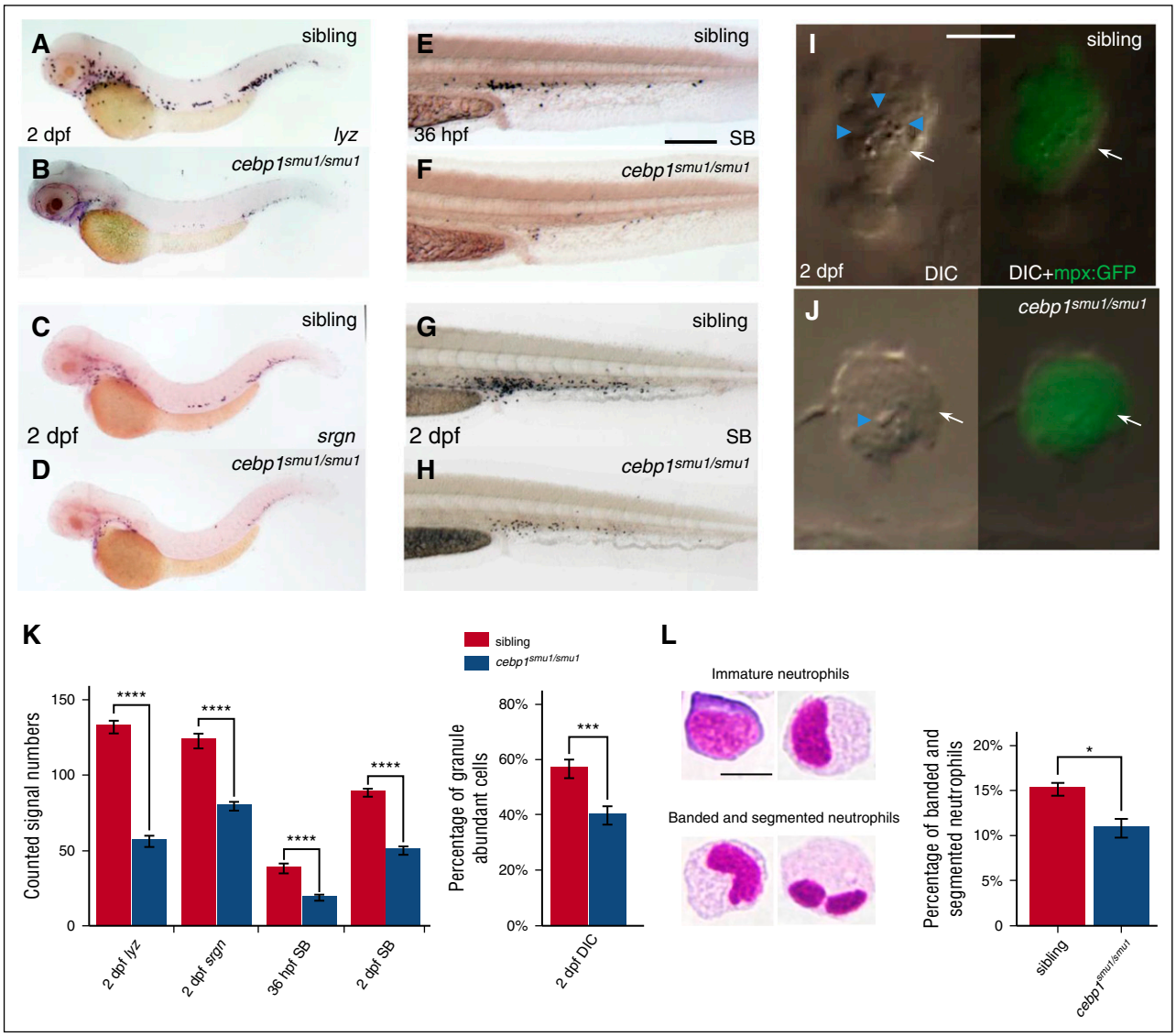


Figure 3. Neutrophil maturation is attenuated in *cebp1*$^{smu1/smu1}$ mutants. (A-B) WISH of *lyz* expression in 2-dpf siblings and *cebp1*$^{smu1/smu1}$ mutants. (C-D) WISH of *srgn* expression in 2-dpf siblings and *cebp1*$^{smu1/smu1}$ mutants. (E-H) SB staining of 36-hpf (E-F) and 2-dpf (G-H) siblings and *cebp1*$^{smu1/smu1}$ mutants. (I-J) In vivo VE DIC microscopy reveals scant granule in neutrophils in 2 dpf *Tg(mpx:GFP);cebp1*$^{smu1/smu1}$ transgenic mutants (J) compared with siblings (I). Left panels, Bright-field DIC images; right panels, overlays of bright-field DIC images with corresponding GFP fluorescent images. Blue arrowheads identify granules contained by neutrophils (white arrows) expressing GFP. (K) Quantifications of WISH, SB staining, and DIC analysis (Student *t* test, ***$P < .001$, ****$P < .0001$; WISH and SB, n ≥ 23; DIC, n ≥ 56). (L) May-Grünwald-Giemsa staining of neutrophils in 3-dpf embryos (left) and neutrophils were quantitated by morphology (right) (Student *t* test, *$P < .05$, n ≥ 500). Scale bars, 200 μm (E-H) and 5 μm (I, J, L).

