**Eafs Control Erythroid Cell Fate by Regulating c-myb Expression through Wnt Signaling**

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**Abstract**

ELL associated factor 1 and ELL associated factor 2 (EAF1/2 factors) are reported to play important roles in tumor suppression and embryogenesis. Our previous studies showed that eaf factors mediated effective convergence and extension (C&E) movements and modulated mesoderm and neural patterning by regulating both non-canonical and canonical Wnt signaling in the early embryonic process. In this study, through knockdown of both eaf1 and eaf2 in embryos, we found that differentiation of primary erythroid cells was blocked, but hematopoietic precursor cells maintained in eafs morphants. Co-injection of c-myb-MO rescued the erythroid differentiation in eafs morphants, as indicated by the restored expression of the erythroid-specific gene, β3 globin. In addition, low dosage of c-myb effectively blocked the β3 globin expression in embryos, and did not affect the expression of markers of hematopoietic progenitor cells and other mesoderm, which was similar to the phenotypes we observed in eafs morphants. We also revealed that knockdown Wnt signaling by transiently inducing dn-Tcf in embryos at the bud stage down-regulated the increased c-myb to normal level and also restored β3 globin expression in eafs morphants. Our evidence points to a novel role for eaf factors in controlling erythroid cell fate by regulating c-Myb expression through canonic Wnt signaling.

**Introduction**

Human hemoglobinopathies are a group of genetic disorders caused by abnormal expression of globin genes, that result in anemia in patients. The molecular mechanism underlying the globin genes induction is evolutionarily conserved among vertebrates, and the process appears to depend on the extensive sharing of lineage-restricted transcriptional factors [1]. Gata1 is the erythroid factor, which is highly expressed in megakaryocytic/erythroid progenitors [2,3,4,5]. It is essential for erythroid specification and differentiation [3,5] and cooperates with runx1 and fli1, which are involved in the divergence of megakaryocytic from the erythroid lineage [6,7]. On the contrary, Pu-1, a myeloid factor, is lineage-restrictively required for granulocyte/macrophage induction [2,5]. Lmo2, gata2 and scl are required earlier in the hematopoiesis process and restrict hemoto-vascular development of lateral mesoderm [8,9]; in addition, they are also in a complex required for full erythroid cell maturation [10].

The c-Myb transcriptional factor is a key regulator of the hematopoietic stem and progenitor cells, one that is essential for the establishment of hematopoiesis as evidenced by the defects in the development of multiple blood lineages in the Myb null mice [11,12,13]. Conditional c-Myb knockout in adult hematopoietic stem cells leads to loss of self-renewal due to impaired proliferation and accelerated differentiation [14]. The c-Myb level must be down-regulated in order for hematopoietic cells terminal differentiation and maturation [15]. Constitutive expression of c-Myb blocks Friend murine erythroleukemia cell differentiation [16]. Expression of c-Myb was repressed by GATA-1 to allow further differentiation of erythrocyte at the onset of terminal differentiation in erythroblast [17,18]. On the contrary, by using microRNAs to knockdown c-Myb expression in primary human erythroid progenitor cells, fetal and embryonic hemoglobin genes displayed elevated expression. This suggests that c-Myb plays an important role in silencing the fetal and embryonic hemoglobin genes [19]. In addition, those microRNAs elevate fetal hemoglobin expression via c-Myb in human trisomy13 [19].

During zebrafish embryogenesis, eaf factors have been revealed to mediate effective C&E movements through maintaining expression of non-canonical Wnt signaling ligands, Wnt5 and Wnt11 [20], and to modulate mesoderm and neural patterning by inhibiting canonical Wnt signaling [21]. In addition, eaf factors form a negative loop with another non-canonical Wnt signaling ligand, Wnt4, in embryos and mammalian cell lines [22]. In the embryos with morpholino-mediated eaf1 and eaf2 knockdown, we observed the high percentage of erythroid differentiation defects, but we still know little about the underlying mechanisms.

