UNIVERSITY OF CALIFORNIA SAN DIEGO

Role of Granulin in Lineage Differentiation during Primitive and Definitive Hematopoiesis

in

Biology

by

Liangdao Li

Committee in charge:

David Traver, Chair
Yury Miller
Emma Farley
Raquel Espin Palazon

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The Thesis of Liangdao Li is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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ABSTRACT OF THE THESIS

Role of Granulin in Lineage Differentiation during Primitive and Definitive Hematopoiesis

by

Liangdao Li

Master of Science in Biology

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Professor David Traver, Chair

Mature blood cells have a limited lifespan and have to be replenished continuously through a group of pluripotent precursors known as hematopoietic stem cells (HSCs). HSCs undergo proliferation and differentiation to achieve this goal. During differentiation, a group of multipotent progenitor cells with lineage-differentiation potential are generated

from HSCs before becoming mature blood cells. The fate of these progenitor cells is regulated by many molecular factors during hematopoiesis. The zebrafish provides a great model to study hematopoiesis during embryonic development. Progranulin a (Grna) was found to be expressed during embryonic development by quantitative PCR (qPCR) and at the sites of hematopoiesis by whole mount *in situ* hybridization (WISH). Using live imaging and flow cytometry, we detected a reduction of macrophages and neutrophils in both Grna morphants and homozygous mutants. This phenotype could be partially rescued through overexpression of *grna*. Further analysis of *grna* transcripts in Pu.1 morphants suggested that Pu.1 acts upstream of Grna, regulating its expression. In addition, we found that Grna downregulated *gata1* expression in Grna morphants. Together, our data suggest that Grna is essential for proper myeloid lineage differentiation.

Introduction

Hematopoiesis, the process of forming blood cells, initiates during embryogenesis and continues throughout adulthood [1]. The multipotent precursors known as hematopoietic stem cells (HSCs) are responsible for the formation and replenishment of all blood cellular components. Even though HSCs have the potential to self-renew and give rise to all blood cell types, they mostly stay in a quiescent state [2, 3]. The active subset thus needs to constantly replenish the system with progenitor cells that possess profound proliferative power, and the ability to differentiate to all lineages. In mammals, HSC-derived progenitors include both lymphoid and myeloid progenitors. Lymphoid progenitors can give rise to B, T, and natural killer cells to boost the immune system, whereas myeloid progenitors differentiate into megakaryocytes, erythrocytes, granulocytes, and macrophages [4]. Antagonistic interactions between Pu.1 and Gata1 have been suggested to regulate the fate outputs of myeloid differentiation. The hematopoietic system is evolutionally conserved in all vertebrates, and the hematopoietic potentials of stem and progenitor cells have been intensively studied across different animal models including drosophila, mouse, and zebrafish [5-8]. Zebrafish (*Danio rerio*) recently has been used as an animal organism to study hematopoiesis due to its availability for live imaging, genetic modification, and chemical screening [9].

Hematopoiesis is first established during embryonic development and can be roughly divided into two distinct waves: primitive and definitive [10]. In the zebrafish, primitive hematopoiesis starts with the formation of primitive macrophages and neutrophils from the anterior lateral mesoderm (ALM) and primitive erythrocytes from the

intermediate cell mass (ICM) at around 18 hours post-fertilization (hpf) [1, 7]. These primitive cells are important for the survival of zebrafish embryos before the emergence of HSCs. The definitive wave of hematopoiesis starts with the emergence of erythromyeloid progenitors (EMPs) in the posterior blood island (PBI) at 24 hpf before the onset of HSCs. These progenitor cells possess the potential to differentiate into erythroid and myeloid cells but have limited proliferative ability [11]. Shortly after the onset of EMPs, at around 36 hpf, HSCs then emerge from the ventral wall of the dorsal aorta (VDA) and migrate to the caudal hematopoietic tissue (CHT). They eventually migrate to the kidney, the adult hematopoietic tissue in fish equivalent to the bone marrow in mammals [12]. Deciphering the molecular and cellular cues required for hematopoietic differentiation is key to develop therapies for the treatment of blood disorders such as leukemia and anemias. In this study, I have utilized the zebrafish to study *in vivo* the role that granulin plays in hematopoiesis.

Progranulin (PGRN), also known as PC cell-derived growth factor (PCDGF), proepithelin, or acrogranin, is classically known as a growth factor involved in neuronal degeneration, tumorigenesis, and autoimmune diseases [13]. It also possesses anti-inflammatory properties that makes it a critical modulator of wound healing and cartilage repair [13]. Despite progranulin being highly expressed by hematopoietic cells, its role in hematopoiesis is unknown. Recent studies showed that inflammatory signaling is required for HSC emergence [14-16] . We have previously demonstrated in our laboratory that tumor necrosis factor alpha (Tnfa) binds tumor necrosis factor receptor 2 (Tnfr2) to initiate HSC specification [14]. PRGN, as an anti-inflammatory factor, can also bind to

TNFR2 [17]. We hypothesized therefore that PGRN may also play a role during HSC development via modulation of the TNFa signaling pathway. Zebrafish have two paralogs of the mammalian PGRN, progranulin (*Grna*) and progranulin *b* (Grnb). *grna* is expressed in hematopoietic sites in the embryo, including the VDA and CHT, and in the adult zebrafish kidney, [18] suggesting that it is involved in embryonic and adult hematopoiesis.

First, we analyzed *grna* and *grnb* expression patterns during zebrafish embryonic development using quantitative PCR (qPCR) and whole mount *in situ* hybridization (WISH). Using double fluorescent *in situ* hybridization (FISH), we identified the myeloid cells responsible for *grna* expression during embryonic development.? To address the potential role of Grna during primitive and definitive hematopoiesis, we performed *grna* knockdown and knockout experiments. Loss of Grna lead to a decrease in macrophage and neutrophil numbers only from 30 hours post-fertilization (hsp) onwards, suggesting that Grna plays a role in myeloid development during definitive hematopoiesis, but not primitive hematopoiesis. We hypothesized that the loss of differentiated myeloid cells could be due to either cell apoptosis or impaired differentiation of myeloid progenitors. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was then used to investigate whether there were apoptotic neutrophils. To rescue the loss myeloid cells due to knock-down of *grna,* co-injection of a *grna* morpholino with an overexpression vector encoding *grna* was used to restore the defective myeloid differentiation back to baseline. We further examined the mechanism of how *grna* regulates early embryonic hematopoiesis. WISH results suggested that the lineage-specific transcription factor Pu.1 acts upstream of *grna*. We also showed that Grna negatively regulated *gata1* expression.

Together, our data indicate that Grna is required for proper lineage differentiation of myeloid progenitors during definitive hematopoiesis.

Materials and Methods

Animal model

In this study, zebrafish adults and embryos were used with the approval of Institutional Animal Care and Use Committee (IACUC). Zebrafish Transgenic lines *Tg(mpx:eGFP)*^{i114} [19], *Tg(gata1:DsRed)*^{sd2} [20], *Tg*(*mpeg:eGFP*)^{gl22} [21], and mutants Grna54a-/- [22] were used in this study.

Quantitative RT-qPCR

RNA was extracted from zebrafish embryos with RNeasy (QIAGEN). cDNA was then synthesized with qScript Supermix (Quanta BioSciences). The primers used for RT-qPCR are listed in **Table 1**. The relative expression levels of genes were calculated with the formula: relative expression = $2^{-(Ct[\text{gene of interest}] - Ct[\text{housekeeping gene}])}$

Table 1: List of primers used for RT-qPCR

Gene	Name	Nucleotide sequence (5'-> 3')	Use
ef1a	F	GAGAAGTTCGAGAAGGAAGC	RT-qPCR
	R	CGTAGTATTTGCTGGTCTCG	
grna	F	AGCCAGACCTTCCCAAATCAT	RT-qPCR
	R	CTCAGCAGGACAGGAAGAGC	
grnb	F	CGGCAAGAGTCTGGAAGAGT	RT-qPCR
	R	CACACGGCCTTGACTAGAGG	

Fluorescent Imaging

Zebrafish embryos collected at each stage of the development were first dechorionated to help observe the embryo under a microscope. The embryos were anesthetized in Tricaine (200 µg/ml) and imaged using Zeiss Axio Zoom v16.

Morpholino Injection

All antisense morpholino oligonucleotides were designed and purchased from Gene Tools. Morpholinos were diluted with molecular grade water at a concentration of 0.2 mM (mismatch-MO), 0.2 mM (Grna-MO), 0.8 mM (Grna-MO2), 0.6 mM (Gata1-MO), and 2 mM (pu1-MO) with phenol red solution and 1nl was microinjected into the embryos at single-cell stage.