the absence of *lyz* expression at early developmental stages in *c-myb*$^{hkz3/hkz3}$ mutants (Figure 1A-B) suggested that inactivation of c-Myb might cause a severe defect in embryonic myelopoiesis because the majority of myeloid cells that emerged before 3 dpf are derived from embryonic myelopoietic tissue, the RBI.[32] We therefore examined embryonic myelopoiesis in *c-myb*$^{hkz3/hkz3}$ mutants with myeloid progenitor and macrophage linage markers and found that the formation of myeloid progenitors and their progression into the macrophage branch were not affected by the *c-myb*$^{hkz3/hkz3}$ mutation as evidenced by normal *pu.1* expression in myeloid progenitors at 21 hpf (Figure 1C-D) and *apoeb* expression in microglia, the brain-resident macrophages at 3 dpf (Figure 1E-F). These observations suggest that embryonic neutrophils, but not macrophages, are severely impaired in *c-myb*$^{hkz3/hkz3}$ mutants.

Previous study has defined embryonic neutrophil development into 3 successive stages: the *cebp1*$^{+}$ early neutrophilic progenitors, the *mpx*$^{+}$/*lyz*$^{+}$ intermediate neutrophilic progenitors, and the SB^{+} granule containing maturing neutrophils.[32] To delineate at which stage neutrophil development is disrupted in *c-myb*$^{hkz3/hkz3}$ mutants, we examined the expression of these different neutrophil markers and the staining of SB. Results showed that the *cebp1*$^{+}$ early progenitors were indistinguishable between *c-myb*$^{hkz3/hkz3}$ mutants and siblings (Figure 1G-H). Likewise, the *mpx*$^{+}$ intermediate progenitors were formed properly in *c-myb*$^{hkz3/hkz3}$ mutants, despite a slight decrease in number (Figure 1I-J). Meanwhile, the expression of *cybb*, which encodes the superoxide-generating enzyme in the neutrophil,[47] is intact in *c-myb*$^{hkz3/hkz3}$ mutants (Figure 1K-L). These observations indicate that in *c-myb*$^{hkz3/hkz3}$ mutants the initiation of the neutrophilic program is intact and the majority of the early neutrophilic progenitors are capable of differentiating into the intermediate neutrophilic progenitors. These results also suggest that the absence of *lyz* expression in *c-myb*$^{hkz3/hkz3}$