In this study, by screening potential repressors of erythroid differentiation in eafs morphants using morpholino-mediated knockdown, we selectively and respectively co-injected morpholinos of genes that had been reported to inhibit erythroid differentiation and also displayed increased expression in eafs morphants. By co-injecting with c-myb-MO [23], we found that knockdown c-myb in eafs morphants could effectively rescue the erythroid differentiation defects, indicated by restored β3 globin expression. Consistently, an over-expressed c-myb in embryos could nearly phenocopy the erythroid differentiation defects shown in...
eafs morphants. In addition, in eafs morphants, by transiently inducing dse-Tcf, a dominant negative form of Wnt signaling, we found that knockdown of canonical Wnt signaling can not only restore the increased expression of c-myb to normal levels, but also restore the reduced β3 globin expression. In our study, we reveal a novel genetic cascade in the process of erythroid differentiation, in which Wnt signaling up-regulates expression of hematopoietic progenitor-restricted transcriptional factors, including c-myb, and the increased c-myb mediates the suppression of erythroid differentiation.

Materials and Methods

Fish Stocks

As previously discussed, wild-type zebrafish (Danio rerio) (AB) maintenance, breeding, and staging were performed [20] and the hsdr/TCF-GFP transgenic line was performed as reported previously [24,25].

Heat-shock Modulation

Embryonic heat-shock experiments were conducted at 38°C for 20 minutes. Genotype was determined by the presence of GFP fluorescence at 3 hours post heat shock, then the non-fluorescent (wild-type) siblings were sorted and used as controls [24,25].

Morpholino and mRNA Synthesis

The translation blocking morpholinos (ATG targeted) eaf1-MO1 and eaf2-MO1, and splicing-blocking morpholinos, eaf1-MO3 and eaf2-MO3, have been described previously [20,21], as the c-myb antisense morpholino have been described previously [23]. All morpholinos were purchased from Gene Tools, LLC (Philomath, Oregon, USA) and their sequences are listed in Table S1.

For mRNA preparation, capped mRNA of c-myb was synthesized using the Amaxap RP6 High Yield message maker kit (Epicenter Biotechnologies). The synthesized mRNAs were diluted into different concentrations and injected into one-cell stage embryos, and the plasmid for c-myb mRNA was described previously [26].

Whole Mount in situ Hybridization and O-Dianisidine Staining

Probes for gata2, fl1, scl, bms2, runx1, gata1, fli1, c-myb and flk1 were reported previously [3,26,27]. Probes for pax2a and myoD were kindly provided by Dr. Schier (Harvard University, Molecular and Cellular Biology), probe for cdh5 was kindly provided by Dr. Wang (Institute of Hydrobiology, Chinese Academy of Science), and probe for ntl was reported previously [20]. The procedures for in situ hybridization and o-dianisidine staining were performed as described previously [20,28].

Western Blot

Western blot was performed as described previously [29]. The embryos at the 24 hpf were washed with ice-cold PBS buffer and then lysed in RIPA (radioimmune precipitation) buffer containing 50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1 mM NaF, 1 mM PMSF (phenylmethylsulphonyl fluoride), 1 mM Na3VO4 (sodium orthovanadate) and 1:100 dilution of protease inhibitor cocktail (Sigma). After homogenizing, lysates were centrifuged for 15 minutes at 12,000 g at 4°C, and the supernatants were boiled with 1 x SDS sample buffer, separated on SDS-PAGE and transferred to PVDF membrane (Millipore). The Western blot analysis was performed as described previously using the indicated anti-β3 globin antibody (Z-FishTM (Zebrafish)) and anti-β-actin antibody (Abcam).

Results

Eafs are Required for Erythroid Differentiation

In our previous studies, we observed C&E movement defects and forebrain defects in eaf1 and eaf2 morphants, revealing that both eaf1 and eaf2 mediate C&E movements by non-canonical Wnt signaling and modulate mesoderm and neural patterning by inhibiting canonical Wnt signaling [20,21]. In the late stage, in eafs morphants at 2 dph, we observed significant defects including heart edema, reduced circular blood cells, and a lack of blood flow or clumps of blood cells located in front of the beating heart in embryos knockdown both of eaf1 and eaf2 (data not shown), suggesting that the eafs morphants displayed hematopoietic defects, as observed in nill morphants [30]. Here, in order to further test the molecular characters underlying the phenotypes, we applied blood markers to detect the hematopoietic defects in eafs morphants.