WISH

Whole mount *in situ* hybridization was used for gene transcript analysis. Zebrafish embryos were first fixed overnight with 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS) and then dehydrated in 100% methanol overnight. Fixed and dehydrated embryos were then rehydrated stepwise in methanol/PBS into 1x PBS with 0.1% Tween 20 (PBT). Then the embryos older than somite stages were incubated (15 minutes for 24hpf, 25 minutes for 36hpf or older) with proteinase K (10ug/mL in PBT). After proteinase K treatment, the embryos were again fixed in 4% PFA in 1x PBS for 20 minutes. Then the embryos were pre-hybridized at least 1 hour at 65°C in hybridization buffer (50% formamide, 5x SSC, 500 mg/mL torula (yeast) tRNA, 50 mg/mL heparin, 0.1% Tween 20, 9 mM citric acid). Embryos were then hybridized in the same buffer with 50-100ng digoxigenin (DIG)-labelled RNA probe overnight at 65°C. Following the hybridization, the embryos were washed stepwise at least 15 minutes each at 65°C in hybridization buffer/2x SSC mix (75%, 50%, 25%). Then the embryos were washed twice at least 30 minutes at 65°C in 0.2x SSC and 5 minutes each at room temperature in 0.2x

SSC/PBT (75%, 50%, 25%). The embryos were then blocked in block solution (2% heat-inactivated goat serum, 2 mg/mL bovine serum albumin (BSA) in PBT) for 1 hour. After blocking, the embryos were incubated overnight at 4°C with alkaline phosphatase conjugated DIG-antibodies (1:5000) in blocking buffer. Finally, the embryos were washed 6 times in 1x PBT and developed with NBT/BCIP (Promega) in AP buffer (100mM Tris, pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20)

Double Fluorescent Whole Mount *in situ* Hybridization

Zebrafish embryos were first fixed overnight with 4% PFA in 1x PBSand then dehydrated in 100% methanol overnight. Fixed and dehydrated embryos were then rehydrated stepwise in methanol/PBS into 1x PBT. The embryos were fixed again for 20 minutes in 4% PFA in PBS and washed briefly twice in PBT. Then the embryos were incubated (15 minutes for 24hpf, 25 minutes for 36hpf or older) with proteinase K (10ug/mL in PBT). After proteinase K treatment, the embryos were again fixed in 4% PFA in 1x PBS for 20 minutes. Then the embryos were pre-hybridized for 5 minutes at HYB- buffer (50% formamide, 5x SSC, 0.1% Tween 20) and 1 hour at 65°C in HYB+ buffer (50% formamide, 5x SSC, 500 mg/mL torula (yeast) tRNA, 50 mg/mL heparin, 0.1% Tween 20, 9 mM citric acid). Embryos were then hybridized in the same buffer with 50-100ng digoxigenin (DIG) labelled and fluorescein (FITC) labelled RNA probe overnight at 65°C. Following the hybridization, the embryos were washed twice for 30 minutes at 65°C in 50% formamide/2x SSC, 15 minutes at 65°C in 2x SSC°C in 50% formamide/2x SSC, and then 30 minutes at 65°C in 0.2x SSC. The embryos were then blocked in 1x maleic acid buffer (150mM maleic acid, 100mM NaCl, (pH 7.5)) plus 2% block solution. After blocking, the embryos were incubated overnight at 4°C with anti-FITC-POD (1:500) in 1x maleic acid

buffer plus 2% blocking buffer. The embryos were then washed 4 times for 20 minutes in 1x maleic acid buffer and twice for 5 minutes in 1x PBS. After the washes, the embryos were incubated for 60 minutes in TSA Plus Fluorescein solution (1:50 in amplification dilution buffer) (PerkinElmer). The embryos were then dehydrated stepwise for 10 minutes each in methanol/PBS mix (30%, 50%, 75%) into 100% methanol. Followed by the dehydration, the embryos were incubated for 30 minutes in 1% H_2O_2 in 100% methanol. Then the embryos were rehydrated stepwise for 10 minutes each in methanol/PBS mix (75%, 50%, 35%) into 1x PBS. The embryos again were blocked in 1x maleic acid buffer (150mM maleic acid, 100mM NaCl, (pH 7.5)) plus 2% block solution. After blocking, the embryos were incubated overnight at 4°C with anti-DIG-POD (1:1000) in 1x maleic acid buffer plus 2% blocking buffer. The embryos were then washed 4 times for 20 minutes in 1x maleic acid buffer and twice for 5 minutes in 1x PBS. After the washes, the embryos were incubated for 60 minutes in TSA Plus CY3 solution (1:50 in amplification dilution buffer) (PerkinElmer). After the incubation, the embryos were washed a few times in 1x PBT and then incubated in DAPI (1:200) in PBT for 20 minutes. The embryos were then stored in 100% glycerol to be imaged.

Flow Cytometry

Zebrafish embryos were dechorionated with pronase (Roche) and dissociated using 0.05 mg/mL Librase TM (Rcohe). The solution was then filtrated with a 40um cell strainer into 1x PBS. The samples were then centrifuged at 300 G's for 10 minutes. The cells were then sorted and analyzed using Fortessa LSRII and FlowJo with Sytox Red (REF) to discriminate dead cells.

TUNEL Assay

Zebrafish embryos were first fixed overnight with 4% PFA in 1x PBS and then dehydrated in 100% methanol overnight. Fixed and dehydrated embryos were then rehydrated stepwise in methanol/PBS into 1x PBT. The rehydrated embryos were then permeabilized for 3 hours in PBSTx+DMSO (1x PBS, 0.5% Triton-X 100, 1% DMSO). Followed by permeabilization, the embryos were washed briefly with PBSTw (1x PBS, 0.1% Tween-20) and fixed for 20 minutes at -20°C in ethanol:acetic acid buffer (2:1). The samples were again briefly washed in 1x PBSTw and blocked overnight at 4°C in 5% bovine seruma lbumin (BSA). The embryos were then washed twice for 10 minutes in PBSTw and blocked using the Biotin Blocking Kit (Vector Laboratories). After brief wash in PBSTw, the embryos were incubated in equilibration buffer (Roche) for 1 hour and followed by another incubation in TdT reaction mix (Roche) overnight at 37°C. The embryos were then washed 6 times for 15 minutes in PBSTw and incubated with anti-GFP antibody (1:500, Aves Lab) overnight at 4°C. Followed by 6 times for 15 minutes wash in PBSTw,the embryos were incubated with secondary antibodies (anti-strepavidin-Alexa647 (1:500, Invitrogen), goat anti-chicken Alexa Flour 488 secondary antibody (1:500, Invitrogen), and DAPI (1:1000, Life Technologies)) for 3 hrs. The embryos were then stored and mounted using Vectorshield (Vector).

Statistical Analysis

All statistical analysis were done using GraphPad Prism. Student's t-test, one-way ANOVA, and post-hoc Tukey test were performed to test significant differences among

different experiment conditions. In all figures, the bar and error bars indicate mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Results

3.1 Zebrafish *grna* and *grnb* expression during early embryo development.

A previous study showed that zebrafish *grna* and *grnb* were expressed during embryonic development in the zebrafish [18]. However, the cell type/s expressing these transcripts have not been identified. While zebrafish *grnb* is mostly expressed in the yolk syncytial layer (YSL), *grna* is expressed by individual cells distributed along the sites of embryonic hematopoiesis [18] We attempted to confirm both *grna* and *grnb* expression patterns by quantitative PCR (qPCR) and whole mount *in situ* hybridization (WISH), as well as identify the *grna* producer cell/s.

3.1.1 RT-qPCR analysis

To confirm *grna* and *grnb* transcript levels at different stages of embryonic development, we performed qPCR for both *grna* and *grnb*. We detected *grna* and *grnb* transcripts at all stages analyzed (2 hpf, 9 hpf, 16 hpf, 24 hpf, 36 hpf, and 48 hpf) (Figure 1A, B). Interestingly, *grna* and *grnb* transcript levels were significantly higher at 2 hpf compared to the rest of stages (Figure 1A, B), indicating that they were maternally deposited. In addition, *grnb* transcript levels were much higher than *grna* at these staes during embryonic development (Figure 1A, B). After 2 hpf, *grna* and *grnb* mRNA levels were significantly reduced, although still detected (Figure 1A, B).