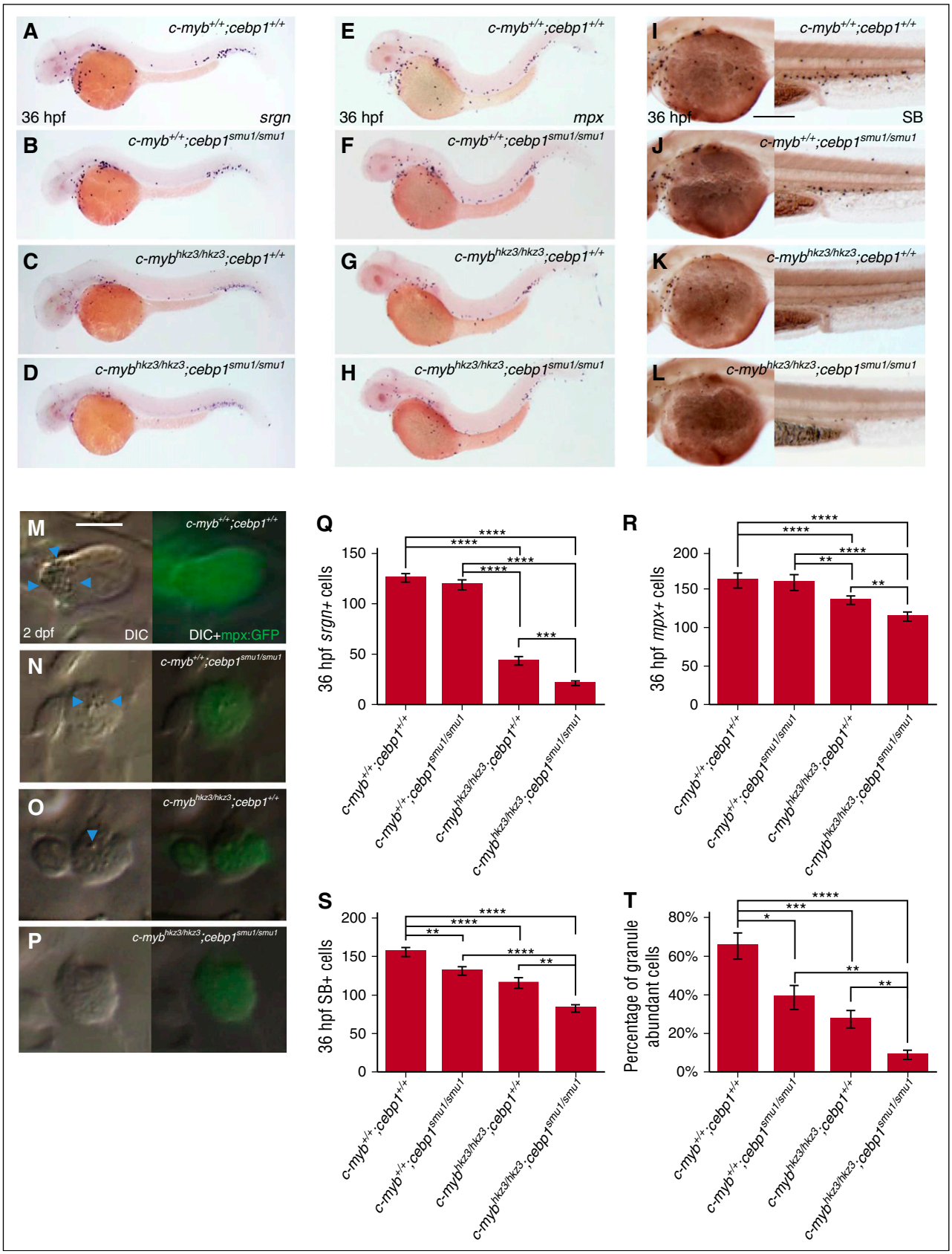


Figure 4. *c-myb* and *cebp1* genetically interact in regulating neutrophil maturation. (A-D) WISH shows further decrease of *srgn* expression in 36-hpf *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{smu1/smu1}$ double mutants (D) compared with *c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$ (B) or *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{+/+}$ (C) single mutants. (E-H) WISH shows further decrease of *mpx* expression in 36-hpf *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{smu1/smu1}$ double mutants (H) compared with *c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$ (F) or *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{+/+}$ (G) single mutants. (I-L) SB staining shows further decrease of SB^{+} cells in 36 hpf *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{smu1/smu1}$ double mutants (L) compared with *c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$ (J) or *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{+/+}$

mutants is largely attributed to the downregulation of gene transcription, implying that c-Myb may govern neutrophil maturation by regulating the transcription of granule protein genes. Indeed, we found that the expression of serglycin (*srgn*), another granule proteoglycan protein highly enriched in neutrophils (supplemental Figure 1A-C, available on the *Blood* Web site),[48] was also significantly downregulated in *c-myb*$^{hkz3/hkz3}$ mutants (Figure 1M-N). The drastic decrease of *srgn* and *lyz* expression indicates that the granule formation in the *c-myb*$^{hkz3/hkz3}$ mutant neutrophils is compromised. This notion was supported by SB-staining analysis, which showed that the SB^{+} neutrophils in 36 hpf *c-myb*$^{hkz3/hkz3}$ embryos were reduced to 50% of sibling counts (Figure 1O-P), and more importantly, the signal intensity per cell for SB staining of the remaining neutrophils in the mutants was profoundly pale compared with those in siblings, suggesting that the *c-myb*$^{hkz3/hkz3}$ neutrophils might have less abundant properly assembled granules. Indeed, VE DIC analysis of live embryos showed that the majority of the neutrophils identified in 2-dpf *c-myb*$^{hkz3/hkz3}$ mutants possessed granules that were scant in their abundance and motility as opposed to the ample motile granule in siblings of the same stage (Figure 1Q-S). Furthermore, May-Grünwald-Giemsa staining showed a significant decrease of mature neutrophils with banded and segmented nuclei[49,50] in 3-dpf *c-myb*$^{hkz3/hkz3}$ mutants compared with that in siblings (Figure 1T). To further confirm that the neutrophil defects in *c-myb*$^{hkz3/hkz3}$ mutants is caused by the loss of c-Myb function, we knocked down *c-myb* expression by morpholino[43] and showed that the *c-myb* morpholino knockdown embryos displayed neutrophil defects similar to *c-myb*$^{hkz3/hkz3}$ mutants (supplemental Figure 2A-H). Moreover, ectopically overexpression of c-Myb by the heat-shock–inducible promoter could restore the *lyz* expression and SB^{+} cell numbers in *c-myb*$^{hkz3/hkz3}$ mutants (supplemental Figure 2I-R). From these results, we conclude that loss of c-Myb function blocks neutrophil differentiation, resulting in the accumulation of immature neutrophils with less segmented nuclei and scant granules, a defect similar to the SGD in mammals.[14-16]