Knockdown of both eaf1 and eaf2 in embryos by injection of either translation-blocking ATG-MO (indicated by eafs-MO1, and shown in Figure 1) or “splicing-blocking” morpholinos (data not shown) caused a striking hematopoietic phenotype. Morpholinos reduced functional eafs message, yielded obviously erythroid defects (Figure 1). Hemoglobin, indicated by o-dianisidine staining, reduced significantly in eafs morphants (Figure 1, A2, A3 and A4), and nearly 100% of morphants displayed dramatically reduced differentiated mature erythrocytes (Figure 1, A3 and A4). By whole mount in situ hybridization (WISH), transcripts of β3 globin, which marked mature erythroid, displayed obviously reduced expression in over half of the detected eafs morphants (59 of 95 morphants, Figure 1, B2); its protein level also significantly in eafs morphants compared to its expression in control embryos (Figure 1, C).

Erythroid Defects in Eafs Morphants are Specific

To determine the specificity of eafs-MO induced erythroid defects, we examined the integrity of non-hematopoietic tissues and other markers which labeling vasculature and hematopoietic progenitors (Figure 2). Eafs morphants displayed obviously anterior neuroectoderm truncation at 24 hpf (Figure 2, A2 and A4, indicated by black arrowhead). These observations were consistent with our previous report that eafs were required for forebrain formation [21]. But the posterior mesoderm, including muscle and notochord, at 24 hpf, were intact but displayed subtle patterning defects in eafs morphants. MyoD displayed more compact expression and striped muscles was irregular (Figure 2, A2), it might be a phenotype of C&E movement defects as we observed previously [20]. Notochord (ntl: Figure 2, A3 and A4) was also normal in a representative morphant which displayed severe anterior truncation (Figure 2, A4, anterior truncation is indicated by black arrowhead). Functional vasculature and primitive hematopoietic progenitors were normal and maintained in eafs morphants. By WISH, eafs-MO injected embryos had a few increased progenitors (c-myb: A5, A6; scl: A7, A8, indicated by black arrow), and had increased vasculature (cdh5: A9, A10; flk1; A11, A12).

In order to test whether the initiation and progress of primitive hematopoiesis were normal in eafs morphants, we detected blood progenitor-restricted transcriptional factors during early somitogenesis. At the 8 somites stage, blood progenitors indicated by scl (Figure 2, B1 and B2), which is required earlier in the
**Figure 1.** Eafs are required for specification of erythroid cells. (A) Erythroid defects in eafs morphants were detected by o-dianisidine staining for hemoglobin. (B) Eafs morphants displayed erythroid defects, indicated by reduced mRNA of βe3 globin. (C) Eafs morphants displayed reduced protein of βe3 globin.

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**Figure 2.** Erythroid defects in eafs morphants are specific. (A) At 24 hpf to 26 hpf, the expression detection of control or eafs-MO injected embryos (8 ng per embryo) processed by WISH for tissue specific genes: somites (A1, A2), notochord (A3, A4), vasculature (cdh5: A9, A10; flk1: A11, A12), and primitive progenitors (c-myb: A5, A6; scl: A7, A8, indicated by black arrow). (B) At the 8 somites stage, primitive hematoposis in eafs morphants, indicated by primitive progenitor genes (scl: B1, B2; lmo2: B3, B4). (C) At the 10 somites stage, progenitors for blood (gata2: C1, C2; lmo2: C3, C4; runx1: C7, C8; c-myb: C15, C16), progenitors for vasculature (flk1: C11, C12), progenitors for both blood and vasculature (scl: C5, C6), more mature erythroid progenitors (gata1: C9, C10), and more mature myeloid progenitors (Pu.1: C13, C14). A1–A12, C13–C16, lateral view, anterior to the left. B1–B4, C1–C12, dorsal view, anterior to the up.

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hematopoiesis and is important for both blood and vessel development, showed normal in eafs morphants. Similarly, bmo2, another important transcriptional factor required earlier in the hematopoiesis process and restricting hemato-vascular development of lateral mesoderm, also displayed normal in eafs morphants (Figure 2, B3 and B4). When the morphants developed to the 10 somites stage (Figure 2, C), however, there was a little overexpression in scl (Figure 2, C5 and C6), and bmo2 (Figure 2, C3 and C4), compared to their respective control; Gata2 (Figure 2, C1 and C2), runx1 (Figure 2, C7 and C8), and pu.1 (Figure 2, C13 and C14) displayed increased expression in eafs morphants from the 10 somites stage; A slight overexpression was also observed in case of b (Figure 2, C3 and C4), gata1 (Figure 2, C9 and C10), and c-myb (Figure 2, C15 and C16).