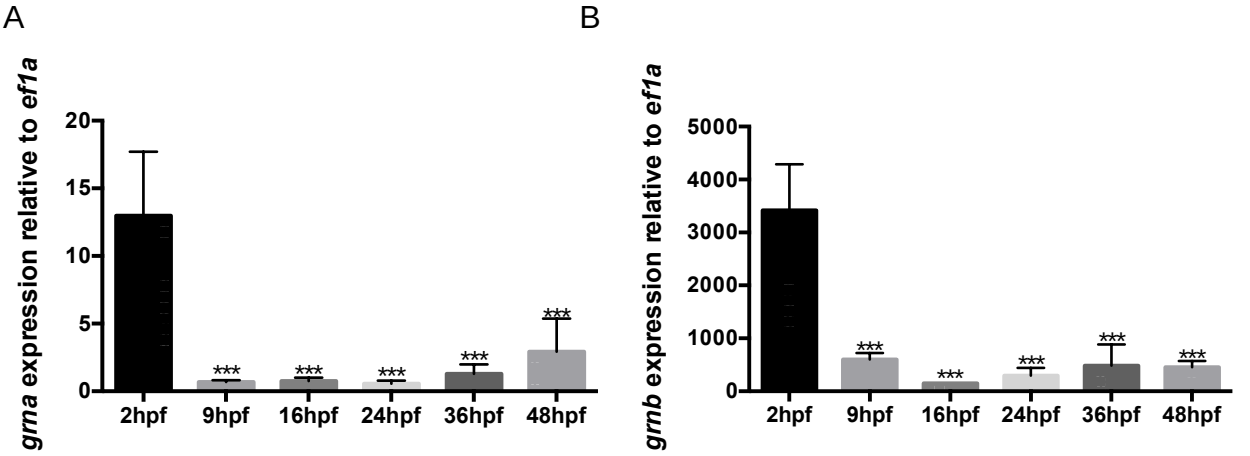

Figure 1. Developmental expression of zebrafish *grna* (A) and *grnb* (B) mRNA by RT-qPCR. Wildtype embryos harvested at 2, 9, 16, 24, 36, and 48 hpf were dissociated for mRNA extraction and RT-qPCR analysis. The transcript levels were normalized using *ef1a*. Bars represent means ± SEM of biological duplicates (***p<0.001).

3.1.2 WISH analysis

To identify where *grna* and *grnb* were expressed, we performed WISH in AB* wildtype (WT) embryos. Ubiquitous *grna* and *grnb* expression could be detected at both 2 hpf and 10 hpf (Figure 2A, B). However, and in agreement with our RT-qPCR data, *grnb* expression at these stages were much stronger than *grna* (Figures 1 and 2). In addition, we detected *grna* expression in the intermediate cell mass (ICM) at 26 hpf. At 36 hpf, we found that *grna* was expressed in the VDA and CHT (Figure 2A, arrows). *grna* transcripts were also detected in the head and yolk sac of the zebrafish embryos starting at 26 hpf (Figure 2A). This expression pattern suggested that *grna* was expressed by myeloid cells during embryonic development. Furthermore, we confirmed the previously described *grnb*

expression at the YSL region at 26 hpf [18] (Figure 2B). Because *grna* was found at the locations of zebrafish hematopoiesis, we focused our research on the Grna paralogue.

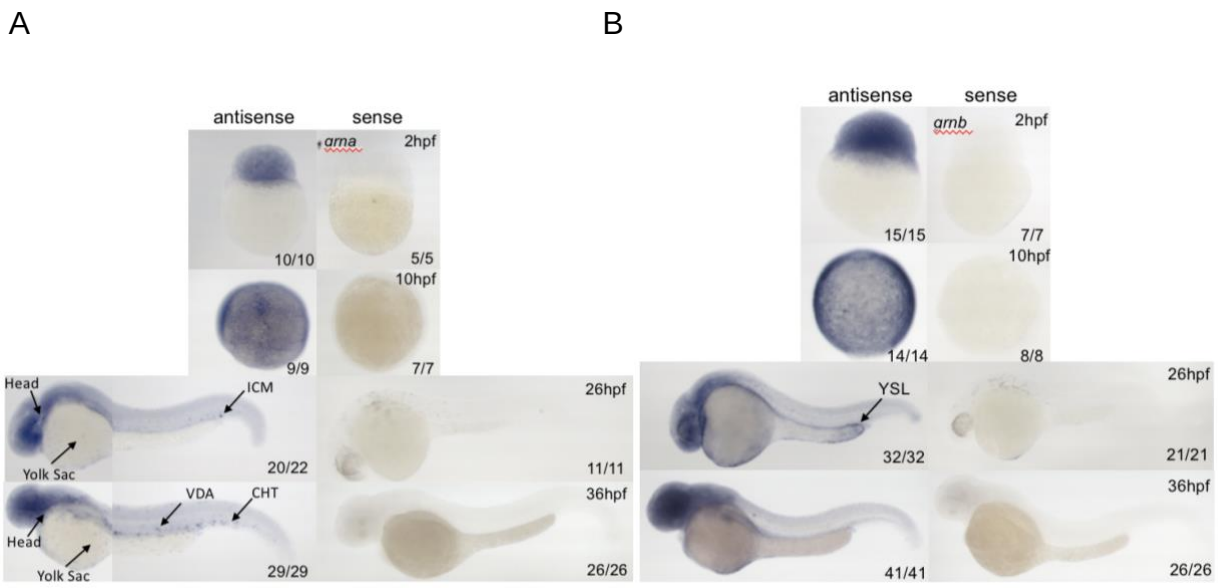

Figure 2. Expression of zebrafish *grna* and *grnb* mRNA during embryo development by WISH. Expression of *grna* (A) or *grnb* (B), in wildtype embryos using WISH at 2, 10, 26, 36 hpf. At 2 hpf and 10 hpf, *grna* is weakly expressed ubiquitously. Starting at 24 hpf, *grna* is expressed by discrete cells in the intermediate cell mass (ICM) (arrows). At 36 hpf, *grna* is express by cells in the head, ventral dorsal aorta (VDA) and caudal hematopoietic tissue (CHT) (arrows). (B) At 2 hpf and 10 hpf, *grnb* is strongly expressed ubiquitously. At 24 hpf, *grnb* expression was found in the yolk syncytial layer (YSL) (arrow), and at 36 hpf, grnb has an ubiquitous expression pattern.

3.2 Identification of the cell type that express *grna*

After validating *grna* expression patterns during embryonic development, we investigated the cell population that was responsible for the synthesis of *grna*. Since *grna* transcripts showed a similar distribution as myeloid cells [18] (Figure 2A). We hypothesized that myeloid cells synthesized *grna* during early embryonic development. We then performed fluorescent *in situ* hybridization (FISH) for *grna* and the myeloid marker *pu.1* at 38 hpf. We observed both *grna* and *pu.1* transcripts within the boundary of same cell (Figure 3). The co-localization of *grna* and *pu.1* transcripts suggested that

myeloid cells were responsible for the synthesis of *grna* during early embryonic development.

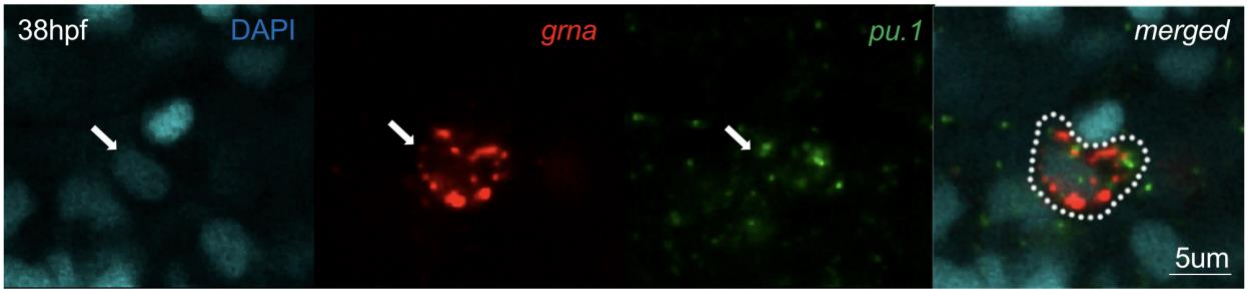

Figure 3. Zebrafish *grna* is expressed by myeloid cells. The co-localization of zebrafish *grna* and *pu.1* transcripts is shown by double fluorescent whole mount *in situ* hybridization in wildtype zebrafish embryos using digoxigenin (DIG)-labeled *grna* RNA probe (red) and fluorescein (FITC)-labeled *pu.1* RNA probe (green) at 38 hpf. Nuclei are stained by DAPI (blue).

