HEMATOPOIESIS AND STEM CELLS

CBFβ and RUNX1 are required at 2 different steps during the development of hematopoietic stem cells in zebrafish

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Key Points

- CBFβ is not required for the emergence of nascent HSCs but is essential for a subsequent step before their release from the AGM.
- RUNX1 is able to drive the emergence of nascent HSCs in the AGM in the absence of its cofactor CBFβ.

CBFβ and RUNX1 form a DNA-binding heterodimer and are both required for hematopoietic stem cell (HSC) generation in mice. However, the exact role of CBFβ in the production of HSCs remains unclear. Here, we generated and characterized 2 zebrafish cbfb null mutants. The cbfb−/− embryos underwent primitive hematopoiesis and developed transient erythromyeloid progenitors, but they lacked definitive hematopoiesis. Unlike runx1 mutants, in which HSCs are not formed, nascent, runx1−/−/c-myb− HSCs were formed in cbfb−/− embryos. However, the nascent HSCs were not released from the aorta-gonad-mesonephros (AGM) region, as evidenced by the accumulation of runx1−/− cells in the AGM that could not enter circulation. Moreover, wild-type embryos treated with an inhibitor of RUNX1-CBFβ interaction, Ro5-3335, phenocopied the hematopoietic defects in cbfb−/− mutants, rather than those in runx1−/− mutants. Finally, we found that cbfb was downstream of the Notch pathway during HSC development. Our data suggest that runx1 and cbfb are required at 2 different steps during early HSC development. CBFβ is not required for nascent HSC emergence but is required for the release of HSCs from AGM into circulation. Our results also indicate that RUNX1 can drive the emergence of nascent HSCs in the AGM without its heterodimeric partner CBFβ. (Blood. 2014;124(1):70-78)

Introduction

Hematopoietic development is evolutionarily conserved among vertebrates. Similar to mammals, zebrafish embryos undertake sequential waves of hematopoiesis at distinct locations during embryonic development. The first wave is primitive hematopoiesis, in which erythroid progenitors arise from the posterior lateral mesoderm and form at later stages the intermediate cell mass, where erythroblasts are produced. In parallel, primitive myeloid progenitors originate from the anterior lateral mesoderm and later differentiate into macrophages. The second wave is definitive hematopoiesis with the generation of hematopoietic stem cells (HSCs), which can differentiate to all definitive blood lineages. Starting at 30 hours postfertilization (hpf), HSCs emerge from the hemogenic endothelium of the ventral wall of the dorsal aorta (DA) in the zebrafish equivalent of the aorta-gonad-mesonephros (AGM) region. High-resolution imaging revealed a stereotyped cell behavior during which endothelial cells from the ventral DA bend into the subaortic space and transdifferentiate into HSCs. This dynamic process has been termed endothelial hematopoietic transition (EHT). The HSCs in the subaortic mesenchyme enter the circulation through the axial vein and colonize the caudal hematopoietic tissue (CHT), which is considered functionally analogous to the mammalian fetal liver. HSCs in the CHT give rise to erythroid and myeloid progenitors and then migrate toward the definitive hematopoietic organs in adult fish, thymus, and kidney. Similar to the mouse, a transient population of erythromyeloid progenitors (EMPs) originates within the posterior blood island and sustains the initiation of the definitive hematopoietic wave in zebrafish.