**Knockdown C-myb in Eafs Morphants Restored β3 globin Expression**

The above observations indicated that blood precursor-restricted transcriptional factors, including gata2, flu, scl, runx1, gata1, bmo2, pu.1, and c-myb increased in eafs morphants from the 10 somites stage (Figure 2), but β3 globin reduced dramatically (Figure 1). Gata1 is essential for erythroid cell fate determination [3,5], and gata2, scl, and bmo2 are factors in the transcriptional complex for erythroid maturation [8,9,10]. However, pu.1, runx1, flu, and c-myb are negative factors that antagonize erythroid differentiation and maturation [6,7]. Thus, we analyzed whether the reduced β3 globin expression might result from the up-regulation of pu.1, runx1, flu, or c-myb in eafs morphants. In order to investigate this hypothesis further, we performed morphorlino-mediated knockdown of those transcriptional factors in eafs morphants respectively. We found that knockdown of c-myb could restore β3 globin expression in eafs morphants to some extent (Figure 3, A3), far fewer embryos (22 of 86 specimen) displayed reduced β3 globin expression after co-injected with c-myb-MO (Figure 3, A3) compared to eafs signal morphants (51 of 93 specimen) (Figure 3, A2). In addition, β3 globin expression also increased in rescued embryos compared to its level in eafs morphants (Figure 2, A3).

Contrary to eafs morphants (Figure 3, B2), c-myb morphants displayed little elevation of expression of β3 globin (Figure 3, B3); the morphants injected with both eafs-MO and c-myb-MO displayed in-between expressions of β3 globin (Figure 3, B4). We also analyzed the morphogenesis of c-myb morphants and defects of non-blood mesoderm in c-myb morphants. As shown in Figure 3C, the c-myb morphants displayed similar phenotypes to those reported previously such as small eyes and small brain (Figure 3, C2) [23], but no obvious defects displayed in the posterior body. In c-myb morphants, the induction of non-blood mesoderm, indicated by pax2a and myoD, also displayed normal expression at the bud stage, although there appear to be subtle patterning defects such as short myoD domain in c-myb morphants (Figure 3, C4).

**C-myb was Required for Maintaining of Hematopoietic Precursors**

Although we found that knockdown c-myb in eafs morphants could rescue β3 globin expression effectively, we still knew little about the genetic cascade between c-myb and eafs factors in this process. In order to further understand the genetic pathway between c-myb and eafs factors in hematopoiesis, we detected more blood markers in c-myb morphants and in morphants co-injected with c-myb-MO and eafs-MO together.

In PLM (posterior lateral mesoderm), the initiation of c-myb was detected around the 4 somites stage (31,32), so we detected the expression of blood markers in c-myb morphants from the 6 somites stage. As shown in Figure 3D, the expression of bmo2 reduced significantly in c-myb morphants from the 6 somites stage (27 of 27 specimen, Figure 3, D2). In embryos co-injected with both c-myb-MO and eafs-MO, bmo2 displayed obviously reduced expression (10 of 13 specimen, Figure 3 D3). In addition, gata1 (14 of 15 specimen, Figure 3, D5) and scl (19 of 21 specimen, Figure 3, D8) also displayed dramatically reduced expression in c-myb morphants. Similarly, in embryos injected with both c-myb-MO and eafs-MO, gata1 (8 of 10 specimen, Figure 3, D6) and scl (13 of 15 specimen, Figure 3, D9) also displayed reduced expression, as we observed in c-myb morphants. All the observations suggested that c-myb might be required to maintain the hematopoietic progenitor cells and could also act downstream of eafs in specification blood cells or regulating expression of blood transcriptional factors.