3.3 Role of zebrafish *grna* in embryonic hematopoiesis

3.3.1 Knock-down and knock-out strategies

Next, we attempted to investigate the role of Grna in the homeostasis of myeloid cells by knock-down and knock-out strategies. Translation blocking morpholinos (MOs) targeting the 5'UTR-ATG region of *grna* (Grna MO1, hereinafter named Grna MO; and Grna MO2) were used to knock down Grna in the zebrafish embryos [23]. A mismatch morpholino with a 5 nucleotide difference from the Grna MO was used as a control (Figure 4) In addition, we obtained Pgrna mutants that had been previously characterized [22]. These mutants had a pre-mature stop codon at Exon 1 in the *grna* gene [22] (Figure 4)

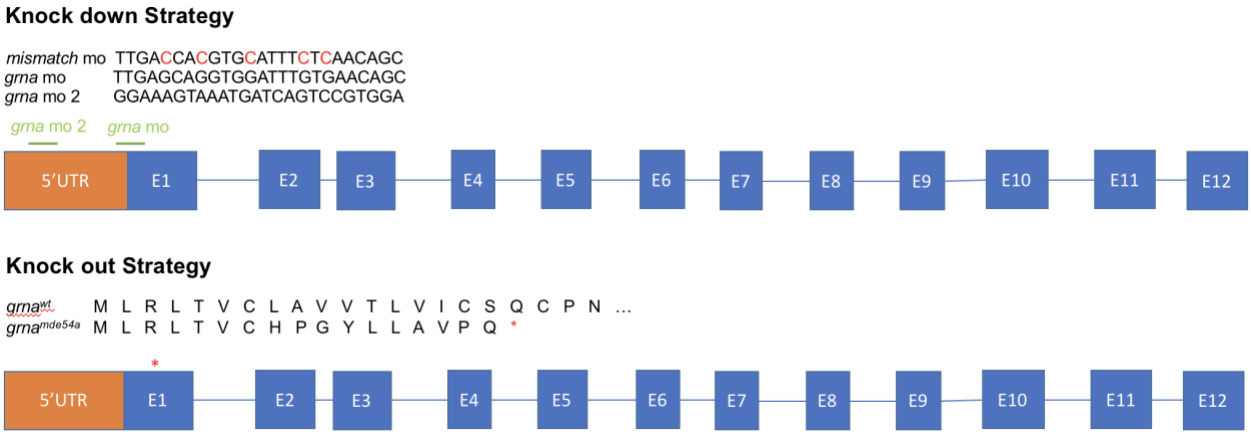

Figure 4. Schematic illustration of the *grna* knock-out and knock-down strategies. Zebrafish *grna* contains 12 exons. Grna mo and Grna mo2 are 5'UTR-ATG translation blocking morpholinos. *mismatch* morpholino is used as the control of the morpholino experiments. *grna* knock-out mutants contain a pre-mature stop codon (asterisk) in exon 1 of *grna*.

3.3.2 *grna* knock-down impaired myeloid differentiation into macrophages

Grna morpholino and mismatch control morpholino were injected into *Tg(mpeg:GFP)* zebrafish embryos at the single cell stage in order to study the role of Grna during macrophage development. We found that there was no statistical differences in the number of macrophages (*mpeg*$^{+}$) at 24 hpf (Figure 5A) between Grna and mismatch morpholino injected embryos, which suggested that primitive macrophages were not affected. However, we did detect a significant decrease in the number of *mpeg*$^{+}$ cells at both 30 hpf and 50 hpf (Figure 5A, B) in *Grna* morphants compared to control embryos. This suggests that myeloid differentiation towards the macrophage lineage during definitive hematopoiesis was impaired in the absence of Grna. We further confirmed our results using flow cytometry. It was found that the percentage of *mpeg*$^{+}$ cells dropped significantly in Grna morphants (Grna MO and Grna MO 2) compared to control siblings (Figure 6A, B). Taken together, these data suggest that Grna is required for myeloid differentiation into macrophages during definitive hematopoiesis.

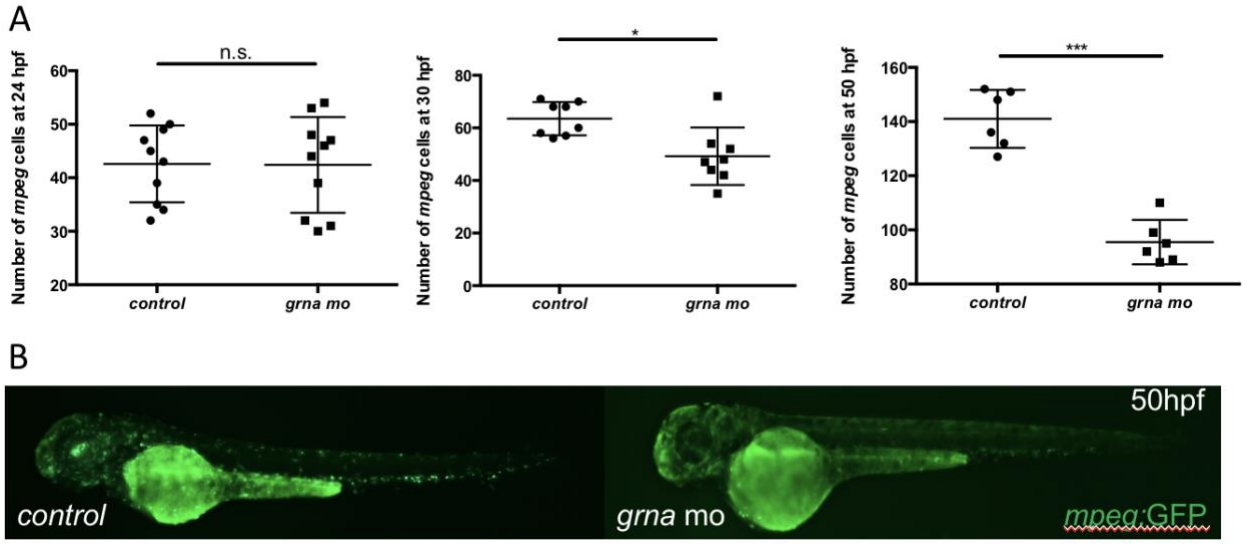

Figure 5. Knock-down of *grna* impairs the numbers of definitive macrophages during embryo development. (A)Quantification of *mpeg* positive cells in the tail?? Of Grna morphants and control (mismatch) siblings) at 24 hpf, 30 hpf and 50 hpf embryos (24 hpf, control, n=10; *grna*, n=9; n.s. p>0.05; 30 hpf, control, n=8; *grna* mo, n=8; *p<0.05; 50 hpf, control, n=6; *grna* mo, n=6; ***p<0.001). (B) Fluorescent microscope images of 50 hpf *mpeg*:GFP embryos injected with *mismatch (control)* and *grna* morpholinos.

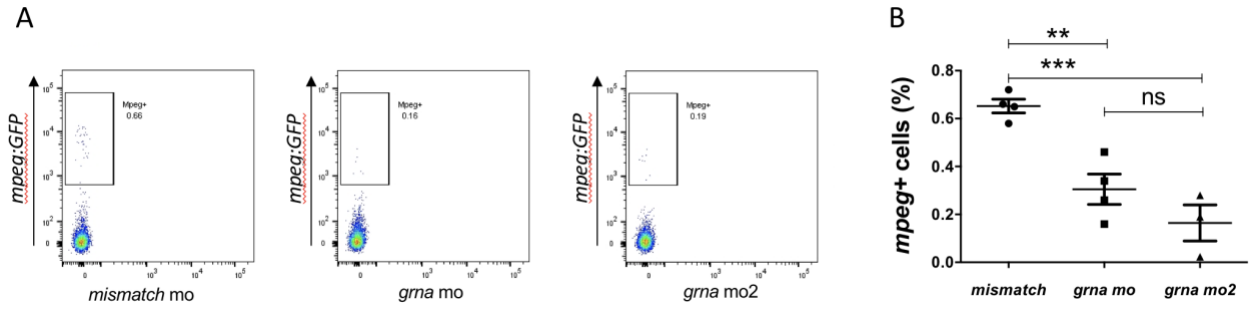

Figure 6. Grna Knock-down impairs macrophage numbers during embryo development by flow cytometry. (A) *mpeg* positive cells of *mismatch* and Grna morphants are analyzed by flow cytometry at 38 hpf. (B) Quantification of *mpeg* positive cells by FlowJo. Each dot represents the percentage of *mpeg*$^{+}$ cells from the total events from 3 embryos, and the bar and error bars indicate means ± SEM (**p<0.01, ***p<0.001).

3.3.3 *grna* knock-down impaired myeloid differentiation into neutrophil

Grna MO was also injected into *Tg(mpx:GFP)* zebrafish embryos at the single cell stage in order to study the role of Grna on neutrophil formation. We found that there were no statistical differences on the number of *mpx*$^{+}$ cells at 30 hpf in Grna morphants compared to control siblings (Figure 7A), which suggested that primitive neutrophils were not affected. However, we did detect a significant decrease in the numbers of *mpx*$^{+}$ cells at 50 hpf in Grna morphants compared to controls (Figure7A, B). This suggested that myeloid differentiation into neutrophils during definitive hematopoiesis was impaired when *grna* expression was knocked down. Decrease of both macrophage and neutrophil numbers indicates that loss of Grna function impairs myeloid differentiation during definitive hematopoiesis.

