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Negative Elongation Factor (NELF) Inhibits Premature Granulocytic Development in Zebrafish

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Abstract: Gene expression is tightly regulated during hematopoiesis. Recent studies have suggested that RNA polymerase II (Pol II) promoter proximal pausing, a temporary stalling downstream of the promoter region after initiation, plays a critical role in regulating the expression of various genes in metazoans. However, the function of proximal pausing in hematopoietic gene regulation remains largely unknown. The negative elongation factor (NELF) complex is a key factor important for this proximal pausing. Previous studies have suggested that NELF regulates granulocytic differentiation in vitro, but its in vivo function during hematopoiesis remains uncharacterized. Here, we generated the zebrafish mutant for one NELF complex subunit Nelfb using the CRISPR-Cas9 technology. We found that the loss of nelfb selectively induced excessive granulocytic development during primitive and definitive hematopoiesis. The loss of nelfb reduced hematopoietic progenitor cell formation and did not affect erythroid development. Moreover, the accelerated granulocytic differentiation and reduced progenitor cell development could be reversed by inhibiting Pol II elongation. Further experiments demonstrated that the other NELF complex subunits (Nelfa and Nelfe) played similar roles in controlling granulocytic development. Together, our studies suggested that NELF is critical in controlling the proper granulocytic development in vivo, and that promoter proximal pausing might help maintain the undifferentiated state of hematopoietic progenitor cells.

Keywords: zebrafish; negative elongation factor; RNA polymerase II promoter proximal pausing; granulocytic development

1. Introduction

Hematopoiesis is a complex process involving the collaboration of intrinsic hematopoietic transcriptional regulators and signaling molecules from the microenvironment [1]. Defects in the formation and differentiation of hematopoietic cells often result in various diseases such as anemia and leukemia.

To ensure that blood cells are properly formed, the expression of key regulatory genes in hematopoiesis is strictly regulated at the transcriptional level. Transcription does not function as a simple ‘on-off’ switch, but instead involves various rate-limiting steps that each contribute to the overall transcription rate and efficiency [2–5]. Increasing studies revealed that transcriptionally engaged RNA polymerase II (Pol II) on many metazoan genes experiences a temporary stalling at 20 to 120 nucleotides downstream of the promoter region after initiation, which is described as the promoter proximal pausing of Pol II [6]. This pausing is widespread in the expressed genome, ranging from 30% to 70% detection among different studies, indicating its critical role in gene regulation [5,7–9].

Promoter proximal pausing stabilization requires two important protein complexes: the pausing negative elongation factor (NELF) and DEB-sensitive inducing factor (DSIF) [10,11].
The NELF complex contains four subunits (A, B, C/D and E) [11,12]. Accumulating evidence has suggested an important role of NELFB in gene expression, and NELFB expression is strictly regulated during development [13–17]. The release of paused Pol II into productive elongation requires the activity of positive transcription elongation factor b (P-TEFb), which phosphorylates Pol II, DSIF, and NELF [6,18]. NELF dissociates from chromatin upon its phosphorylation, whereas DSIF switches to a positive elongation factor [19–21]. Despite extensive biochemical studies, limited research exists regarding the function of these complexes in vivo. A recent study using in vitro hematopoietic cell culture suggested that NELF plays negative roles in granulocytic differentiation [17], but, its in vivo functions in other hematopoietic lineage differentiation remain unknown.

As a powerful genetic model, zebrafish have increasingly been used to study hematopoietic development, with many advantages. They provide a convenient and fast tool to study gene regulation and functions in vivo [1]. Although hematopoiesis in zebrafish occurs in a spatially unique manner compared to other vertebrates, the developmental processes and genetics programs are still highly conserved [1]. Similar to the mammals, hematopoiesis in zebrafish also involves a primitive wave and definitive wave [22]. Starting at approximately 12 h post-fertilization (hpf), primitive hematopoietic cells are born in the anterior lateral plate mesoderm (ALPM) and the intermediate cell mass (ICM) [1,23]. During this wave, the cells in the ALPM generate mainly myeloid cells, including macrophages and neutrophils, while the cells in ICM (also known as the posterior lateral plate mesoderm) mainly give rise to erythrocytes and potentially neutrophils [24–27]. The definitive wave of hematopoiesis initiates at around 30 hpf [22]. The definitive hematopoietic stem cells (HSCs) emerge from the aorta-gonad-mesonephros (AGM) region, and these HSCs migrate to the caudal hematopoietic tissue (CHT) in the tail, finally residing in the kidney marrow (equivalent to mammalian bone marrow) [22]. Different from primitive hematopoiesis, the definitive wave generates all lineage blood cells, including myeloid cells and lymphoid cells [22].