c-Myb directly regulates the transcription of the granule-related gene *lyz*

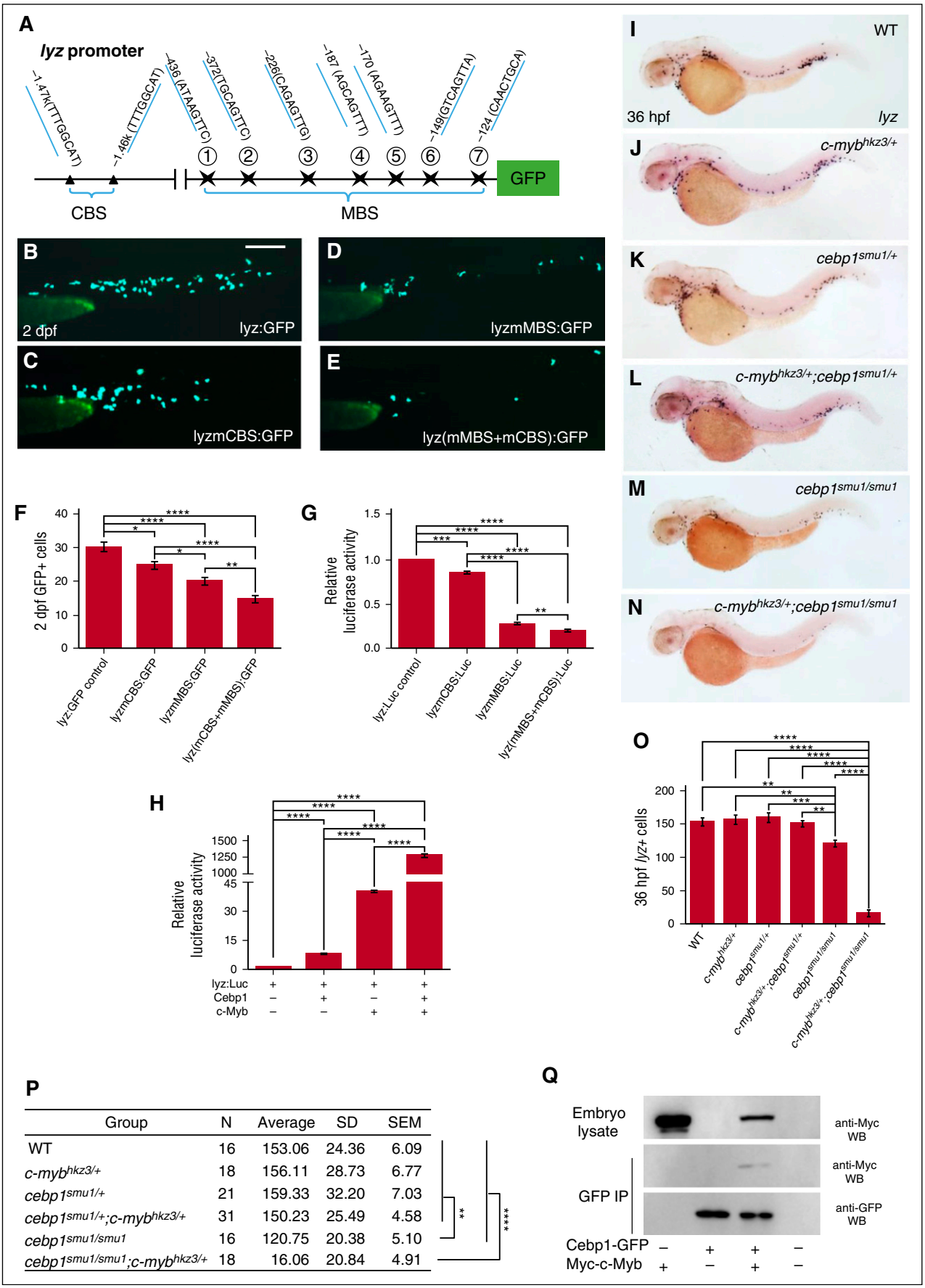
The blockage of neutrophil differentiation and the downregulation of several granule proteins in *c-myb*$^{hkz3/hkz3}$ mutants (Figure 1) suggest that c-Myb function is required for the transcription of certain granule proteins. We therefore focused on *lyz*, the most widely expressed granule proteins found in all subtypes of neutrophil granules.[44-46] To ascertain whether c-Myb directly regulates *lyz* expression, we first carried out bioinformatics analysis and identified 7 putative c-Myb–binding sites (MBSs) within the proximal promoter region (−0.5∼0 kb) of the *lyz* promoter (Figure 2A),[51] raising the possibility that c-Myb might regulate *lyz* transcription by directly binding to the −0.5∼0 kb proximal promoter region. To support this notion, we performed chromatin immunoprecipitation (ChIP) assays using embryos injected with *Myc*-tagged *c-myb* mRNA to test whether recombinant Myc-tagged c-Myb proteins could bind the proximal promoter region of the *lyz* promoter. As shown in Figure 2B, pulling down recombinant c-Myb protein with anti-Myc antibody coprecipitated the −0.5∼0 kb proximal region of the *lyz* promoter, but not the −2.5 kb∼-2 kb distal region of the *lyz* promoter or 0∼0.5 kb within the coding region of actin gene, indicating that the −0.5∼0 kb proximal promoter region of the *lyz* promoter is occupied by c-Myb. To further determine which MBS is critical for c-Myb binding, we performed electrophoretic mobility shift assay (EMSA) and results showed that MBS-6 had the strongest binding ability with c-Myb, whereas MBS-4 and -7 exhibited moderate binding ability and the other MBSs (1, 2, 3, and 5) displayed weak or no binding ability (Figure 2C). In concordance with the electrophoretic mobility shift assay (EMSA) results, transient GFP reporter assay in zebrafish embryos and luciferase reporter assay in 293T cells showed that disruption of MBS-6 and MBS-7 dramatically reduced the *lyz* promoter activity as well (Figure 2D-J). Taken together, these data demonstrate that c-Myb regulates the *lyz* transcription by directly binding to its promoter region, especially MBS-6 and -7.