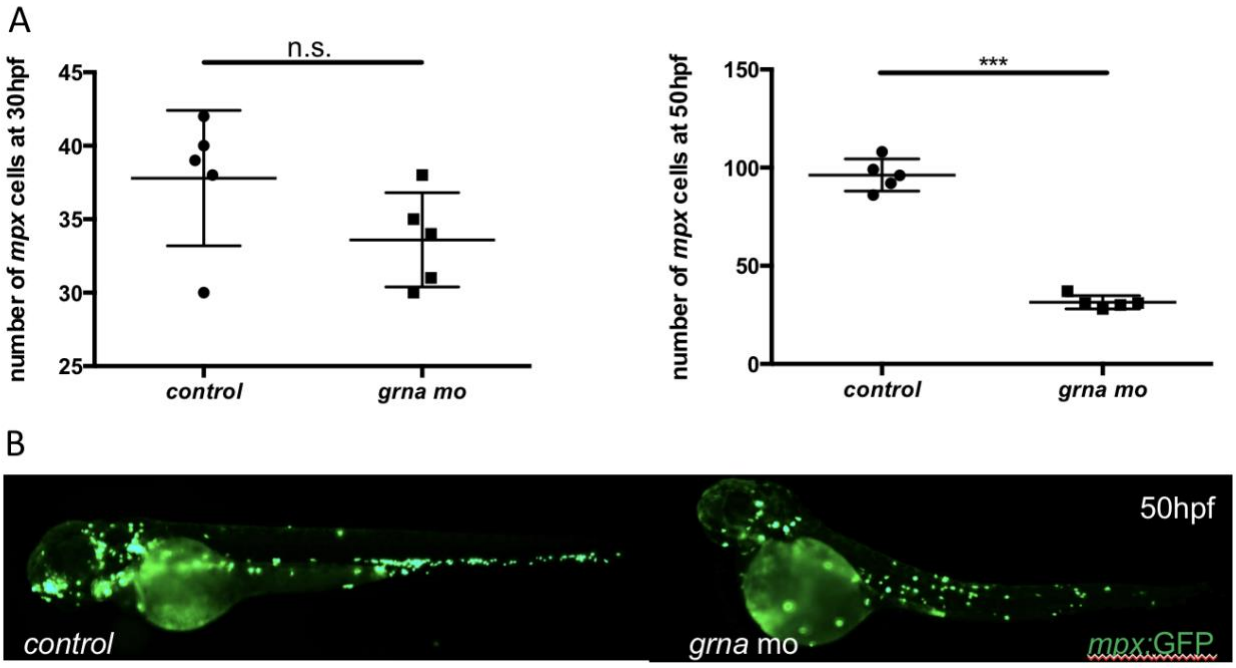

Figure 7. Grna knock-down impairs neutrophil numbers from 50 hpf embryo development. (A) Quantification of *mpx* positive cells in the tail at 30 hpf and 50 hpf embryos (30 hpf, control, n=5; *grna* mo, n=5; n.s. p>0.05; 50 hpf, control, n=5; Grna mo, n=5; ***p<0.001) (B) Fluorescent microscope images of *mpx*:GFP at 50 hpf embryos injected with *mismatch (control)* and Grna morpholino.

3.3.4 Grna mutants recapitulate the myeloid defects observed after Grna knockdown

After showing that *grna* knock-down had a significant impact on myeloid differentiation, we investigated if the loss of macrophages and neutrophils was also present in *grna* mutants. We then performed WISH for *mpx* of *grna*^{wt} and *grna*$^{mde54a-/-}$ embryos at 48 hpf. There was a statistically significant decrease of *mpx*$^{+}$ cells in the *grna*$^{mde54a-/-}$ mutants compared to *grna*^{wt} embryos (Figure 8A, B). This result validated our Grna knockdown data, supporting the hypothesis that Grna is essential for myeloid development.

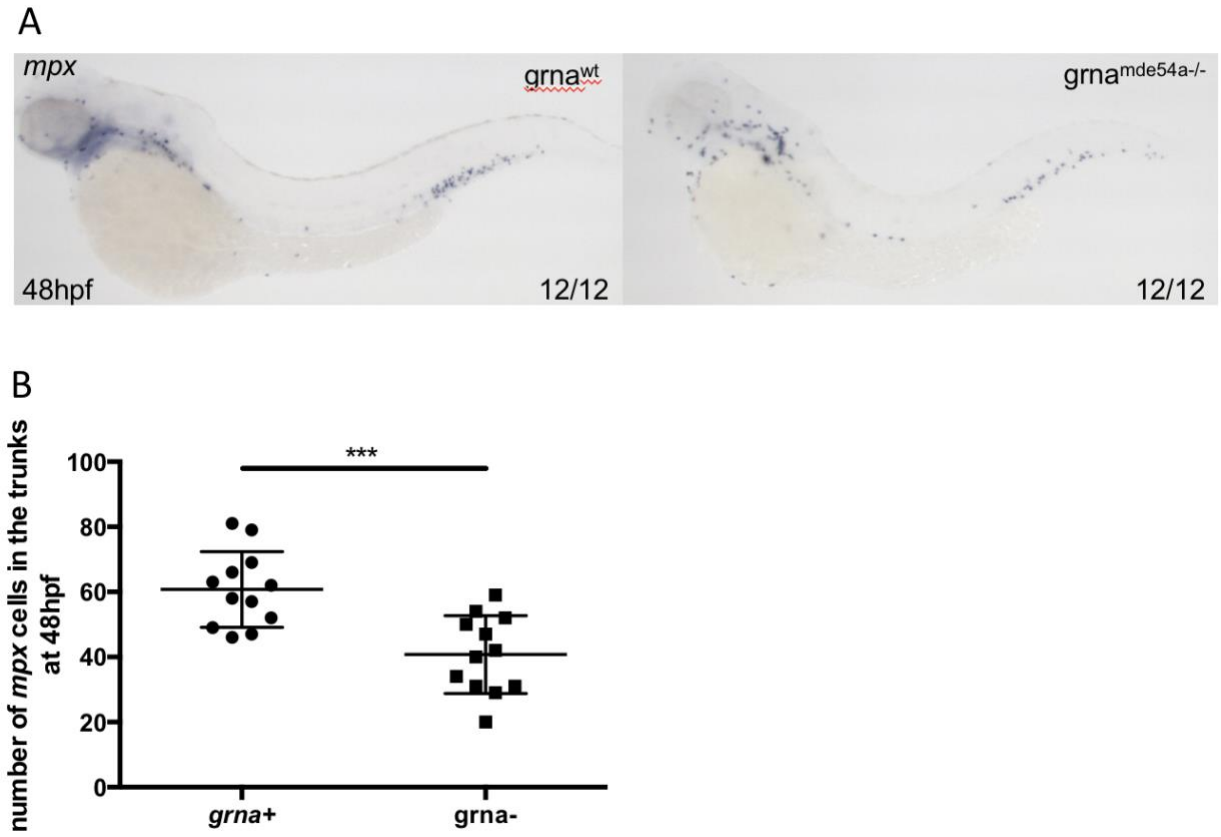

Figure 8. Knock-out of *grna* reduces the amount of neutrophils. (A) Representative images of *mpx* expression assessed by WISH of *grna*^{wt} *and grna*$^{mde54-/-a}$ embryos at 48 hpf. (B) Quantification of neutrophil numbers from (A). Each dot represents the number of neutrophils per embryo in the trunk. Bars and error bars indicate means ± SEM. ***p<0.01.

3.4 Loss of neutrophils is not due to cell apoptosis

After determining that *grna* is required for proper myeloid differentiation from 30 hpf on but not earlier, we investigated if the loss of neutrophils and macrophages was due to cell apoptosis. We performed TUNEL assays on *Tg(mpx:GFP)* and *Tg*(*mpeg:GFP*) embryos injected with *grna* and mismatch morpholinos at 30 hpf. We did not find any TUNEL^{+} *mpx*$^{+}$ cells, orTUNEL^{+} *mpeg*$^{+}$ cells (Data not shown), in both control embryos and *grna* morphants at 30 hpf (Figure 9). This data suggested that the loss of neutrophils and macrophages was not due to apoptosis.