In this study, we used zebrafish as a model system to investigate the role of Nelfb and other NELF subunits during hematopoietic development. Our results demonstrated that Nelfb specifically controls primitive and definitive granulocytic development at the appropriate time by regulating the expression of lineage genes through Pol II pausing. We further demonstrated that other NELF subunits (Nelfa and Nelfe) play similar roles in granulopoiesis. Together, these results indicate that NELF plays an important role in preventing premature granulopoiesis.

2. Results
2.1. Expression of nelfb during Development and Generation of Zebrafish nelfb Mutants

NELF is composed of four subunits (NELFA, B, C/D, and E) that are interdependent [28]. One of the most extensively studied subunits is NELFB. To investigate the roles of Nelfb during embryonic development, we first examined the expression of nelfb at different development stages using quantitative reverse transcription PCR (q-RT-PCR). nelfb was abundantly expressed at early developmental stages, but decreased when embryos proceeded to later stages (Figure 1A). To analyze the expression of nelfb in blood cells, we purified draculin (drl)+-hematopoietic cells from Tg(drl:GFP) fish by fluorescence-activated cell sorting (FACS). The draculin element is active during early development in all lineages derived from the anterior and posterior hematopoietic population; thus; Tg(drl:GFP) covers all hematopoietic cells [29,30]. Compared with the whole embryo lysates, nelfb was more abundantly expressed in hematopoietic cells (Figure 1B), supporting its potential function in hematopoiesis.
We first examined the function of Nelfb in primitive hematopoietic development. To do this, whole-mount in situ hybridization (WISH) was performed at different stages to detect scl expression (Figure 2A,D). However, scl expression was dramatically reduced at later stages (22 hpf) (Figure 2A,D). The embryos that survived developed into adults (Figure 1G). The adult Zebrafish hematopoiesis, which mainly proceeds through two successive waves (primitive wave and definitive wave), is highly conserved when compared to that of mammals [1]. We first examined the function of Nelfb in primitive hematopoietic development. To do this, whole-mount in situ hybridization (WISH) was performed at different stages to detect primitive hematopoiesis markers.

The loss of nelfb had no apparent effects on hematopoietic progenitor cell development at 7- and 14-somite stages, as indicated by normal scl expression (Figure 2A,D). However, nelfb deficiency led to accelerated granulocytic development during primitive hematopoiesis.
scl expression was dramatically reduced at later stages (22 hpf) (Figure 2A,D). Similarly, the expression of pu.1 in nelfb−/− mutants at the 14-somite stage was comparable to WT embryos, with a decrease at 22 hpf, suggesting an abnormal myeloid progenitor development (Figure 2B,D). These data indicated that, although primitive hematopoiesis before the 14-somite stage is not affected by nelfb knockout, defects in primitive hematopoietic progenitors, such as the myeloid progenitors, initiates from the later stage, around 22 hpf.

Figure 2. Nelfb deficiency leads to accelerated granulocytic development during primitive hematopoiesis. (A) WISH for scl in WT and nelfb−/− embryos at 7-, 14-somite stages and 22 hpf. (B) WISH for pu.1 in WT and nelfb−/− embryos at 14-somite stage and 22 hpf. (C) WISH for mpx, mfap4, and gata1 in WT and nelfb−/− embryos at 22 hpf. (D) Quantification of WISH results in (A–C) (n = 40–60 embryos per group). (E) Q-RT-PCR analysis of gene expression in nelfb−/− and WT embryos at 22 hpf. Gene expression is normalized to β-actin and presented as fold-change relative to WT. (F) May–Grünwald–Giemsa staining of peripheral blood in WT and nelfb−/− embryos at 28 hpf. Green arrowheads indicate precursors; red arrowheads indicate granulocytes. (G) WISH for scl, pu.1, mpx, mfap4, and gata1 in WT and nelfb morphants at 22 hpf. (H) Quantification of WISH results in (G) (n = 20–40 embryos per group). All results are presented as the mean ± SD from three independent experiments (t test, * for p < 0.05, ** for p < 0.01, *** for p < 0.001). Grey arrowheads, yellow arrowheads, and red arrowheads in (A–C,G), respectively, indicate ICM, ALPM, and PLPM. “+++” and “+++” in (D,H) respectively represent strong staining and weak staining. WISH, whole-mount in situ hybridization; ICM, intermediate cell mass; ALPM, anterior lateral plate mesoderm; PLPM, posterior lateral plate mesoderm.
At 22 hpf, nelfb knockout also resulted in a slight loss of the macrophages expressing marker mfp4, but it led to a striking increase in the expression of neutrophil marker mpx, which encodes a proinflammatory enzyme myeloperoxidase mainly expressed in neutrophils [31]. Meanwhile, erythroid development appeared to be relatively normal in the nelfb−/− mutant, as comparable gata1 mRNA expression was observed (Figure 2C,D). These results were also verified by q-RT-PCR (Figure 2E). Moreover, no significant change of blood cell proliferation or apoptosis, but a slight inflammatory activation, was observed (Supplementary Figure S1). Together, these results suggested that nelfb depletion selectively causes premature granulocytic development at the cost of hematopoietic progenitor cells during primitive hematopoiesis.