c-myb and *cebp1* genetically interact in regulating neutrophil maturation

Previous studies have shown that, similar to the role of C/EBPε in neutrophil development in mammals,[14-16,52,53] transient knockdown of *cebp1* expression by morpholino in zebrafish leads to the reduction of granule protein expression including *lyz*, without affecting the formation of macrophages and neutrophils.[51,54,55] These findings prompted us to speculate that, in zebrafish, Cebp1 and c-Myb may form a genetic network to regulate neutrophil development.

To unravel the genetic relationship of these 2 factors during zebrafish neutrophil development, we generated 2 frameshift *cebp1* mutant fish by transcription activator-like effector nucleases (TALENs; *cebp1*^{smu1} and *cebp1*^{smu2}) (supplemental Figure 3A-B) and asked whether inactivating Cebp1 would lead to the impairment of neutrophil development. We found that, although myeloid progenitors and macrophages remained unaffected (supplemental Figure 3C-H), the *lyz*$^{+}$ and *srgn*$^{+}$ neutrophils were clearly reduced in 2-dpf *cebp1*$^{smu1/smu1}$ mutants (Figure 3A-D). The reduction of *lyz* and *srgn* staining in 2-dpf *cebp1*$^{smu1/smu1}$ mutants was likely due to the decrease of *lyz* and *srgn* expression because the *mpx*$^{+}$ neutrophils in 2-dpf *cebp1*$^{smu1/smu1}$ mutants were comparable to those in control siblings (supplemental Figure 3I-J). As expected, the development of neutrophils in *cebp1*$^{smu1/smu1}$ mutants was compromised as indicated by the accumulation of neutrophils with less abundant granules and unsegmented nuclei in *cebp1*$^{smu1/smu1}$ mutants (Figure 3E-L). Likewise, the neutrophil development in adult *cebp1*$^{smu1/smu1}$ mutants as indicated by the decreased expression of neutrophil markers in the kidney marrow cells was also impaired, whereas macrophages developed normally (supplemental Figure 3K). The other allele of *cebp1* mutant, *cebp1*^{smu2}, exhibited similar *lyz*$^{+}$ reduction phenotype as *cebp1*$^{smu1/smu1}$ (supplemental Figure 3L-M). These data reveal that, similar to the function of mammalian C/EBPε,[56-58] Cebp1 plays a critical role in neutrophil differentiation in zebrafish.

Figure 4 (continued) (K) single mutants. (M-P) In vivo VE DIC microscopy reveals further reduction of granules in neutrophils in 2-dpf *Tg(mpx:GFP);c-myb*$^{hkz3/hkz3}$*;cebp1*$^{smu1/smu1}$ double mutants (P) compared with *Tg(mpx:GFP);c-myb*$^{+/+}$*;cebp1*$^{smu1/smu1}$ (N) or *Tg(mpx:GFP);c-myb*$^{hkz3/hkz3}$*;cebp1*$^{+/+}$ (O) single mutants. Left panels, Bright-field DIC images. Right panels, Overlays of bright-field DIC images with corresponding GFP fluorescent images. Blue arrowheads indicate granules contained by neutrophils. (Q) Quantifications of *srgn*$^{+}$ cells in 36 hpf *c-myb*$^{hkz3/hkz3}$*;cebp1*$^{smu1/smu1}$ double and single mutants. No statistical difference is found between *c-myb*$^{+/+}$*;cebp1*$^{+/+}$ and *c-myb*$^{+/+}$*;cebp1*$^{smu1/smu1}$. An asterisk indicates a statistical difference (ANOVA, Dunnett T3, n ≥ 16). (R) Quantifications of *mpx*$^{+}$ cells in 36-hpf WT, *c-myb*$^{hkz3/hkz3}$*;cebp1*$^{smu1/smu1}$double and single mutants. No statistical difference is found between *c-myb*$^{+/+}$*;cebp1*$^{+/+}$ and *c-myb*$^{+/+}$*;cebp1*$^{smu1/smu1}$. An asterisk indicates a statistical difference (ANOVA, Dunnett T3, n ≥ 20). (S) Quantifications of SB^{+} cells in 36-hpf WT, *c-myb*$^{hkz3/hkz3}$*;cebp1*$^{smu1/smu1}$ double and single mutants. An asterisk indicates a statistical difference (ANOVA, Bonferroni, n ≥ 18). (T) Percentage of neutrophils with abundant granules in 2-dpf WT, *c-myb*$^{hkz3/hkz3}$*;cebp1*$^{smu1/smu1}$ double and single mutants (ANOVA, Dunnett T3, n ≥ 16). An asterisk indicates a statistical difference (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$). Scale bars, 200 μm (I-L) and 5 μm (M-P).