**C-myb Suppressed β3 globin Expression, but Had no Influence on Hematopoietic Precursors**

With all the above observations taken together, we assumed that c-myb might be required for hematopoietic precursor cells, but would block erythroid differentiation in zebrafish, thus functioning similarly with its mammalian orthologs [14,17,19]. To further detect its roles in hematopoiesis in vivo, we over-expressed c-myb in embryos by mRNA injection. We injected the embryos with different dosages of c-myb mRNA, from 50 pg per embryo to 200 pg per embryo, and the embryos did not display defects of morphology (data not shown). We analyzed the development of hematopoietic cells in embryos injected with different dosages of c-myb mRNA respectively. Most embryos injected with c-myb mRNA displayed obviously reduced expression of β3 globin, either the dosage was 50 pg per embryo (Figure 4, A2) or was 200 pg per embryo (Figure 4, A4), however, gata1, displayed normal expression in all the detected embryos injected with different dosages of c-myb mRNA (Figure 4, A6 and A8). The observations here suggested that, c-myb could effectively suppress β3 globin expression in vivo even at a very low dosage (50 pg per embryo). In order to further detect the roles of c-myb in hematopoiesis in zebrafish embryos, we detected more molecular markers labeling non-hematopoietic tissues or hematopoietic cells, with all embryos injected with the same dosage of c-myb mRNA (50 pg per embryo). At this low dosage (50 pg per embryo), c-myb could effectively suppress β3 globin expression in vivo (37 of 70 specimen, Figure 4, B2), and the corresponding injected c-myb mRNA displayed ubiquitously distribution (Figure 4, B4). We wondered whether reduced β3 globin expression resulted from mesoderm pattern defects, then we detected the expression of other molecular markers, including markers labeling hematopoietic precursors and axis mesoderm, in embryos with ectopic c-myb expression (50 pg per embryo). At the 10 somites stage, the erythroid precursor marker, gata1, displayed similar expression levels in embryos with ectopic expression of c-myb (Figure 4, C2) as in control embryos (Figure 4, C1). Similarly, scl, another erythroid precursor marker, also displayed normal expression in embryos injected with c-myb mRNA (Figure 4, C3 and C4). In addition, non-blood mesoderm markers, pax2a and myoD, showed normal expression in embryos with ectopic c-myb expression (Figure 4, C6) when compared to control embryos (Figure 4, C5). All the data suggested that c-myb specifically inhibited β3 globin expression, but had no influence on expression of axis mesoderm and hematopoietic precursor cells in embryos.
Eafs Control Erythroid Cell Fate

Eafs Regulate Erythroid Cell Differentiation by Modulating C-myb Expression through Wnt Signaling

The above observations suggested that the increased expression of c-myb might mediate the suppression of erythroid differentiation in eafs morphants. High Wnt signaling was revealed in eafs morphants [21], and Wnt signaling was reported as being required for stem cell renewal and for inhibiting terminal multi-image cells differentiation [33,34,35]. In addition, down-regulating Wnt activities resulted in significantly reduced expression of gata1 in intermediate cell mass (ICM) [36], and c-myb, in aorta-gonad-mesonephros (AGM) [25], in treated embryos.

In this study, by applying hs:dnTCF-GFP transgenic embryos [24,25,36], which is a stable line that express a dominant negative of TCF/LEF, we down-regulated Wnt activities by transiently inducing dn-Tcf expression in embryos, and detected some hematopoietic cell markers including gata1, c-myb and erythroid specific markers. The scheme of knockdown Wnt signaling in embryos using hs:dnTCF-GFP fish was shown in Figure 5A. We heat-shocked the embryos at the bud stage, there was no obvious morphology defects observed in the GFP positive embryos at the 16 somites stage or later (data not shown), which differs from the previous report that the embryos showed malformation after heat-shocking at 75% epiboly stage [36]. We detected the expression of hematopoietic precursors and erythroid markers in dn-Tcf transgene induced embryos. Compared to their control offsprings (Figure 5, B1 and B3), embryos with expression of transiently induced dn-Tcf, both c-myb (Figure 5, B2) and gata1 (Figure 5, B4) displayed obviously reduced expression, the observations here were consistent with previous reports [25,36].