Core binding factor (CBF) is a heterodimeric DNA-binding complex that consists of a DNA-binding α-subunit (encoded in mammals by RUNX1, RUNX2, or RUNX3) and a non-DNA-binding β-subunit, encoded by CBFB. RUNX1 and CBFβ, encoded by RUNX1 and CBFB, respectively, are both required for the development of definitive hematopoiesis. Mice with targeted disruption of either Runx1 or Cbfb show essentially identical phenotypes with complete lack of definitive hematopoiesis and lethality between embryonic days 11.5 and 13.5. The observations suggest that RUNX1 and CBFβ function together in vivo, which is consistent with biochemical studies that RUNX1 and CBFβ form a heterodimer for binding DNA and regulating the expression of downstream target genes. The absence of all definitive hematopoietic lineages in both Runx1−/− and Cbfb−/− embryos also suggests that both genes are required at the stage of HSC specification. Subsequent studies demonstrated that Runx1 is required for the emergence of HSCs from the hemogenic endothelium within the AGM region in the mouse. Our group previously generated a zebrafish runx1 mutant with a nonsense mutation (W84X) within the RUNT domain, resulting in a
prematurely truncated RUNX1 protein. Homozygous runx1<sup>W8X/W8X</sup> mutants lack expression of the HSC marker c-myb and do not develop definitive blood lineages in the CHT and thymus. Further studies from other groups demonstrated that, in zebrafish, runx1 is also required for the emergence of HSCs from the hemogenic endothelium in the AGM. On the other hand, relatively little is known about the exact role of Cbfb during the early stages of HSC development in the mouse, although it is assumed that Cbfb plays a similar role as Runx1 does. Even though a highly conserved cbfb gene (the encoded protein is 87% identical to the mammalian CBFβ proteins) has been identified in the zebrafish, the role of the zebrafish cbfb during HSC production in definitive hematopoiesis remains to be investigated.

In this study, we generated and characterized 2 independent zebrafish cbfb knockout mutant lines (cbfb<sup>-/-</sup>), which revealed a previously unknown role of cbfb during definitive hematopoiesis, and showed that the function of RUNX1 and CBFβ during HSC development could be uncoupled.

**Methods**

**Zebrafish lines and maintenance**

Zebrafish were maintained and used following approved National Human Genome Research Institute Animal Care and Use Committee protocols. Zebrafish handling and breedings were performed as described previously. The following strains were used: wild-type (WT) EK (Ekkwill), runx1<sup>W8X/W8X</sup>, the transgenic line tg(spaghetti:GFP),<sup>29</sup> the transgenic line tg(aas:NICD), and tg(hsp70:gal4)<sup>29</sup> which were kindly provided by Ajay Chitnis.

**Generation of cbfb mutants and genotyping**

Sixteen pairs of CompoZr zinc-finger nucleases (ZFNs) targeting the first half of the open reading frame of the cbfb gene were designed and evaluated for in vitro activity by Sigma-Aldrich (St. Louis, MO), and messenger RNA (mRNA) from the pair with the highest in vitro activity was chosen for subsequent targeted mutagenesis. Injection of mRNA, founder screening, and identification of cbfb heterozygous adult fish has been described in detail previously.<sup>30</sup> For each experiment, cbfb-Del4 and cbfb-In4 were genotyped by fluorescent polymerase chain reaction (PCR) using a mixture of M13F-tailed (5′-TGTAAAACGACGGCCAG-3′) cbfb-specific forward primer (5′-AGTCGGGCGGCTCCCTTCC3′), 6-FAM-labeled M13F primer, and Pig-tailed (5′-GTTGGCCT-3′) cbfb-specific reverse primer (5′-AGGGCCGCTTAGGAG-3′) in the PCR mix.<sup>30</sup> Genotyping of fixed samples has been performed as above but using a different PCR mix (Sigma RED Extract-N-Amp PCR Ready Mix R4775 REDExtract-N-Amp PCR Ready Mix, R4775; Sigma-Aldrich).

**WISH, o-dianisidine staining, and imaging**

Whole-mount in situ hybridization (WISH) was carried out essentially as described by Thissen and Thissen.<sup>31</sup> The cbfb antisense mRNA probe has been described as generated by hybridization by Blake et al.<sup>32</sup> The following DIG-labeled antisense mRNA probes were generated by using UTP-digoxigenin (Roche): cbfb, gata1, ace1-l-globin (bbacl), l-plastin, mpx, ikaro, rag1, runx1, and c-myb. Zebrafish embryos were stained in o-dianisidine staining solution for 15 minutes in the dark, as previously described.<sup>1</sup> The embryos were observed with a Leica MZ16F stereomicroscope, and the pictures were taken with a Leica DC500 camera using Leica FireCam (version 1.7.1).