P

Group	N	Average	SD	SEM
WT	16	153.06	24.36	6.09
c-myb$^{hkz3/+}$	18	156.11	28.73	6.77
cebp1$^{smu1/+}$	21	159.33	32.20	7.03
cebp1$^{smu1/+}$;*c-myb*$^{hkz3/+}$	31	150.23	25.49	4.58
cebp1$^{smu1/smu1}$	16	120.75	20.38	5.10
cebp1$^{smu1/smu1}$;*c-myb*$^{hkz3/+}$	18	16.06	20.84	4.91

Figure 5.

To delineate the genetic relationship of c-Myb and Cebp1 during neutrophil differentiation, we first investigated whether there was a mutual dependency of the expression of *c-myb* and *cebp1* at around 22 hpf when embryonic neutrophils begun to undergo differentiation. Results showed that *c-myb* expression was unchanged in *cebp1*$^{smu1/smu1}$ mutant embryos (supplemental Figure 3N-P), and likewise, *cebp1* expression was also unaltered in *c-myb*$^{hkz3/hkz3}$ embryos (Figure 1G-H). The independence of *c-myb* and *cebp1* transcriptional regulation suggests that they may act in parallel and perhaps cooperatively to regulate neutrophil differentiation. To test this possibility, we generated *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{smu1/smu1}$ double mutants and asked whether inactivating both c-Myb and Cebp1 spontaneously would lead to a more severe neutrophil phenotype. Indeed, *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{smu1/smu1}$ double mutants displayed a much greater reduction of *srgn*, *mpx*, and SB staining compared with *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{+/+}$ or *c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$ single mutants (Figure 4A-D,E-H,I-L,Q-S). Moreover, the percentage of immature neutrophils in *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{smu1/smu1}$ double mutants was significantly increased compared with *c-myb*$^{hkz3/hkz3}$;*cebp1*$^{+/+}$ or *c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$ single mutants (Figure 4M-P,T). Collectively, these genetic data suggest that c-Myb and Cebp1 act in parallel and cooperatively to regulate neutrophil terminal maturation.

c-Myb and Cebp1 synergistically regulate *lyz* transcription activation

The genetic interaction of c-Myb and Cebp1 led us to hypothesize that the 2 factors govern neutrophil differentiation by coregulating the expression of downstream target genes, especially the genes associated with granule formation. To support this idea, we again focused on the transcriptional regulation of *lyz*, a key granule protein known to be regulated by both c-Myb (Figure 2) and Cebp1.[51] Previous study has shown that a 2.4-kb DNA fragment upstream of the *lyz* transcription initiation site is sufficient to direct GFP reporter expression in a manner similar to that of the endogenous *lyz*,[51] indicating that this 2.4-kb DNA fragment likely contains the critical *cis* elements for the regulation of *lyz* expression. Indeed, a cluster of putative MBSs and another cluster of Cebp1-binding sites (CBSs) were found within this 2.4-kb fragment (Figure 5A) and these clusters have been shown to be recognized by c-Myb (Figure 2) and Cebp1,[51] respectively. To examine the cooperative effect of c-Myb and Cebp1 on *lyz* transcription, we injected wild-type (WT) 2.4-kb *lyz* promoter reporter construct and the mutated 2.4-kb *lyz* promoter reporter construct into zebrafish embryos and assayed the promoter activity. Our results showed that the disruption of either MBS or CBS alone reduced the activity of *lyz* promoter as indicated by the decrease of GFP expression in the myeloid cells in the embryos injected with respective construct (Figure 5B-D,F). Consistent with the genetic analysis, when MBS and CBS were mutated simultaneously, GFP expression was further reduced (Figure 5E-F), indicating that the binding of c-Myb and Cebp1 to the *lyz* promoter contributes to the activation of *lyz* transcription. A similar conclusion was obtained with *lyz* promoter luciferase reporter assay (Figure 5G). These data prompted us to speculate that c-Myb and Cebp1 might regulate *lyz* transcription synergistically. To support this notion, we compared the *lyz* promoter activity in 293T cells overexpressing c-Myb and Cebp1 individually or simultaneously. As anticipated, results showed that, when c-Myb and Cebp1 were cotransfected, the luciferase activity was much higher than the combined values with c-Myb or Cebp1 alone (Figure 5H), indicating that the 2 transcription factors act synergistically to regulate *lyz* transcription.