The differentiated erythroid markers, βe3 globin (Figure 5, B6 and B10) and band3 (Figure 5, B8), however, showed small increase in the embryos with induced dn-Tcf expression. We could not detect the expression change of non-blood axis mesoderm, such as pax2a and myoD, in the embryos with transiently induced dn-Tcf expression (Figure 5, B11 and B12), consistent with the observations that no obvious morphology defects displayed in the heat-shocked GFP positive embryos (data not shown).

We then contemplated whether down-regulating Wnt signaling in eafs morphants could rescue the erythroid defects and down-regulate the expression of precursor markers. Figure 6A shows the scheme of using hs:dnTCF-GFP fish to do rescue experiments. As expected, transiently inducing expression of dn-Tcf in eafs morphants could restore the expression of βe3 globin significantly (Figure 6, B and C). In a total of 27 eafs morphants, 48.2% of morphants displayed strongly reduced βe3 globin expression, 22.2% of morphants displayed mildly reduced βe3 globin expression, and 29.6% of morphants showed normal (Figure 6, C); but after heat-shocking to induce dn-Tcf expression in eafs morphants, we found that in a total of 29 detected embryos, only 6.9% of morphants showed strongly reduced expression of βe3 globin. Another 34.5% of morphants even showed increased expression of βe3 globin (Figure 6, B and C), suggesting that dn-Tcf might act downstream of eafs and be very effective to rescue differentiation defects of erythroid cells in eafs morphants. In addition, the increased expression of c-myb was also restored to a normal level by transiently inducing dn-Tcf expression in eafs morphants (Figure 6, B and C).
Discussion

Erythroid Differentiation is Blocked in Eafs Morphants, and the Phenotype is Specific

Eafs factors play important roles in tumor suppression and embryogenesis. In zebrafish, by morpholino-mediated knockdown, we revealed that eafs patterned the embryonic axis by regulating both non-canonical and canonical Wnt signaling at early developmental stages [20,21]. At later stages, we observed significant defects including reduced circular blood cells and a lack of blood flow in eafs morphants. By further detecting the molecular markers of hematopoietic cells in eafs morphants, obvious defects of erythroid differentiation were revealed, indicated by reduced mRNA and protein level of \( \beta e3 \) globin and significant reduced o-dianisidine staining hemoglobin in eafs morphants (Figure 1). Normal vasculature (Figure 2, A9–A12), the integrity of posterior non-hematopoietic tissues indicated by myoD and ntl (Figure 2, A1–A4), and increased expression of precursors of blood and vessel, indicated by increased expression of gata1, scl, lmo2, gata2, pu.1, runx1, fli1, and c-myb in eafs morphants from the 10 somites stage (Figure 2, A5–A8 and C), both suggested that defects of erythroid specification in eafs morphants were highly specific and not due to the expense of nearby tissues. Morphants at 24 hpf displayed severe anterior truncation (Figure 2, A2 and A4, indicated by black arrowhead), the observations here were consistent with our previous reports that eafs are required for forebrain formation in embryos [20,21,22]. In eafs morphants, the specification and formation of the erythroid progenitors were normal, but erythroid differentiation was blocked, suggested that the cells in the primary hematopoietic process might keep and accumulate on the precursor stage. The following observations help to support this point.

Firstly, in our study, all precursor markers, including gata1, scl, lmo2, gata2, c-myb and other genes, displayed increased expression from the 10 somites stage (Figure 2, A and C), but their expressions

Figure 4. C-myb suppressed specification of mature erythroid cells, and the phenotype of c-myb gain of function is specific. (A) Different dosage of c-myb on specification of mature erythroid cells (\( \beta e3 \) globin: A2, 50 pg per embryo; A4, 200 pg per embryo) and on erythroid progenitors (gata1: A6, A8). (B) In situ hybridization of \( \beta e3 \) globin showed that erythroid differentiation was blocked in embryos with ectopic c-myb expression (50 pg per embryo) (B2), the number of embryos displayed reduced \( \beta e3 \) globin was shown in (B2), and the in situ hybridization of c-myb shown its ectopic expression in corresponding embryos (B4). (B) Hematopoietic progenitor cells including gata1 (C1, C2) and scl (C3, C4), and other mesoderm cells including pax2a and myoD (C5, C6) specified and maintained normally in embryos with ectopic c-myb expression. A1–A8, B1, B2, C1–C4, dorsal view, anterior to the up; B3, B4, C5, C6, lateral view, anterior to the left. doi:10.1371/journal.pone.0064576.g004
were still normal, even when detected by the 8 somites stage (Figure 2, B), and it was impossible that some factors could generally up-regulate all the precursor markers in such a short time. Second, it is reported that the initiation of erythroid began around the 8–10 somites stage in zebrafish embryos [37] (personal communication with Jared J. Ganis). In our study, the increased expression of precursor markers began shortly after the initiation of erythroid differentiation (Figure 2, B and C), this might only happen after the blocking of erythroid differentiation. If we could count the whole number of blood cells and the ratio of precursor cells and differentiated erythroid cells in both control embryos and in eaf knockdown embryos, we might perceive more clear mechanism clues underlying the phenotypes in eaf morphants. Of course, we still could not remove the possibility that the increased expression of precursor markers might come from the accelerated proliferation of progenitor cells or from their increased expression in a single cell.