**Time-lapse experiments and statistical analysis**

Dechorionated embryos obtained from cbfb<sup>-/-</sup>tg(spaghetti:GFP) incrosses were anesthetized with tricaine, mounted in 0.8% low-melting agarose, and imaged from 48 to 63 hpf. Z stacks were collected every 5 minutes for 15 hours. The embryos were then recovered and genotyped. Tg(bkl:neselin1-eGFP) embryos were treated with dimethylsulfoxide (DMSO) (0.1%) or Ro5-3335 at 5 μM from 24 hpf and at 48 hpf were mounted for imaging as described above and covered with 4 mL of DMSO (0.1%) or Ro5-3335 at 5 μM. Treated embryos were imaged every 5 minutes for 10 hours. Details about the imaging systems used are available in the supplemental Methods on the Blood Web site.

**Results**

**Generation of zebrafish cbfb<sup>-/-</sup> mutants**

To determine the role of CBFβ in the formation of HSCs, we generated 2 independent cbfb mutant lines by ZFN-mediated targeted mutagenesis.<sup>32,33</sup> The selected CompoZr ZFN pair targeted a specific region within cbfb exon 3 (Figure 1A). Among 9 mutations identified from 6 germline-transmitting founders, we selected 2 mutations predicted to cause frameshifts with premature terminations, cbfb<sup>h810</sup> (c.215delACCT, p.N72IfsX25) and cbfb<sup>h11</sup> (c.215insACCT, p.N72IfsX25), denoted here as cbfb<sup>del4</sup> and cbfb<sup>ins4</sup> (Figure 1B). In order to test whether the mutations lead to loss of cbfb expression, we evaluated the presence of cbfb transcripts in cbfb<sup>del4del4</sup> and cbfb<sup>ins4ins4</sup> mutants by WISH. At 36 hpf, cbfb expression in the ventral DA was detectable in WT embryos (Figure 1C), but not in cbfb<sup>del4del4</sup> (Figure 1D) or cbfb<sup>ins4ins4</sup> (Figure 1E) embryos, suggesting that the mutant mRNA was degraded in both cbfb<sup>del4del4</sup> and cbfb<sup>ins4ins4</sup> mutants.

Embryos heterozygous for the mutations in cbfb were indistinguishable from their WT clutchmates and presented normal hematopoiesis (data not shown). cbfb<sup>del4del4</sup> and cbfb<sup>ins4ins4</sup> embryos did not show any obvious morphologic or developmental defects and were indistinguishable in appearance from their WT or heterozygous clutchmates. cbfb<sup>del4del4</sup> and cbfb<sup>ins4ins4</sup> embryos presented identical hematopoietic phenotypes and died around 14 dpf; therefore, they are frequently referred collectively as cbfb<sup>-/-</sup> mutants in this report.

**Loss of cbfb does not affect primitive hematopoiesis and EMP formation**

In a previous report, we showed that in WT embryos cbfb expression is detectable in the posterior lateral mesoderm, where primitive hematopoietic progenitors are formed, and in the intermediate cell mass, where primitive erythroid cells arise. To determine if cbfb<sup>-/-</sup>
mutants had any primitive hematopoietic defects, we tested the expression of several hematopoietic markers in \( \text{cbf}^{+/−} \) mutants and WT siblings by WISH. The expression of the erythroid marker \( \text{gata}1 \) appeared unaltered in \( \text{cbf}^{del4/del4} \) and \( \text{cbf}^{ins4/ins4} \) mutants at 16 somites and 24 hpf (supplemental Figure 1A-F), suggesting that the development of primitive erythroid cells was unaffected in \( \text{cbf}^{+/−} \) mutants. Primitive erythroblasts differentiated into erythrocytes in \( \text{cbf}^{del4/del4} \) and \( \text{cbf}^{mod4/mod4} \) embryos as whole-embryo \( \alpha\)-dianisidine staining appeared normal at 48 hpf (supplemental Figure 1J-L), and the expression of the hemoglobin gene \( \text{ael}-\text{globin} \) (\( \text{hbae}1 \)) was unaffected (supplemental Figure 1M-O). The expression of the myeloid marker \( \text{l-plastin} \) was also maintained in both \( \text{cbf} \) null mutants at 24 hpf (supplemental Figure 1G-I), suggesting that primitive myeloid cells were unaffected. In addition, \( \text{cbf} \) null embryos had normal expression of \( \text{gata}1 \), \( \text{l-plastin} \), and \( \text{mpx} \) in the posterior blood island at 36 hpf, indicating that the EMP progenitors were correctly specified (supplemental Figure 2A-I).