Differentiated neutrophils are characterized by banded and segmented nuclei [32]. Therefore, blood cells were collected at 28 hpf to stain nuclei with May–Grünwald–Giemsa stain (Figure 2F). The results showed a significant increase in differentiated granulocytic cells in the nelfb−/− mutants compared with WT at 28 hpf, supporting that primitive neutrophil development is indeed accelerated by nelfb mutation.

To verify the results obtained from the nelfb−/− mutants, we used a previously published morpholino [33] to knock down nelfb and examined primitive hematopoiesis (Supplementary Figure S2A,B). At 22 hpf, although the nelfb morphants showed similar levels of scl and pu.1 expression with WT, the mpx stained neutrophils were significantly increased in morphants, with decreased macrophages marked by mfp4 (Figure 2G). Meanwhile, primitive erythroid development was normal (Figure 2G). Due to the potential for limited efficiency of morpholino-mediated knockdown of nelfb, these results also supported that nelfb deficiency accelerates granulocytic differentiation.

2.3. Inhibition of Pol II Elongation Rescues Primitive Hematopoiesis in nelfb−/− Embryos

Losing Pol II pausing may lead to premature release of Pol II into the elongation phase. As one of the subunits of P-TEFb, CDK9 is critical for promoting Pol II into productive elongation [34]. To examine if the defects in nelfb−/− were caused by premature Pol II elongation, WT and nelfb−/− embryos were treated with CDK9 inhibitor flavopiridol from the 2-somite stage to 22 hpf. The results showed that progenitor cells and myeloid precursors were greatly increased, while neutrophils were notably decreased, in nelfb−/− embryos by treatment with flavopiridol at the concentration of 2.5–5 µM (Figure 3A–D). The progenitor cells and myeloid precursors were also increased, and neutrophils were decreased in WT embryos with the same flavopiridol treatment, but to a much lower degree. In addition, we used a splicing-blocking morpholino to knock down Cdk9 in the embryos [33]. Both low- and high-doses of morpholino reversed the phenotypes in nelfb−/− embryos, and both showed mild similar effects in WT embryos, consistent with the phenotypes in the flavopiridol treatment (Figure 3E–H). Collectively, these results suggested that primitive progenitor formation and myeloid development require a tightly controlled release of paused Pol II. Losing pausing factors like Nelfb leads to premature granulocytic differentiation, which can be reversed by inhibiting Pol II elongation.