To further support the results obtained from *lyz* promoter reporter assay, we generated a combination of double mutants expressing different levels of c-Myb and Cebp1 and subsequently profiled *lyz* expression at 36 hpf. *c-myb*$^{+/+}$;*cebp1*$^{+/+}$, *c-myb*$^{hkz3/+}$;*cebp1*$^{+/+}$, *c-myb*$^{+/+}$;*cebp1*$^{smu1/+}$, *c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/+}$, *c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$, and *c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/smu1}$ embryos expressed different levels of c-Myb and Cebp1 and profiled *lyz* expression at 36 hpf. As shown in Figure 5I-P, single-heterozygous (*c-myb*$^{hkz3/+}$;*cebp1*$^{+/+}$ and *c-myb*$^{+/+}$;*cebp1*$^{smu1/+}$) and double-heterozygous (*c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/+}$) mutant embryos had comparable *lyz* staining with the WT (*c-myb*$^{+/+}$;*cebp1*$^{+/+}$) embryos (Figure 5I-L,O-P), whereas *cebp1* single-homozygous (*c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$) displayed a slight reduction in *lyz* staining (reduced by ~20%) (Figure 5M,O-P) (*c-myb* single-homozygous *c-myb*$^{hkz3/hkz3}$ contained a very low level of *lyz* expression and was hardly detectable by WISH; Figure 1B). However, as we expected, a dramatic decrease of *lyz* expression (reduced by ~90%) was seen in the *cebp1*-homozygous plus *c-myb*-heterozygous (*c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/smu1}$) double-mutant embryos compared with the *cebp1* single-homozygous (*c-myb*$^{+/+}$;*cebp1*$^{smu1/smu1}$) mutant embryos (Figure 5M-P), showing that the suppression of *lyz* expression in *cebp1*$^{smu1/smu1}$ was further exacerbated by *c-myb*$^{hkz3/+}$ heterozygosity.

The cooperative effect of c-Myb and Cebp1 on the *lyz* promoter suggests that the 2 factors might be part of the activation complexes critical for *lyz* transcription. To test this hypothesis, we performed a coimmunoprecipitation experiment in transgenic zebrafish embryos with heat-shock–induced Cebp1-GFP and Myc-c-Myb. Immunoprecipitating GFP-tagged Cebp1 with an anti-GFP antibody indeed resulted in coprecipitation of the Myc-tagged c-Myb (Figure 5Q), suggesting that Cebp1 and c-Myb are physically interacted, directly or indirectly, to regulate *lyz* transcription in a cooperative manner. Taken together, the genetic epistasis and biochemical analysis conclude that c-Myb and Cebp1 act in parallel and cooperatively to regulate

Figure 5. c-Myb and Cebp1 synergistically regulate *lyz* transcription. (A) Schematic diagram of *lyz* promoter region. The transcription initiation site is designated as 0. Seven putative c-Myb (marked by star) and 2 Cebp1 (marked by triangle) consensus sites are identified using promo 3.0 online software and their positions relative to the transcription initiation site are shown. (B-E) Transient reporter assay demonstrates that c-Myb and Cebp1 synergistically regulate *lyz* transcription. Embryos were injected at 1-cell stage with pTol-GFP reporter constructs driven by an intact 2.4-kb *lyz* promoter (lyz:GFP), c-Myb sites mutated *lyz* promoter pTol-lyz(mMBS):GFP, Cebp1 sites mutated *lyz* promoter pTol-lyz(mCBS):GFP or both sites mutated pTol-lyz(mMBS+mCBS):GFP, and *lyz* promoter activity were measured at 2 dpf by scoring the number of GFP$^+$ cells. Scale bars represent 200 μm. (F) Quantification of GFP$^+$ cells in 2 dpf pTol-lyz:GFP, pTol-lyz(mCBS):GFP, pTol-lyz(mMBS):GFP, and pTol-lyz(mCBS+mMBS):GFP-injected embryos (ANOVA, Dunnett T3, n ≥ 160). (G-H) Luciferase reporter assay. Bars showed the relative luciferase activity of transfection with *lyz*(mMBS):Luc, *lyz*CBS (mCBS):Luc or both sites mutated *lyz*(mMBS+mCBS):Luc compared with unmutated *lyz*:Luc control (G). Synergistic effect on transactivation of *lyz* promoter by c-Myb and Cebp1 (H). Bars showed the relative luciferase activity with overexpression of *cebp1*, *c-myb*, or both genes compared with control (H). The statistical significance was calculated using 1-way ANOVA followed by Dunnett T3 correction. The asterisk indicates a statistical difference (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, triplicated). (I-N) WISH shows that *lyz* expression is further decreased in 36-hpf *c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/smu1}$ double (*cebp1* homozygous and *cmyb* heterozygous) mutants (N) compared with *cebp1*$^{smu1/smu1}$ single mutants (M). (O-P) Quantifications of *lyz*$^+$ cells in 36-hpf WT (*c-myb*$^{+/+}$;*cebp1*$^{+/+}$), single-heterozygous (*c-myb*$^{hkz3/+}$ and *cebp1*$^{smu1/+}$), double-heterozygous (*c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/+}$) mutant embryos, *cebp1*$^{smu1/smu1}$ single mutants and *c-myb*$^{hkz3/+}$;*cebp1*$^{smu1/smu1}$ double mutants shown in the statistical figure (O) and table (P). The statistical significance was calculated using 1-way ANOVA followed by Bonferroni correction. The asterisk indicates a statistical difference (**$P < .01$, ***$P < .001$, ****$P < .0001$, n ≥ 16). (Q) Coimmunoprecipitation experiment shows a physical interaction between c-Myb and Cebp1. Embryos overexpressed with *Myc*-tagged *c-myb*, and *GFP*-tagged *cebp1* by heat-shock induction were immunoprecipitated with anti-GFP antibody. The immunoprecipitates were subjected to western blotting with anti-Myc and anti-GFP antibody.

neutrophil maturation by synergistically regulating granule protein gene transcription.