**The zebrafish \( \text{cbf}^{+/−} \) embryos lack definitive hematopoiesis**

It is known that loss of either \( \text{Runx1} \) or \( \text{Cbfb} \) abolishes the onset and the development of definitive hematopoietic cells in mouse embryos.\(^{12,13}\) Because the zebrafish \( \text{runx1}^{+/−} \text{cbf}^{+/−} \) mutant embryos also lack definitive hematopoiesis,\(^{21}\) we evaluated the presence of definitive blood lineages in the \( \text{cbf}^{+/−} \) mutants (Figure 2 and supplemental Figure 3). At 3 dpf, the expression of \( \text{l-plastin} \) in definitive myeloid progenitors within the CHT was almost undetectable in \( \text{cbf}^{del4/del4} \) (Figure 2B) and \( \text{cbf}^{mod4/mod4} \) (supplemental Figure 3B) mutants when compared with WT siblings (Figure 2A and supplemental Figure 3A). The expression of \( \text{hbae}1 \) in erythroid precursors within the CHT was also abrogated in \( \text{cbf}^{del4/del4} \) (Figure 2C-D) and \( \text{cbf}^{ins4/ins4} \) (supplemental Figure 3C-D) embryos at 6 dpf. The lymphoid markers \( \text{ikaros} \) and \( \text{rag}1 \) in the developing thymus were absent in \( \text{cbf}^{del4/del4} \) (Figure 2E-H) and \( \text{cbf}^{mod4/mod4} \) (supplemental Figure 3E-H) mutants at 5 dpf as well. Moreover, circulating thrombocytes were also almost undetectable in \( \text{cbf}^{del4/del4} \) (\( \text{cd41}-\text{GFP} \)) embryos, in which the expression of green fluorescent protein is driven by the promoter of a thrombocyte-specific gene, \( \text{cd41} \) (supplemental Movies 1 and 2). Therefore, our results are consistent with a complete failure of definitive blood lineages in the \( \text{cbf}^{+/−} \) zebrafish mutants.

**The emergence of nascent HSCs is unaffected in \( \text{cbf}^{+/−} \) embryos**

To investigate whether loss of \( \text{cbf} \) affected the onset of definitive hematopoiesis, we analyzed HSC development by testing the expression of \( \text{runx1} \) and \( \text{c-myb} \) by WISH. Nascent \( \text{runx1}^{+/−} \text{c-myb}^{+/−} \) HSCs emerge from the hemogenic endothelium in the ventral wall of the DA around 30 hpf.\(^{34,35}\) In \( \text{runx1}^{+/−} \text{c-myb}^{+/−} \) mutants, \( \text{c-myb}^{−/−} \) HSCs were absent.\(^{21}\) However, at 36 hpf, \( \text{c-myb} \) expression was observed along the ventral wall of the DA in both \( \text{cbf}^{del4/del4} \) (Figure 3B-E) and \( \text{cbf}^{mod4/mod4} \) (Figure 3C-E′) embryos at similar levels to their WT clutchmates (Figure 3A-A′). Similarly, expression of the early HSC marker, \( \text{runx1} \), was intact or even slightly increased in the ventral DA region of the \( \text{cbf}^{del4/del4} \) (Figure 3E-E′) and \( \text{cbf}^{mod4/mod4} \) (Figure 3F-F′) embryos at 36 hpf, as compared with the controls (Figure 3D-D′). Given the normal expression of \( \text{c-myb} \) and the strong expression of \( \text{runx1} \) in \( \text{cbf}^{+/−} \) mutants at 36 hpf, we evaluated the presence of a compensatory mechanism involving other \( \text{runx} \) family members. However, in \( \text{cbf}^{+/−} \) embryos between 36 hpf and 3dpf, the expression pattern and level of \( \text{runx2a} \), \( \text{runx2b} \), and \( \text{runx3} \) were normal (in the pharyngeal arches and cartilage) and no ectopic expression was detectable, especially in the hematopoietic tissues (data not shown).