To further test if the loss of nelfb indeed affects the transcription elongation of hematopoietic genes, we assayed blood gene transcripts near the 5′ or 3′ end using q-RT-PCR to study Pol II position (Figure 3I), as suggested in the previous study [35]. The expression of 5′ ends and 3′ ends of scl, pu.1, gata1, and mfp4 gene transcripts were all slightly decreased or unaffected, and there was a slightly greater increase in transcription products in the 3′ ends than in the 5′ ends for these genes (Figure 3I). However, we detected a strongly increased expression of the 5′ ends of mpx gene transcripts in nelfb−/−. Moreover, the 3′ ends of mpx gene transcripts in nelfb−/− showed a dramatical increase, which was reduced by cdk9 morpholino (Figure 3I). These results suggested that the loss of Pol II pausing leads to an increased Pol II elongation, particularly at the loci of the neutrophil granulocyte specific gene mpx, as well as a subsequent increased expression, and that the increased mpx expression can be reversed by inhibiting the elongation.
Figure 3. Inhibition of Pol II elongation rescues primitive hematopoiesis in nelfb−/− embryos. (A–C) WISH for scl (A), pu.1 (B) or mpx (C) at 22 hpf in WT and nelfb−/− embryos treated with DMSO, 2.5 µM flavopiridol, or 5 µM flavopiridol. (D) Quantification of WISH results in (A–C) (n = 30–50 embryos per group). (E–G) WISH for scl (E), pu.1 (F), or mpx (G) at 22 hpf in WT and nelfb−/− embryos without or with cdk9 MO injection (1.2 ng or 4 ng). (H) Quantification of WISH results in (E–G) (n = 30–50 embryos per group). (I) Upper panel shows the position of primers used in Q-RT-PCR analysis. Primers for the 5′ transcripts are located within 120 bp from transcription start site (TSS), and primers for the 3′ transcripts are in the 3′ coding region or 3′UTR. Lower-left panel shows Q-RT-PCR analysis of 5′ and 3′ transcripts of hematopoiesis-related genes in WT and nelfb−/− embryos at 22 hpf. Lower-right panel shows Q-RT-PCR analysis of 5′ and 3′ transcripts of mpx gene in 22 hpf nelfb−/− embryos without or with cdk9 MO injection (1.2 ng or 4 ng). Gene expression is normalized to the 5′ transcript of β-actin and shown as fold-change relative to WT, following the methods in the previous study [35]. All results are presented as the mean ± SD from three independent experiments (t test, * for p < 0.05, ** for p < 0.01, *** for p < 0.001, **** for p < 0.0001). Yellow arrowheads and red arrowheads in (A–C) and (E–G), respectively, indicate ALPM and PLPM. “+++” and “+” in (D,H) respectively represent strong staining and weak staining. MO, morpholino.
2.4. Expression of Human NELFB in Hematopoietic Cells Partially Rescues Granulopoiesis in nelfb−/− Embryos

Since it is possible that the loss of Nelfb in adjacent tissues affects hematopoietic development in nelfb−/−, we next studied if Nelfb functions directly in hematopoietic cells to regulate primitive granulopoiesis. The zebrafish Nelfb protein displays a 76.8% identity to the human NELFB protein and a 76.9% identity to the mouse NELFB protein, indicating that NELFB is highly evolutionarily conserved in vertebrates (Figure 4A). To investigate the cell autonomous regulation of Nelfb in blood, we constructed a stable transgenic line with the plasmid containing the human NELFB (hNELFB) gene under the control of the draculin promoter [30], Tg (drl:hNELFB-2A-GFP) to overexpress hNELFB in blood cells (Figure 4B).

Figure 4. Expression of human NELFB in hematopoietic cells partially rescues granulopoiesis in nelfb−/− embryos. (A) Alignment of human, mouse, and zebrafish NELFB proteins. Identical and similar amino acids are shaded in gray. (B) Generation of Tg(drl:hNELFB-2A-GFP) fish and GFP expression. (C) WISH for scl or pu.1 in WT and Tg(drl:hNELFB-2A-GFP) embryos at 22 hpf. (D) Quantification of WISH results in (C) (n = 40–60 embryos per group). (E) WISH for mpx in WT, Tg(drl:hNELFB-2A-GFP), nelfb−/− and nelfb−/−; Tg(drl:hNELFB-2A-GFP) embryos at 22 hpf. (F) Quantification of WISH results in (E) (n = 20–30 embryos per group). “+++” and “+” in (D,F) respectively represent strong staining and weak staining. All results are presented as the mean ± SD from three independent experiments (t test, * for p < 0.05). hNELFB, human NELFB.

Tg(drl:hNELFB-2A-GFP) overexpression in WT embryos only led to a mild increase in stem cells and myeloid progenitors and did not affect neutrophil development (Figure 4C–F). However, Tg(drl:hNELFB-2A-GFP) overexpression in nelfb−/− partially inhibited the premature granulocytic development, supporting the idea that NELFB likely functions in blood cells to control granulopoiesis (Figure 4E,F).

2.5. Other NELF Subunits Play Similar Roles in Primitive Granulocytic Development

NELF is a complex containing 4 subunits (NELFA, NELFB, NELFC/D, and NELFE) [12]. Only one copy of each NELF subunit is found in zebrafish, and all NELF subunits are highly conserved across different species. The individual zebrafish NELF subunit displays a more than 70% identity to the corresponding human homologous protein. We analyzed
whether Nelfa and Nelfe play similar functions in primitive hematopoiesis. We first examined the expression of nelfa and nelfe at different development stages. nelfa and nelfe showed decreased mRNA expression as the embryos progressed, similar to the nelfb expression dynamics (Figure 5A). The results are also consistent with previous studies suggesting that the expression of NELF subunits is interdependent [28].