In eaf2 knockout mice, Xiao et al also found hematopoiesis defects [38,39]. Very small number of eaf2 knockout mice developed extramedullary hematopoiesis, they suggested that eaf2 inactivation may disrupt the normal hematopoiesis in bone marrow, causing a compensatory response from spleen and liver in the eaf2 knockout mice [38,39]. In addition, eaf2 deletion enhanced B-cell lymphoma development in eaf2 knockout mice [39], and eaf1 and eaf2 have been implicated in human hematopoietic cancers [40,41]. Our data here showed more a detail character of the hematopoiesis defects cause by eaf1 and eaf2 knockdown in vivo, and the data here also suggested that the roles of eaf factors in regulating hematopoiesis might be conserved from zebrafish to mice.

C-myb was Downstream of Eaf in Hematopoietic Developmental Process

We observed increased expression of precursor markers but dramatically suppressed erythroid differentiation in eaf morphants (Figure 1, Figure 2). Through a morphorlino-mediated knockdown of c-myb in eaf morphants, we found that knockdown c-myb could significantly restore βe3 globin expression in eaf morphants (Figure 3, A and B). C-myb morphants displayed a morphogenesis phenotype as reported previously [23], characterized by a small brain but normal body axis formation (Figure 3, C2). Consistent with its normal formation of posterior body axis by morphology, we also observed normal pattern of mesoderm markers in c-myb morphants, indicated by pax2a and myoD expression (Figure 3, C4). But the primary hematopoietic wave failed in c-myb morphants, indicated by reduced expression of progenitor markers and accelerated erythroid differentiation before 24 hpf (Figure 3, B and D).

Figure 5. Knockdown Wnt signaling in hs:dnTCF-GFP embryos by heat shock at the bud stage resulted in reduced progenitor cells and accelerated differentiation of erythroid cells. (A) Scheme of using hs:dnTCF-GFP fish to knockdown Wnt signaling in embryos. (B) Reduced progenitor blood cells, labeled by c-myb (B1, B2) and gata1 (B3, B4), but accelerated erythroid cells differentiation, labeled by βe3 globin (B5, B6, B9, B10) and band3 (B7, B8) displayed in hs:dnTCF-GFP positive embryos, and black arrow indicate the increased expression of βe3 globin expression in hs:dnTCF-GFP positive embryos (B10) compared to its control siblings (B9). Other mesoderm, labeled by pax2a and myoD (B9, B10, B11, B12), was normal in hs:dnTCF-GFP positive embryos. B1, B2, B7–B12, lateral view, anterior to the left; B3–B6 dorsal view, anterior to the up. doi:10.1371/journal.pone.0064576.g005
On the contrary, embryos with ectopic expression of \textit{c-myb} displayed reduced expression of erythroid markers but normal expression of hematopoietic precursors in fish (Figure 4). It is reported that \textit{c-Myb} is required for the development of hematopoietic precursors [12,13], and conditional \textit{c-Myb} knockout in adult hematopoietic stem cells leads to impaired proliferation and accelerated differentiation in mouse[14]. In addition, \textit{c-Myb} suppresses erythroid differentiation [16] and plays an important role in silencing the fetal and embryonic hemoglobin genes [19]. Our data here supported that \textit{c-myb} is required for maintaining hematopoietic precursor markers, but blocks erythroid differentiation, and suggests that the roles of \textit{c-myb} in hematopoiesis were conserved from zebrafish to mammalian.