Discussion

In this study, we uncover transcription factor c-Myb as a new molecular determinant involved in neutrophil terminal differentiation during zebrafish embryonic myelopoiesis. Since discovered as the cellular counterpart of v-MYB, an oncogene presented in 2 avian retroviruses associated with myeloid leukemia,[18] c-MYB was thought to have a critical role in myeloid lineage development. Despite a number of genes found to be regulated by c-MYB and overexpression of c-MYB blocking monocyte/macrophage differentiation in cultural cell lines,[59,60] in vivo knockout studies in mice have revealed that c-MYB is mainly involved in the development of HSCs and the formation of eosinophils.[24,61] Whether c-MYB has a role in the regulation of the development of 2 main myeloid lineages, macrophages, and neutrophils, remains uncertain. Our present study provides evidence demonstrating that c-Myb is essential for terminal differentiation, especially granule formation, of neutrophilic granulocytes during zebrafish embryonic myelopoiesis. Intriguingly, inactivation of c-Myb appears to have little impact on the development of myeloid progenitors and macrophages, despite its enriched expression in these cells. Why c-Myb is required for neutrophil differentiation but is dispensable for myeloid progenitor and macrophage lineage development remains completely unknown; further in-depth analysis will be required to clarify this issue. Another interesting question, which cannot be addressed in current study because of the block of adult hematopoiesis at the HSC level in *c-myb*$^{hkz3/hkz3}$ mutants,[37] is whether c-Myb has a similar role during adult myelopoiesis. Generation of a myeloid-specific *c-myb*–deficient mutant fish will be necessary to answer this question. Finally, our findings prompt us to speculate that perhaps mammalian c-MYB also plays a similar role in neutrophil differentiation. Thus, it will be of great interest to reinvestigate the role of c-MYB in murine embryonic and adult myelopoiesis.

Our study also shows that the neutrophilic phenotype in *c-myb*$^{hkz3/hkz3}$ mutants is largely attributed to the downregulation of differentiation-related genes such as *lyz* and *srgn*, the key components of granule proteins.[44-46,48] This result is somewhat surprising in view of a well-documented role of c-MYB in cell-cycle regulation.[62] It may reflect a disparate requirement for c-Myb in distinct cellular contexts, which is also supported by our previous findings showing that c-Myb regulates the migration of newly born HSCs by modulating the expression of *sdf1a*.[37] Consistent with previous in vitro cell line studies showing that c-MYB could directly interact with C/EBPε and cooperatively activate transcription of several myeloid-specific promoters (such as elastase, *mim-1*, and *mpo*),[63-67] our genetic and biochemistry analysis indicates that c-Myb interacts with Cebp1, but they act in parallel and cooperatively to regulate neutrophil terminal differentiation, presumably through coactivating the granule-forming related gene transcription. Intriguingly, the loss of c-Myb function appears to have more profound impacts on neutrophil development than the loss of Cebp1 function, suggesting that c-Myb plays a more important role in the neutrophil maturation pathway. Besides the synergistic effect of c-Myb and C/ebp1 we reported here, other combined effects on *lyz* transcriptional regulation by different transcription factors (such as Runx1[51] together with c-Myb or Cebp1) remain currently unknown and need future study.

Finally, the neutrophilic defect in *c-myb*$^{hkz3/hkz3}$ mutants is reminiscent of the pathological features of SGD, which is characterized by recurrent bacterial infections due to disabled neutrophils. The neutrophils in SGD patients display typical bilobed nuclei and lack the expression of at least 1 primary granule or/and all secondary and tertiary granule proteins.[16] To date, the mutations in *C/EBPε* are the only known genetic lesions associated with SGD, but several classic SGD cases are shown to have intact *C/EBPε*.[17] Our study strongly suggests that *c-MYB* could be an unidentified gene mutated in those SGD patients with intact *C/EBPε* alleles and it would be of great interest to investigate whether these human patients carry mutations in the *c-MYB* locus.

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Authorship

Contribution: H.J., Y.Z., Z.W., W.Z., and L.L. designed the experiments; H.J. and Z.H. designed and performed experiments; M.W., Y.C., and Y.L. performed the reporter assay; M.W. performed the rescue assay and generated the *cebp1*^{smu2} mutant; L.Z. generated the *cebp1*^{smu1} mutant; R.Z. performed blood cell staining and generated constructs; Y.Z., Z.H., and M.W. performed biochemistry experiments; L.L. characterized the *c-myb*^{hkz3} mutant; H.J., Z.H., Y.Z., and Z.W. wrote the manuscript; and J.X., F.Z., L.L., W.Z., Z.W., and Y.Z. discussed the results and commented on the manuscript.

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