Figure 5. Other NELF subunits play similar roles in primitive granulocytic development. (A) Q-RT-PCR analysis of nelfa and nelfe expression at different stages. The relative gene expression was normalized to β-actin. (B) WISH for scl, pu.1, mpx, mfap4, or gata1 in WT, nelfa, or nelfe morphants at 22 hpf. Yellow arrowheads and red arrowheads, respectively, indicate ALPM and PLPM. (C–G) Quantification of WISH results in (B) (n = 30–50 embryos per group). All results are presented as the mean ± SD from three independent experiments t test, * for p < 0.05, ** for p < 0.01, *** for p < 0.001, **** for p < 0.0001). “++” and “+” in (C–G) respectively represent strong staining and weak staining.

Next, we used nelfa and nelfe morpholinos as previously used [33] to respectively knock down their expression in the embryos (Supplementary Figure S2). Although morphants showed no significant difference in primitive progenitor and precursor development,
indicated by scl and pu.1 expression, excessive neutrophil differentiation in nelfa and nelfe morphants was observed, as suggested by increased mpx expression (Figure 5B–E). In accordance with the primitive macrophage development in nelfb−/− mutants, nelfa and nelfe morphants showed decreased mfap4 expression (Figure 5B,F). Primitive erythroid development was not affected, as indicated by the comparable expression of gata1 with WT (Figure 5B,G). As a whole, the results suggested that other NELF subunits function similarly to Nelfb, and they may work together to specifically regulate granulopoiesis.

2.6. Granulocytic Differentiation at Late Developmental Stages and Adulthood Also Shows Defects in nelfb−/− Zebrafish

Next, we want to examine if nelfb also regulates granulopoiesis at later development stages and during adulthood. We analyzed the expression of hematopoietic genes in WT and nelfb−/− mutants. Previous studies have shown that the disruption of Pol II pausing causes a reduction of hematopoietic stem cell (HSC) specification [33]. Our WISH results also revealed a loss of HSCs (runx1 stained cells) from 26 hpf in nelfb−/−, which became more severe at 36 hpf. Lymphoid progenitors indicated by rag1 that were closely related to HSC development also showed a dramatical decrease at 4 dpf (Figure 6A). The cmyb-stained definitive myeloid progenitors were also obviously reduced at 2 dpf (Figure 6B). However, the expression of mpx, mfap4, and hbbe1 at 3 dpf showed no significant difference between the mutants and their WT siblings (Figure 6C). To test if transcription elongation regulates granulopoiesis during these later stages, we treated the embryos with flavopiridol and cdk9 morpholino to prevent elongation. Consistent with the hematopoietic phenotypes during the early embryogenesis, the flavopiridol-treated embryos or cdk9 morphants, with either WT or nelfb−/− mutant backgrounds, showed increased HSCs and reduced neutrophils, indicating that granulocytic differentiation is similarly regulated during this stage (Supplementary Figures S3 and S6E–H). At 5 dpf, although mpx expression was not yet significantly increased in nelfb−/−, our studies show that the loss of nelfb led to a strongly increased release of Pol II at the mpx gene loci compared with the control actin loci, as demonstrated by the Q-RT-PCR analysis, which demonstrated that the 3′ end of the mpx gene transcripts displayed a higher expression level than the 5′ end gene transcripts (Figure 6I). These results suggested that granulocytic differentiation is similarly regulated by transcription proximal pausing and elongation during later developmental stages.

To further investigate myelopoiesis along the course of development, we tested the hematopoietic genes expression in 26-day-old zebrafish using Q-RT-PCR. The expression level of runx1, cmyb, and rag1 remained slightly or significantly decreased in nelfb−/−. However, the mpx expression was strongly increased, supporting the idea that granulopoiesis is likely increased in nelfb−/− as the development progresses, and that granulopoiesis is also enhanced during definitive hematopoiesis in nelfb−/− (Figure 6J).

To study the hematopoietic phenotypes in adult zebrafish, a cytological assay of the whole kidney marrow (WKM) was performed. The overall kidney morphology between WT and nelfb−/− were similar (Figure 6K). For the hematopoietic cells, the population of lymphoid/stem cells and the myeloid progenitor cells was slightly decreased. The intermediate myelomonocytes were significantly decreased, but the population of mature neutrophils was strongly expanded in nelfb−/− (Figure 6L). These results implied that the loss of nelfb also induces the pre-maturation of granulocytic cells in adults.