Overall, the presence of \( \text{runx}1^{+/−} \) and \( \text{c-myb}^{+/−} \) cells within the hemogenic endothelium of \( \text{cbf}^{del4/del4} \) and \( \text{cbf}^{mod4/mod4} \) mutants indicates that the emergence of nascent HSCs does occur in \( \text{cbf}^{+/−} \) embryos.

**HSCs do not reach the CHT and kidney in the \( \text{cbf}^{+/−} \) embryos**

Starting from 30 hpf, HSCs asynchronously egress from the ventral DA into the subaortic space and intravasate into the axial vein to seed the CHT.\(^{7}\) Consistent with the translocation of HSCs, at 2 dpf, \( \text{c-myb}^{+/−} \) embryos were correctly specified in both \( \text{cbf}^{del4/del4} \) (D) and \( \text{cbf}^{mod4/mod4} \) (E) embryos.
expression is observed in both AGM and CHT regions of WT embryos (Figure 4A-A'). However, c-myb expression in cbfb−/− mutants appeared strongly reduced and was detectable only in the AGM (Figure 4B-C'). At 3 dpf, when c-myb+ cells were found only in the CHT of WT embryos (Figure 4D-D'), no c-myb expression was detected in any hematopoietic region of cbfb del4/del4 (Figure 4E-E') and cbfb ins4/ins4 (Figure 4F-F') mutants. At 5 dpf, c-myb expression in CHT and kidney was detectable in WT (Figure 4G-G'), but not in cbfb−/− (Figure 4H-H'), embryos.

Apoptosis was not likely the reason for the reduction in c-myb expression, because terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining was not increased in cbfb−/− embryos between 36 and 48 hpf (supplemental Figure 4A). Moreover, the expression of the proliferating cell nuclear antigen (PCNA)β in hematopoietic progenitors within the AGM of cbfb−/− mutants between 36 and 48 hpf appeared comparable to WT controls (supplemental Figure 4B). Similarly, anti-phosphohistone H3 staining between 36 and 48 hpf appeared comparable to WT controls (supplemental Figure 4C), whereas no differences in proliferation of the hematopoietic progenitors (eGFP+ cells) within the AGM (supplemental Figure 4C-F).

On the other hand, the expression of the early HSC marker, runx1, was maintained in the AGM of cbfb−/− mutants (Figure 5B-C') at 48 hpf as compared with the WT embryos (Figure 5A-A'). In WT embryos at 3 dpf, HSCs were located in the CHT and runx1 expression was no longer detectable in the AGM (Figure 5D-D'). At the same stage of development, however, strong runx1 expression was still detectable in the AGM region in cbfb del4/del4 and cbfb ins4/ins4 mutants (Figure 5E-F'). Because cbfb del4/del4 and cbfb ins4/ins4 mutants presented normal blood circulation (supplemental Movies 3-5), these findings suggest that HSCs could not leave the AGM in cbfb−/− mutant embryos.

**HSCs are not released from the AGM in cbfb−/− embryos**

In order to demonstrate directly the behavior of HSCs in live embryos, we incrossed cbfb−/del4/tg(c-myb:eGFP) mutants and counted the number of eGFP+ cells that were released from the AGM into the circulation by performing time-lapse imaging analysis of multiple cbfb−/del4/tg(c-myb:eGFP) and WT tgg(c-myb:eGFP) siblings between 48 and 63 hpf (Figure 5G-I and supplemental Movies 6 and 7). We observed an average of 12 eGFP+ cells leaving the

![Figure 3. HSCs emerge from hemogenic endothelium in cbfb−/- embryos.](image-url)

Expression of the HSC markers c-myb and runx1 in cbfb−/- and WT embryos at 36 hpf by WISH. Compared with WT siblings (A-A'), the HSC marker c-myb was normally expressed in the hemogenic endothelium of the ventral DA of cbfb del4/del4 (B-B') and cbfb ins4/ins4 (C-C') embryos at 36 hpf. Similarly, the expression of runx1, another HSC marker, was also unaffected in cbfb del4/del4 (E-E') and cbfb ins4/ins4 (F-F') embryos, as compared with WT embryos (D-D'). Panels A'-F' depict the boxed regions in panels A-F.