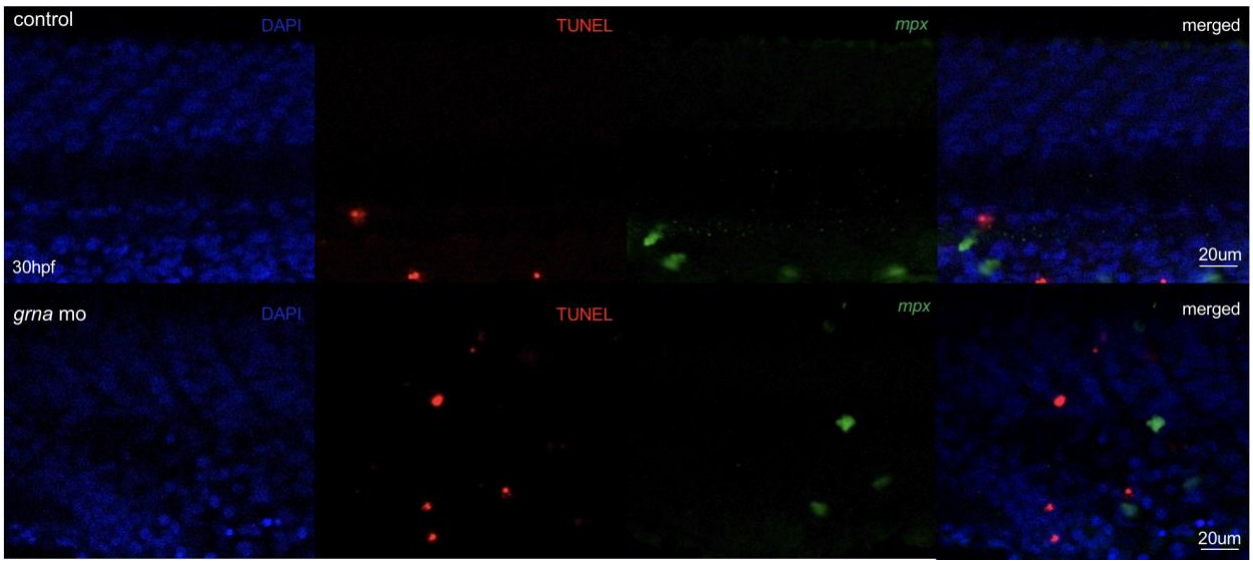

Figure 9. Knock-down of *grna* did not cause loss of neutrophils through apoptosis. PBI region of 30 hpf *mpx:*GFP *mismatch* (control), and *grna* morphant embryos assayed for TUNEL (red) and GFP expression (green).

3.5 Loss of neutrophil numbers in Grna morphants was rescued by *grna* overexpression

After observing impaired myeloid differentiation during hematopoiesis, we attempted to rescue the phenotype by overexpressing *grna*. We co-injected *grna* and mismatch MOs with an overexpression vector containing the cassette *hsp70:grna-nlsGFP* into WT embryos. The heat shock promoter *hsp70* was activated by heat shock (three one-hour heat shocks at 24, 30, and 36 hpf at 37°C). The embryos were then harvested at 38 hpf to be analyzed by WISH. As expected, we observed a reduction in *mpx*$^{+}$ cells in the *grna* morphants compared to control embryos (Figure 10A, B). Furthermore, we observed that *grna* overexpression resulted in a significant increase in the number of *mpx*$^{+}$ cells (Figure 10A, B). There was a partial rescue of neutrophil numbers in *grna* morphants after *grna* overexpression compared to *grna* morphants alone (Figure 10A, B).

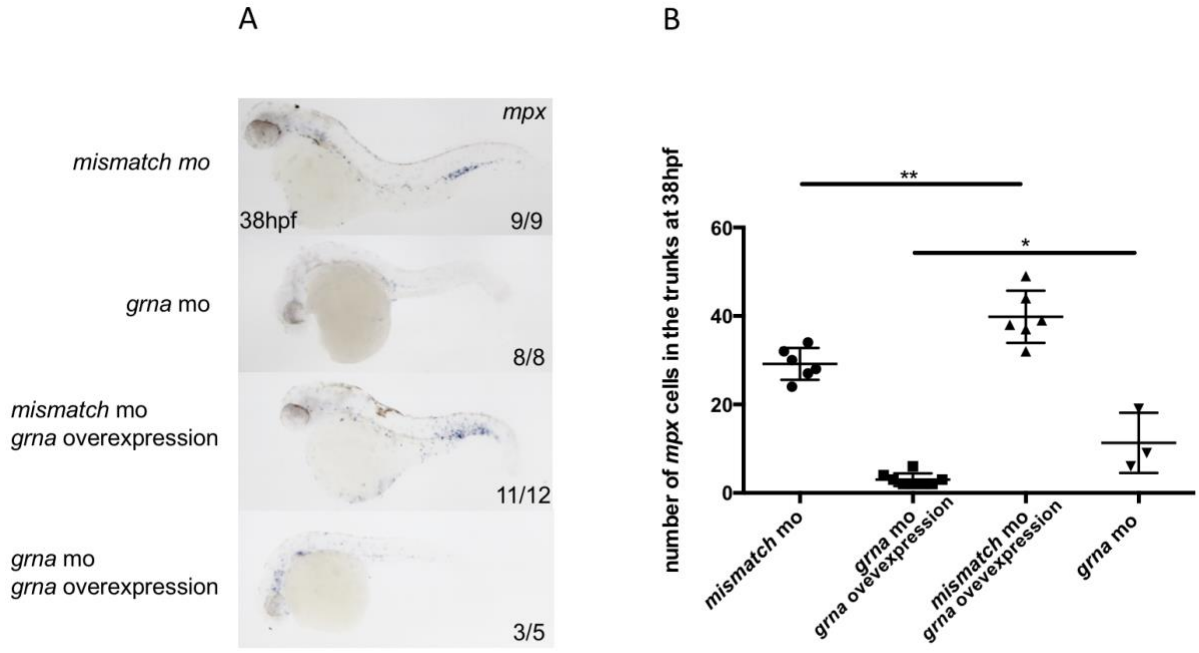

Figure 10. *grna* overexpression partially rescues the loss of neutrophils observed after Grna knockdown. (A) Representative images of WISH for the neutrophilic marker *mpx* of 38 hpf wildtype embryos injected with *mismatch* and *grna* morpholinos in combination with *hsp70:grna-nlsGFP*. (B) Quantification of neutrophils from (A). Each dot represents the number of neutrophils per embryo in the trunk. Bars and error bars indicate means ± SEM. *p < 0.05, **p<0.01.

3.6 Grna is essential for myeloid specification from EMPs.

To investigate the role of Grna during definitive hematopoiesis, we attempted to check if EMP-derived macrophages were reduced when *grna* was knocked down. Because EMPs have *gata1* expression from 24-48 hpf and can lineage differentiate into both myeloid ($Mpeg^+$) and erythroid ($Gata1^+$) cells [11], we can then distinguish EMP-derived macrophages ($Mpeg^+$ $Gata1^+$) from primitive macrophages ($Mpeg^+$ $Gata1^-$). We injected both mismatch and *grna* morpholinos into double transgenic embryos *Tg(mpeg:GFP; gata1:DsRed)*. Embryos were harvested at 36 hpf to be analyzed by flow cytometry. We found a reduction of $Mpeg^+Gata1^+$ cells when the embryos were injected

with *grna* MO but no significant change of Mpeg^{+}Gata1^{-} cells compared to the control (Figure 11A, B). This further confirmed that primitive macrophages were not affected when Grna was knocked down. In addition, we detected a reduction of EMP-derived macrophages in the absence of Grna.

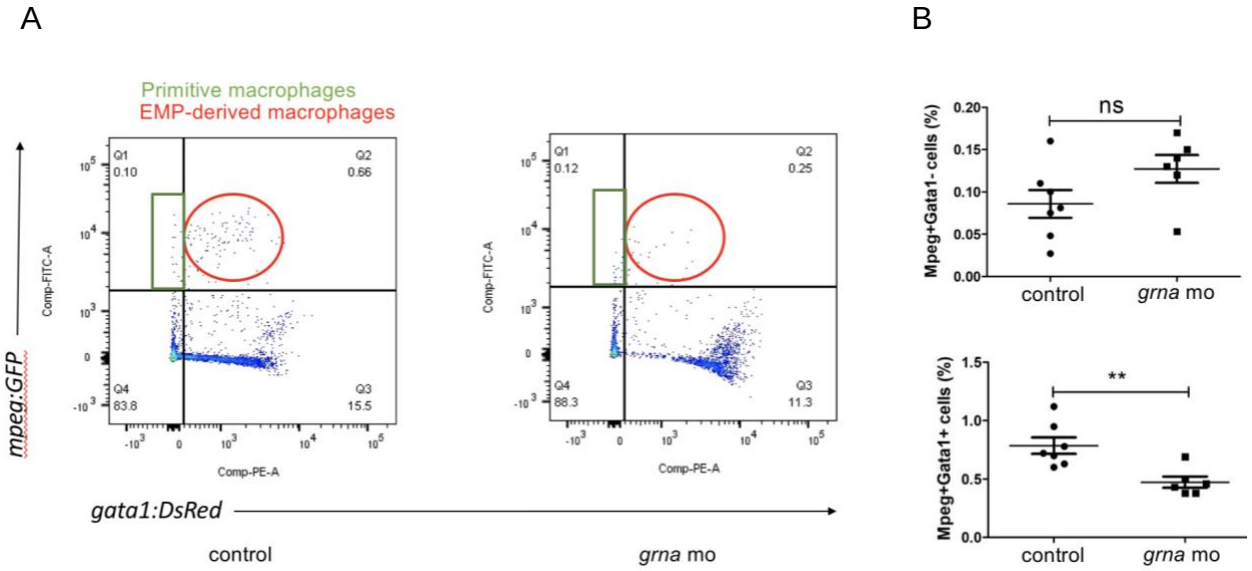

Figure 11. EMP-derived macrophages were reduced in the absence of Grna. (A) Flow cytometry data of mpeg^{+}gata1^{+} cells (red) and mpeg^{+}gata1^{-} cells (green) from *Tg(mpeg:GFP; gata1:DsRed*) embryos injected with mismatch (control) and grna morpholinos at 36 hpf. (B) Quantification of mpeg^{+}gata1^{-} cells (upper panel), and mpeg^{+}gata1^{+} cells (lower panel) from (A). Each dot represents the percentage of cell from the total event of 3 embryos. Bars and error bars indicate means ± SEM. **p<0.01; ns: not significant.