Taken together, our findings support that Nelfb plays a consistent role in primitive and definitive granulocytic differentiation, likely through the regulation of Pol II pausing at the granulocyte lineage genes.
Figure 6. Granulocytic differentiation at late developmental stages and in adulthood also shows defects in nelfb<sup>−/−</sup> zebrafish. (A) WISH for runx1 or rag1 in WT and nelfb<sup>−/−</sup> embryos at 26 hpf and 4 dpf. (B) WISH for cmyb in WT and nelfb<sup>−/−</sup> embryos at 2 dpf. (C) WISH for mpx, mfap4, and hbbe1 in WT and nelfb<sup>−/−</sup> embryos at 3 dpf. Blue arrowheads, white arrowheads, and red arrowheads, respectively, indicate AGM region, thymus, and CHT. (D) Quantification of WISH results in (A–C) (n = 30–40 embryos per group). (E) WISH for mpx at 3 dpf in WT and nelfb<sup>−/−</sup> embryos treated with flavopiridol. (F) Quantification of WISH results in (E) (n = 20–30 embryos per group). (G) WISH for mpx at 3 dpf in WT and nelfb<sup>−/−</sup> embryos injected with 1.2 ng cdk9 MO. (H) Quantification of WISH results in (H) (n = 20–30 embryos per group). (J) Q-RT-PCR analysis of 5′ and 3′ transcripts of mpx gene in WT and nelfb<sup>−/−</sup> embryos at 5 dpf. Gene expression is normalized to the 5′ transcript of β-actin and shown as fold-change relative to WT. (J) Q-RT-PCR analysis of hematopoiesis-related genes in WT and nelfb<sup>−/−</sup> mutant fish at 26 dpf. Gene expression is normalized to β-actin and presented as fold-change relative to WT. (K) May–Grünwald–Giemsa staining of WKM from WT and nelfb<sup>−/−</sup> fish at 8 mpf. Blue arrowheads, precursors; green arrowheads, intermediate myelomonocytes; yellow arrowheads, mature neutrophils; black arrowheads, lymphocytes and HSCs; red arrowheads, erythrocytes. (L) Quantification of WKM staining results. All results are presented as the mean ± SD from a representative of three independent experiments (t test, * for p < 0.05, ** for p < 0.01, *** for p < 0.001). “+++” and “+” in (D,F,H) respectively represent strong staining and weak staining. Note: dpf, days post-fertilization; mpf, months post-fertilization; AGM region, aorta-gonad-mesonephros region; CHT, caudal hematopoietic tissue; WKM, whole kidney marrow; HSCs, hematopoietic stem cells.
3. Discussion

Hematopoiesis is a very dynamic process, and multiple signals cooperate to control cell proliferation and differentiation [35]. To achieve the normal function of the hematopoietic system, the specific cell types must be generated at the correct time and place. The molecular circuitries underlying blood cell fate choice must enable cells to respond appropriately to the external stimuli [36]. Hematopoietic gene expression has been extensively studied at the transcription initiation step that is controlled by cell-specific transcription factors including SCL, GATA1, and PU.1 [37]. Recently, Pol II promoter proximal pausing has been reported as another critical step in various gene regulation [17]. This suggests that Pol II pausing allows for the integration of signals, but also maintains an active chromatin architecture, thus playing an essential role in a rapid and synchronized activation of gene expression on developmental or environmental changes [38,39], but whether Pol II pausing regulates hematopoietic lineage differentiation remains largely unknown. Previous in vitro studies suggested a role of NELF in hematopoietic differentiation [17]. Due to the embryonic lethality of NELF subunits (like NELFB) in knockout mice, the function of NELF during hematopoiesis remains uncharacterized [40]. Here, using the zebrafish model, we provide in vivo evidence that the NELF complex, an important promoter-proximal pausing factor, plays an essential role in regulating proper granulocytic development.

Interestingly, NELF-mediated pausing displays a lineage-specific regulation. It was previously shown that NELF protein levels remained unchanged during erythropoiesis [17]. Consistent with this study, our data showed that the loss of nelfb did not affect erythroid development, despite aberrant granulocyte formation. In humans, the half-life of circulating neutrophils is less than 1 day, while erythrocytes have an average life span of 120 days [41,42]. Moreover, as a crucial element of the immune system, granulocytes must be sensitive to environmental stress, capable of rapidly responding to multiple microbial and sterile challenges [42]. Granulopoiesis is a complex process that requires both lineage-specific transcription factors (such as PU.1 and C/EBPα) and ubiquitous transcription factors (such as STATs) [43]. Despite intrinsic factors, the development of granulocytic cells is also regulated by extrinsic factors such as cytokines and metabolic signaling [44,45]. The cooperation of these factors is the essential driving force in mediating granulopoiesis. Previous studies suggested that pausing is particularly prevalent and required in genes that are involved in stimulus-responsive networks [39]. Thus, we hypothesize that NELF-mediated Pol II pausing at granulocyte specific genes might initially suppress their expression, but then poise them to respond rapidly to appropriate environmental cues under steady-state and emergency conditions. It will be interesting to investigate the expression of NELF proteins in different lineage cells during hematopoiesis and to analyze the chromatin binding targets of NELF together with Pol II and hematopoietic lineage-specific transcription factors. Such studies will help reveal the precise mechanism of NELF in more dynamic cell-like granulocytes during cell differentiation. Moreover, the characterization of the role of NELF-mediated pausing during granulopoiesis under environmental stress, such as in cancer and injury, may also provide valuable information.