![Figure 4. HSCs do not translocate from the AGM to the CHT and kidney in cbfb−/- embryos.](image-url)

Expression of the HSC marker c-myb in the CHT and kidney between 48 hpf and 5 dpf by WISH. At 48 hpf, c-myb+ HSCs had started to populate the CHT in WT embryos (A-A'), whereas they did not in cbfb del4/del4 (B-B') or cbfb ins4/ins4 (C-C') embryos. At 3 dpf, c-myb+ hematopoietic cells could readily be detected in the CHT in WT embryos (D-D'), whereas no c-myb expression was detectable in the CHT in cbfb del4/del4 (E-E') or cbfb ins4/ins4 (F-F') embryos. At 5 dpf, c-myb expression in the CHT and kidney was detectable in WT (G-G'), but not in cbfb del4/del4 (H-H') or cbfb ins4/ins4 (I-I') embryos. Panels A'-F' depict the boxed regions in panels A-F. Panels G', H', and I' depict the regions in the left boxes in panels G-I. Panels G''-I'' depict the regions in the right boxes in panels G-I.
AGM and entering the circulation through the axial vein per
tg(c-myb:eGFP) embryo (n = 5) during the recorded period (15 hours; Figure 5J). On the other hand, on average we observed only 1 eGFP⁺ cell leaving the AGM per cbfβ⁺/−/tg(c-myb:eGFP) embryo (n = 5) in the same recorded period (Figure 5J), which was significantly lower than the control (P < .001).

Thus, unlike runx1⁺/− mutants, where HSC formation was completely abrogated, nascent HSCs were formed in the cbfβ⁻/⁻ embryos, but they could not leave the AGM. Taken together, our results indicate that CBFβ is dispensable for the emergence of nascent HSCs but is necessary for their release from the AGM.
Pharmacologic inhibition of RUNX1-CBFβ interaction phenocopies the hematopoietic defects in cbfb<sup>−/−</sup> embryos

Recently, we identified a specific inhibitor of the RUNX1-CBFβ interaction, Ro5-3335. Zebrafish embryos treated with Ro5-3335 from 24 hpf to 6 dpf showed defects in the development of definitive hematopoiesis as demonstrated by the reduction of circulating thrombocytes in the transgenic line tg(cd41:GFP). Moreover, tg(cd41:GFP) embryos carrying 1 allele of the runx1 truncation mutation (runx1<sup>1/W84X</sup>) are more sensitive to Ro5-3335 treatment (for developing hematopoietic defects) than WT transgenic embryos. Because Ro5-3335 induces defects in definitive hematopoiesis by blocking the RUNX1-CBFβ interaction, we reasoned that its inhibition would reproduce the early HSC phenotype observed in cbfb<sup>−/−</sup> mutants, but not the one in the runx1 mutants.

Therefore, we treated WT embryos with different concentrations of Ro5-3335 from 24 hpf to 36 hpf, or from 24 hpf to 3 dpf, and then evaluated the effect of Ro5-3335 treatment on HSC markers by WISH. WT embryos treated with Ro5-3335 at 5 μM, 2.5 μM, and 0.25 μM from 24 hpf to 36 hpf showed normal expression of c-myb and runx1 within the ventral DA (Figure 6A and supplemental Figure 5A-D). At a higher concentration (5 μM), we observed only a slight reduction in c-myb expression in 12% of the embryos when compared with their DMSO controls (Figure 6A). Neither cbfb<sup>+/del4</sup> nor runx1<sup>1/W84X</sup> embryos showed more reduction in c-myb expression than WT embryos at 36 hpf after Ro5-3335 treatment (data not shown). On the other hand, WT embryos treated with Ro5-3335 at these same concentrations showed a dose-dependent reduction of c-myb expression in the CHT region at 3 dpf, similar to the phenotype in the cbfb<sup>−/−</sup> embryos (Figure 6B). In addition, cbfb<sup>+/del4</sup> mutants were more sensitive to Ro5-3335 treatment than WT embryos for the absence of c-myb expression in the CHT, as more cbfb<sup>+/del4</sup> embryos developed the phenotype than WT embryos at a given concentration (compare Figure 6B with supplemental Figure 5E).