3.7 *pu.1* acts upstream of *grna*, and *grna* negatively regulates *gata1* expression

It was previously shown that the lineage-specific transcription factors GATA1 and PU.1 negatively regulate each other. While high *GATA1* expression leads to erythroid differentiation, high *PU.1* expression levels trigger myeloid differentiation [24]. These roles of GATA1 and PU.1 in hematopoietic differentiation are conserved across vertebrates, including the zebrafish [8]. Since Pu.1 is a key transcription factor for myeloid differentiation, we wanted to know if Pu.1 was activating *grna* transcription in zebrafish. We therefore performed WISH for *grna* after *pu.1* knockdown using a specific morpholino.

We observed that *grna* expression was completely ablated in *pu.1* morphants at 40 hpf (Figure 12A, B). These data suggested that *pu.1* is upstream of *grna* expression. As expected, ablation of Gata1 using a specific MO led to increased *grna* expression (Figure 12A, B), perhaps due to the *pu.1* upregulation observed after Gata1 knockdown [8, 24, 25]. In addition, to investigate if Grna reduced *gata1* expression, we injected the *grna* morphlino into *Tg (Gata1:DsRed)* embryos at the single cell stage. We observed an increase in DsRed intensity in *grna* morphants compared to the control siblings (Figure 13A). Flow cytometry data also indicated that $Gata1^{+}$ cells from *grna* morphants had higher DsRed intensity compared to controls (Figure 13B). This suggested that *grna* also had inhibitory effect on *gata1* expression.

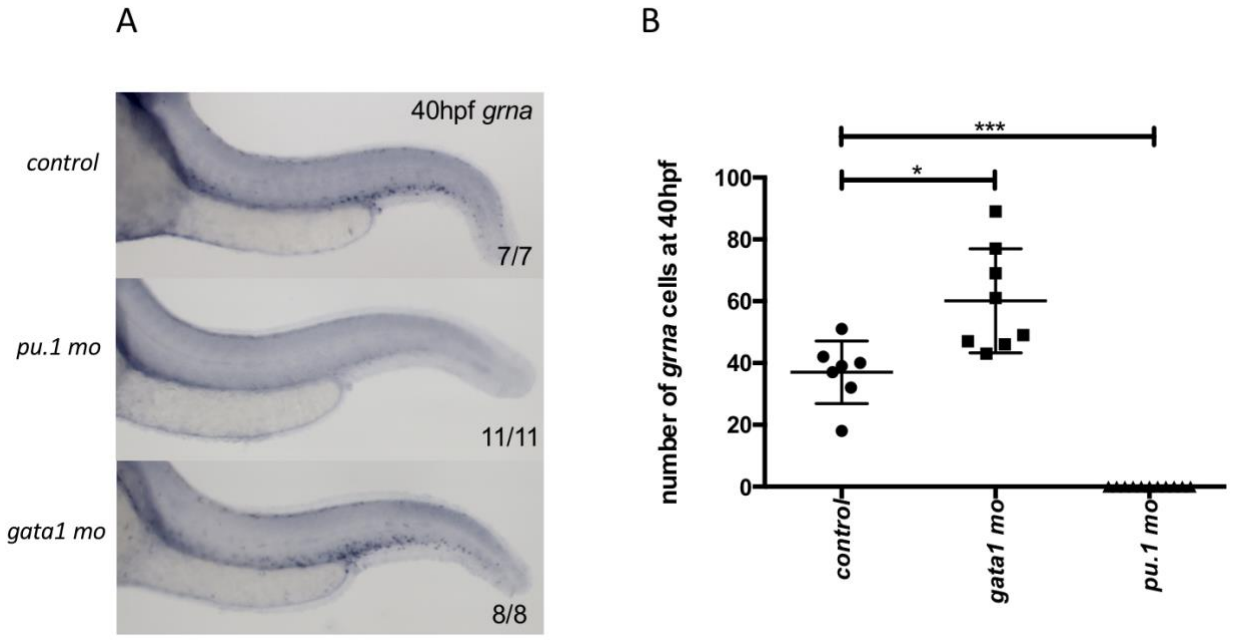

Figure 12. Knock-down of Gata1 increased *grna* expression and knock down of *pu.1* decreased the number of cells expressing *grna*. (A) WISH for *grna* at 40 hpf in embryos injected with standard (std), *pu.1*, and *gata1* morpholinos. Numbers indicate the number of embryos with the shown phenotype. (B) Quantification of *grna* positive cells from (A). Each dot represents the number of *grna* positive cells per embryo in the trunk..Bars and error bars indicate means ± SEM.*p<0.05, ***p<0.01

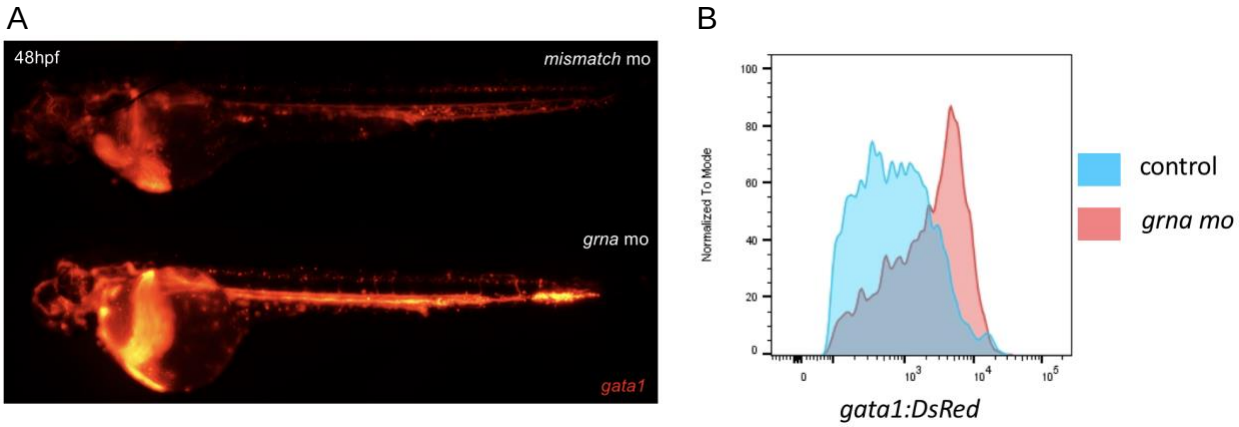

Figure 13. Grna knock-down enhanced *gata1* expression. (A) Fluorescent microscope images of *Gata1*:DsRed embryos injected with *mismatch* (control) and Grna morpholinos at 48 hpf. (B) Percentage of *gata1* positive cells (y-axis) are presented with *gata1:DsRed* intensity (x-axis) from *mismatch* (blue) and *grna* (red) morphants at 38 hpf by flow cytometry.

Discussion

Previous studies mainly focused on the role of GRN in frontotemporal dementia and tumorigenesis, but the role that GRN played in hematopoiesis was unknown. In this study, we have characterized *in vivo* the role of GRN in the regulation of myeloid differentiation in primitive and definitive hematopoiesis. Our results suggest that *grna* is dispensable for primitive myeloid differentiation but has an important role in myeloid differentiation from EMPs in the definitive wave of hematopoiesis. WISH data further suggested that Grna acts downstream of the lineage-specific transcription factor Pu.1. In addition, we demonstrated that Grna downregulates the expression of the erythroid transcription factor *gata1*.

We first characterized both the temporal and spatial expression of zebrafish *grna* and *grnb* during embryonic development. Both *grna* and *grnb* transcripts were found to be expressed during the first days of embryo development. The detection of *grna* and *grnb* transcripts during the first four hours of embryonic development is due to maternally-loaded RNA transcripts during oogenesis [26]. Additionally, *grna* transcripts were detected specifically at the locations of hematopoiesis (ICM, VDA, and CHT). This suggested that *grna* might be involved in embryonic hematopoiesis. Identification of myeloid progenitors that expressed *grna* during embryonic hematopoiesis further suggested that *grna* might be required during myeloid differentiation.