Our experiments prove that NELF plays a negative role in granulocytic development in vivo. Previous in vitro studies also showed that the forced expression of NELF subunits repressed granulocytic differentiation and enhanced hematopoietic progenitor cells [17]. Our data showed that the overexpression of human NELF in blood cells led to a slight increase in hematopoietic progenitor cells, but did not interfere with normal granulocyte formation. It is possible that human NELFB may not express or function efficiently in zebrafish, despite the 77% identity between the two proteins. Nonetheless, it is possible that high expression of NELF may interfere with normal myeloid differentiation, leading to immature myeloid cells proliferation and accumulation, which is the main feature of myeloid leukemia. Interestingly, acute myeloid leukemia (AML) patients with both high NELFA or NELFB expression display low survival rates (Supplementary Figure S4). Thus, we provide evidence that inhibiting pausing or promoting elongation might be a new potential therapy for promoting myeloid differentiation in leukemia.
The promoter proximal pausing complex contains NELF and DSIF, which likely function together [6]. Previous studies have shown that the loss of DSIF pausing function had no effects on primitive hematopoiesis in zebrafish [33]. In our studies, the loss of **nelfb** led to pre-released Pol II elongation, resulting in decreased hematopoietic progenitor cells and excessive granulocytic development during primitive hematopoiesis. Thus, our studies suggest that there might be different pausing subtypes to control gene expression at different stages of development. Further studies focused on DSIF function during different stages, including definitive hematopoiesis, may help unveil more details about the differences between NELF and DSIF in regulating lineage specification.

In summary, we report that NELF-mediated transcription promoter proximal pausing plays an important role in preventing the premature development of granulocytic cells. It provides an in vivo model for future mechanistic studies on related processes and for understanding human hematopoietic diseases associated with dysregulated transcription pausing and elongation.

### 4. Materials and Methods

#### 4.1. Zebrafish Maintenance and Embryo Handling

The wild type (WT) AB and transgenic zebrafish were maintained, handled, and bred according to standard protocols from the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. Adult zebrafish were raised in a circulating water system under a 14 h/10 h light/dark cycle at 26–28 °C and fed two times per day. Adult male and female zebrafish were kept separated using a transparent barrier in the same mating tank in the evening and mated the following morning. The embryos were collected and kept at 28.5 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) with a density of 80–120 embryos per 10 cm diameter Petri dish. Embryos were staged by hours post-fertilization (hpf) and days post-fertilization (dpf).

#### 4.2. Generation of nelfb Knockout Mutants

**nelfb** knockout mutants were generated through the CRISPR-Cas9 system. **nelfb** specific guided RNA (gRNA) (Figure 1C) was designed to target on the exon 2. One-cell stage WT embryos were injected with 1 nl of the solution containing 100 ng/µL Cas9 mRNA and 20 ng/µL gRNA. Injected F0 fish were raised to adulthood and outcrossed with WT fish. F1 mutant offspring were identified using a T7 endonuclease I (T7E1) assay (M0302S, NEB, Bellingham, WA, USA) using primers around the target loci. Target loci was amplified by PCR from the genomic DNA, and the mutation was detected by Sanger DNA sequencing. F1 fish were outcrossed with WT to obtain stable F2 mutant lines. Primers for genotyping are listed in Supplementary Table S1.

#### 4.3. Generation of NELFB Overexpression Lines

To overexpress NELFB in the blood system, the plasmid driven under the **drl** promoter, which contains the full-length of the human **NELFB** sequence, the zebrafish GFP sequence, and Tol2 mRNA, were co-injected into WT embryos at the one-cell stage. Injected embryos showing a positive expression of GFP were selected and raised to adults (F0 founder). F0 fish was outcrossed to WT fish, and GFP positive embryos was raised to adults (F1). F1 fish was outcrossed to WT again to obtain the stable transgenic line.