\textit{C-myb} is a transcriptional factor which is important in hematopoiesis; it can bind with a vast number of different proteins in specifying different lineage of blood cells. The conditional \textit{c-myb} knockout mice displayed hematopoietic defects in bone marrow [14], totally opposite to hematopoietic defects in a \textit{c-myb} M303 mutant [42]. Similarly with the observations in mice, in this study, the hematopoietic defects occurred in \textit{c-myb} morphants, which are different from what Thompson et al observed in \textit{c-myb} h316 mutants; they found that \textit{gata2}, \textit{bro2}, and \textit{gata1} displayed normal expression in the mutants around 18 hpf [26]. We speculate, \textit{c-myb} b316 mutants, similar to mice \textit{c-Myb} M303 mutant, maybe only lost binding ability with some specific protein or lost some specific roles important for embryogenesis development, but its ability in regulating primary hematopoiesis still exists. The limitation of this mutant model can not truly or really tell us about the hematopoietic specification as the \textit{c-myb} gene is disrupted by morpholino mediating knockdown in embryos. \textit{C-Myb} specifically inhibited erythroid specification was convinced in this study (Figure 4), although we still know little about the underlying mechanisms. Countless literatures report that \textit{c-Myb} is a myeloid lineage regulator, cooperates with C/EBP\alpha, to activate transcription of myeloid genes [43,44]. Recently, hematopoietic genes, especially myeloid other than erythroid genes, have been identified as direct targets of \textit{C-Myb} by ChIP-Seq (chromatin immunoprecipitation followed by massively parallel sequencing) [45,46].

Figure 6. Knockdown Wnt signaling in \textit{eafs} morphants by transiently inducing \textit{dn-Tcf} expression rescued defects of \textit{c-myb} expression and erythroid cells specification. (A) Scheme of rescuing experiments in \textit{eafs} morphants by using hs:dnTCF-GFP embryos. (B) Increased \textit{c-myb} expression and reduced \textit{\(\beta\)e3 globin} expression were restored in \textit{eafs} morphants by transiently inducing \textit{dn-Tcf} in embryos at the bud stage. B1–B8, dorsal view, anterior to the up. doi:10.1371/journal.pone.0064576.g006

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Myeloid and erythroid lineage cell fate is influenced by both Wnt and serum mediators. The Wnt/Jun signaling pathway is crucial for regulating hematopoietic differentiation. Here, we investigate the role of Wnt signaling in the regulation of hematopoietic lineage commitment.

**Eaf Control Erythroid Cell Fate by Regulating C-myb Expression through Wnt Signaling**

Eaf factors have been revealed to regulate the development of mesoderm and neural patterning by inhibiting canonical Wnt signaling, and high activity of canonical Wnt/β-catenin is revealed in eaf morphants [21]. Wnt signaling was reported to be required for renewal of stem cells and progenitor cells and for inhibiting terminal differentiation [33,34,35]. In addition, the expression of a hematopoietic precursor marker, gata1, reduced significantly in ICM in embryos with down-regulating Wnt activities [36].

In this study, we down-regulated Wnt activities in embryos by transiently inducing expression of dn-Traf transgene, and revealed that including the reported gene gata1 (Figure 5, B3 and B4) [36], other precursor markers, specially c-myb (Figure 5, B1 and B2) [25] and pu.1 (data not shown), all displayed reduced expression. Our data was not only consistent with the previous study [25,36], but also suggested that down-regulating Wnt activities in embryos could result in general down-regulating expression of most precursor markers. How Wnt signaling acts on those cells in primitive hematopoiesis? Is this specific or direct? Since Goessling et al. found that, in morphants in which knockdown of c-myb expression was restored to normal level (Figure 6, B and C), some morphants even showed increased expression of β3 globin after heat-shocking (Figure 6, C), suggesting that dn-Traf might act downstream of eaf factors in hematopoietic differentiation.

**Supporting Information**

**Table S1** Morpholinos used in this study.

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**Author Contributions**

Conceived and designed the experiments: XM JXL. Performed the experiments: XM JXL. Analyzed the data: XM JXL. Wrote the paper: XM JXL.

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