To characterize the role of Grna in the regulation of myeloid differentiation, we utilized *Mpx:GFP* and *Mpeg:GFP* transgenic zebrafish lines to image the activity of early neutrophils and macrophages, respectively, *in vivo*. Primitive macrophages emerge from

cephalic mesoderm in zebrafish embryos between 18-24 hpf and provide transient immunity before the onset of definitive hematopoiesis. In our study, live imaging and quantification of primitive macrophages and neutrophils before the definitive waves indicated that *grna* is dispensable for proper primitive myelopoiesis. In contrast, Grna knockdown and knockout experiments led to decreased macrophage and neutrophil numbers from 30 hpf onwards, a time when EMPs have already emerged. In addition, analysis of primitive (*mpeg*$^{+}$*, gata1*$^{-}$) and definitive (*mpeg*$^{+}$*, gata1*$^{+}$) macrophages by flow cytometry in Grna knockdown experiments showed that Grna participates in myeloid differentiation from definitive, but not primitive hematopoiesis.

Even though neutrophil numbers were increased by *grna* overexpression, we did not obtain a full recovery of differentiated neutrophils. In contrast to mRNA overexpression, injection of DNA plasmid generates mosaic expression. However, mRNA is less stable and can degrade within the first 24 hpf in the embryo, while DNA is more stable. To overcome this caveat, we have generated a stable *hsp70:grna-nlsGFP* transgenic line that we hope will give a more robust rescue for the loss of function phenotypes described here for Grna. This line is currently growing in our zebrafish facilities.

Most studies have shown that GRN is a secreted growth factor [27, 28]. However, it has also been reported to regulate intracellular activities when it was localized inside the cells [29]. A previous study found that granulin interacted with cyclin T1 to inhibit transcription [30], suggesting a role for GRN in the nucleus. Although we addressed here a role for Grna during embryonic hematopoiesis, more experiments should be conducted

to address if Grna acts in the nucleus or as a secreted protein in the context of hematopoiesis.

The lineage-specific transcription factors PU.1and GATA1 antagonize each other to inhibit either erythroid or myeloid lineage differentiation, respectively [31, 32]. PU.1 was found to auto-regulate itself via an upstream regulatory element (URE) to control the levels of PU.1 to regulate myeloid-erythroid differentiation [33]. In this study, we found that zebrafish Pu.1 acts upstream of *grna*. Additionally, we were able to predict nine Pu.1 and one Gata1 binding sites within the *grna* promoter using ConSite (http://consite.genereg.net/). These data suggest that Pu.1 and Gata1 might have a direct regulatory effect on *grna* expression, and ChIP-qPCR experiments should be performed to test this hypothesis.

Interestingly, granulin was also expressed in adult HSCs in mice [34]. It was found that leukemic bone marrow cells had high levels of GRN expression, and these GRN^{+} cells are capable of promoting tumor progression [34]. In addition, samples from breast cancer patients revealed a positive correlation between levels of GRN and progression of breast cancer patients [34]. The data suggested that GRN plays an important role in carcinogenesis. On the other hand, the balance of PU.1 and GATA1 is critical for the myeloid and lymphoid lineage differentiation. In mice, it was discovered that disruption of the balance of PU.1 and GATA1 activity can lead to leukemogenesis [24]. One study found that reduction of PU.1 expression in mice can lead to the onset of acute myeloid leukemia [35]. It would be interesting to investigate if the role of Grn in embryonic

hematopoiesis is also conversed in adult hematopoiesis, and it might be an attractive therapeutic target for the treatment of carcinogenesis and hematological disorders.

In conclusion, we discovered a new function of Grna as an important regulator of myeloid lineage differentiation during hematopoiesis. With the discovery of the role of Grna during hematopoiesis and many more molecular factors in the future, we can fill the gap of our understandings of the hematopoietic process. Furthermore, knowing the spatial and temporal relationships of these important molecular factors can help us understand the fate of lineage differentiation of HSC-derived progenitor cells. These inputs can help develop therapeutic target for treating blood disorders in the future.

References

1. Orkin, S.H. and L.I. Zon, *Hematopoiesis: an evolving paradigm for stem cell biology.* Cell, 2008. **132**(4): p. 631-44.

2. Pietras, E.M., M.R. Warr, and E. Passegue, *Cell cycle regulation in hematopoietic stem cells.* J Cell Biol, 2011. **195**(5): p. 709-20.

3. Nakamura-Ishizu, A., H. Takizawa, and T. Suda, *The analysis, roles and regulation of quiescence in hematopoietic stem cells.* Development, 2014. **141**(24): p. 4656-66.

4. Kondo, M., *Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors.* Immunol Rev, 2010. **238**(1): p. 37-46.

5. Lanot, R., *Postembryonic hematopoiesis in Drosophila.* Dev Biol, 2001. **230**(2): p. 243-57.

6. Muller, A.M., *Development of hematopoietic stem cell activity in the mouse embryo.* Immunity, 1994. **1**(4): p. 291-301.

7. Davidson, A.J. and L.I. Zon, *The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis.* Oncogene, 2004. **23**(43): p. 7233-46.

8. Rhodes, J., *Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish.* Dev Cell, 2005. **8**(1): p. 97-108.

9. Jing, L. and L.I. Zon, *Zebrafish as a model for normal and malignant hematopoiesis.* Dis Model Mech, 2011. **4**(4): p. 433-8.

10. Galloway, J.L. and L.I. Zon, *Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo.* Curr Top Dev Biol, 2003. **53**: p. 139-58.

11. Bertrand, J.Y., *Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo.* Development, 2007. **134**(23): p. 4147-56.

12. Bertrand, J.Y., *Haematopoietic stem cells derive directly from aortic endothelium during development.* Nature, 2010. **464**(7285): p. 108-11.

13. Liu, C.J. and X. Bosch, *Progranulin: a growth factor, a novel TNFR ligand and a drug target.* Pharmacol Ther, 2012. **133**(1): p. 124-32.

14. Espin-Palazon, R., *Proinflammatory signaling regulates hematopoietic stem cell emergence.* Cell, 2014. **159**(5): p. 1070-1085.

15. Sawamiphak, S., Z. Kontarakis, and D.Y. Stainier, *Interferon gamma signaling positively regulates hematopoietic stem cell emergence.* Dev Cell, 2014. **31**(5): p. 640-53.

16. He, Q., *Inflammatory signaling regulates hematopoietic stem and progenitor cell emergence in vertebrates.* Blood, 2015. **125**(7): p. 1098-106.

17. Tang, W., *The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice.* Science, 2011. **332**(6028): p. 478-84.

18. Cadieux, B., *The zebrafish progranulin gene family and antisense transcripts.* BMC Genomics, 2005. **6**: p. 156.

19. Renshaw, S.A., *A transgenic zebrafish model of neutrophilic inflammation.* Blood, 2006. **108**(13): p. 3976-8.

20. Traver, D., *Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants.* Nat Immunol, 2003. **4**(12): p. 1238-46.

21. Ellett, F., *mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish.* Blood, 2011. **117**(4): p. e49-56.

22. Solchenberger, B., *Granulin knock out zebrafish lack frontotemporal lobar degeneration and neuronal ceroid lipofuscinosis pathology.* PLoS One, 2015. **10**(3): p. e0118956.

23. Li, Y.H., *Progranulin A-mediated MET signaling is essential for liver morphogenesis in zebrafish.* J Biol Chem, 2010. **285**(52): p. 41001-9.

24. Burda, P., P. Laslo, and T. Stopka, *The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis.* Leukemia, 2010. **24**(7): p. 1249-57.

25. Palis, J., *Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.* Development, 1999. **126**(22): p. 5073-84.

26. Preuss, K.M., *Identification of maternally-loaded RNA transcripts in unfertilized eggs of Tribolium castaneum.* BMC Genomics, 2012. **13**: p. 671.

27. Zanocco-Marani, T., *Biological activities and signaling pathways of the granulin/epithelin precursor.* Cancer Res, 1999. **59**(20): p. 5331-40.

28. Bateman, A. and H.P. Bennett, *The granulin gene family: from cancer to dementia.* Bioessays, 2009. **31**(11): p. 1245-54.

29. Daniel, R., *Cellular localization of gene expression for progranulin.* J Histochem Cytochem, 2000. **48**(7): p. 999-1009.

30. Hoque, M., *The growth factor granulin interacts with cyclin T1 and modulates P-TEFb-dependent transcription.* Mol Cell Biol, 2003. **23**(5): p. 1688-702.

31. Zhang, P., *PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding.* Blood, 2000. **96**(8): p. 2641-8.

32. Burda, P., *GATA-1 Inhibits PU.1 Gene via DNA and Histone H3K9 Methylation of Its Distal Enhancer in Erythroleukemia.* PLoS One, 2016. **11**(3): p. e0152234.

33. Okuno, Y., *Potential autoregulation of transcription factor PU.1 by an upstream regulatory element.* Mol Cell Biol, 2005. **25**(7): p. 2832-45.

34. Elkabets, M., *Human tumors instigate granulin-expressing hematopoietic cells that promote malignancy by activating stromal fibroblasts in mice.* J Clin Invest, 2011. **121**(2): p. 784-99.

35. Rosenbauer, F., *Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1.* Nat Genet, 2004. **36**(6): p. 624-30.