#### 4.4. Whole-Mount In Situ Hybridization (WISH)

Whole-mount in situ hybridization was performed using digoxigenin-UTP labeled RNA probes (**scl**, **pu.1**, **runx1**, **cmyb**, **mpx**, **rag1**, **mfap4**, **gata1**, **hbbe1**). Embryos at the desired time point were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, bleached, and dehydrated in methanol at −20 °C for at least two hours. Further processing of the embryos was conducted according to the previous protocol [46]. The stained embryos were imaged with a SZX16 stereomicroscope (Olympus, Tokyo, Japan).
4.5. May–Grünwald–Giemsa Staining of Embryo Blood Cells and Adult Whole Kidney Marrow Cells

Twenty-eight hpf embryos were dechorionated with pronase (11459643001, Roche, Mannheim, Germany) and anesthetized in PBS containing 10% FBS and 0.02% tricaine. After tail clipping with a syringe needle, blood cells were collected by pipetting and cytopspun onto slides by centrifugation at $450 \times g$ rpm for 3 min using Cytospin 4 (Sigma-Aldrich, St. Louis, MI, USA). Each group was collected from a pool of 80–100 embryos. The adult fish kidney marrow was dissected and placed into PBS containing 10% FBS. The single hematopoietic cells from the kidney marrow were generated by pipetting and filtration through 40-µm filters. Single-cell suspension was diluted to 15,000–30,000 cells/mL and cytocentrifuged at $300 \times g$ rpm for 4 min using the Cytospin 4.

Blood smears were processed using May–Grünwald–Giemsa double stain (63590/48900, Sigma-Aldrich, St. Louis, MI, USA) for morphological analysis and differential cell counts.

4.6. Flow Cytometry and Cell Sorting

We sorted $drl^+$ cells from Tg ($drl$:GFP) WT and from Tg ($drl$:GFP) $nefb^{-/-}$ embryos at 22 hpf. The embryos were dechorionated with pronase and washed with E3 solution. Single cells were collected by shredding the larvae with a blade, and then the cells were incubated for 20 min ($37 \degree C$) with 38 µg/mL Liberase (05401119001, Roche, Basel, Switzerland). A total of 10% FBS was added to stop the reaction, followed by filtration (40 µm filter) and centrifugation ($1200 \times g$ rpm, 4 °C, 5 min). The supernatant was removed, and the cells were resuspended with PBS containing 1% FBS. GFP negative and positive cells were sorted respectively using FACS Aria III (Becton, Dickinson and Company, San Jose, CA, USA).

4.7. Gene Expression Tested by Real-Time qPCR

Gene expression was evaluated using real-time qPCR. Briefly, the total RNA was extracted from embryos with TRIzol reagent (10296028, Thermo Fisher Scientific, Waltham, MA, USA). The cDNAs were synthesized from the total RNA using the HiFai® II 1st Strand cDNA Synthesis Super Mix (11123ES60, Yeasen, Shanghai, China). Hieff® qPCR SYBR Green Master Mix (11203ES08, Yeasen, Shanghai, China) was used for qPCR analysis. Each target gene was calculated using the $2^{-\Delta\Delta CT}$ method [47]. The primers for different target genes and β-actin (the reference gene) are listed in Supplementary Table S2.

4.8. Flavopiridol Treatment

The 2-somite embryos were continuously exposed to flavopiridol (146426-40-6, Aladdin, Shanghai, China) until 22 hpf. For later-stage treatment, 5-somite embryos were continuously exposed to flavopiridol until 36 hpf or until 3 dpf. The control groups were treated with DMSO. The embryos were fixed in 4%PFA and used for WISH.

4.9. Morpholino Microinjection

Morpholinos (gene tools) were injected into 1- to 2-cell-stage embryos. See Supplementary Table S3 for morpholino information. The efficiency of each morpholino was verified (Supplementary Figure S2).

4.10. Statistical Analysis

GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com, accessed on 2 April 2016) was used to analyze all data. The values of all triplicate experiments are presented as mean ± SD. The statistical significance was displayed as “ns” for no statistical significance, “*” for $p < 0.05$, “**” for $p < 0.01$, “***” for $p < 0.001$, and “****” for $p < 0.0001$. The unpaired 2-tailed Student’s $t$-test was used for data analysis. For statistical analysis with WISH and other staining results, the groups with strong staining are used in the $t$-tests.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms23073833/s1. References [33,48,49] are cited in the supplementary materials.

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Institutional Review Board Statement: The wild type (WT) AB and transgenic zebrafish were maintained, handled, and bred according to standard protocols from the Institutional Animal Care Committee of Shanghai Jiao Tong University.

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