To confirm that Ro5-3335 fully recapitulated the cbfb<sup>−/−</sup>-mutant hematopoietic phenotype, we used the transgenic line...
tg(flk1:moesin1-eGFP), which expresses the Moesin1-eGFP fusion protein from the promoter of flk1, a gene specifically expressed in endothelial cells. The tg(flk1:moesin1-eGFP) embryos were treated with DMSO or Ro5-3335 at 5 μM from 24 hpf, and their AGM regions were monitored between 48 and 58 hpf with time-lapse imaging (Figure 6D-F and supplemental Movies 8 and 9). The inhibition of RUNX1-CBFβ interaction by Ro5-3335 resulted in a significant impairment of HSC release from the AGM into the circulation in the recorded period (10 hours; *P < .001; Figure 6C).

Taken together, our results showed that treatments with the RUNX1-CBFβ inhibitor Ro5-3335 phenocopied the phenotype observed in cbfb−/− mutants and confirmed that the function of RUNX1 and CBFβ during HSC development could be uncoupled.

cbfβ acts downstream of the Notch pathway

The Notch-Runx1 pathway is critical for the initial specification of HSCs during definitive hematopoiesis.34,38 Transient overexpression of an activated form of notch (NICD) in zebrafish embryos has been shown to induce ectopic expression of runx1 and expand definitive HSCs.34 Conversely, runx1 expression in HSCs is abrogated in the mind bomb mutant, where an E3 ubiquitin ligase essential for Notch signaling is mutated.34 Based on these observations, we evaluated whether cbfb was also controlled by Notch activity. We confirmed that 36-hpf mind bomb mutants lacked the expression of c-myb and runx1 in the artery (data not shown). Interestingly, we observed that cbfb expression within the hematopoietic progenitors in the ventral wall of the DA was also abolished in 36-hpf mind bomb mutants (Figure 7A-B'). We then examined the expression of c-myb, runx1, and cbfb by WISH in 36-hpf hsp70:gal4; uas:NICD embryos, which were heat shocked between 8 and 20 somites. We confirmed that the expression of c-myb and runx1 was expanded in the heat-shocked embryos (Figure 7C-F'). We observed that cbfb expression was also expanded in the aorta and ectopically expressed in the vein (Figure 7G-H'), similar to both c-myb and runx1 (Figure 7C-F'). These results suggest that cbfb expression is regulated by Notch activity.

Discussion

The Cbfβ gene has been demonstrated as a key regulator of definitive hematopoiesis during embryogenesis in mice.12,15 Cbfβ−/− embryos lacked definitive hematopoiesis, whereas some EMPs remained.19 In a recent study, lineage specific expression of a Cbfβ transgene in Cbfβ knockout mice showed that EMPs and HSCs differentiate from distinct populations of hemogenic endothelial cells.32 However, there have been no reported studies on the exact roles of Cbfβ for the emergence of HSCs from hemogenic endothelium.

In this study, we generated 2 independent zebrafish cbfb knockout mutants (cbfb−/−), which presented identical hematopoietic phenotypes. cbfb−/− embryos retained primitive hematopoiesis and EMPs but completely lacked all definitive blood lineages. Studies in both mouse and zebrafish clearly demonstrated that Runx1 is required for the EHT of the hemogenic endothelium into HSC during the early phases of definitive hematopoiesis.5,18,19 Therefore, because CBFβ is considered the obligate partner of RUNX1, the impairment of all definitive hematopoietic lineages in both Runx1−/− and Cbfβ−/− mice suggested that the CBF heterodimer is required for HSC formation. Our present data, however, suggest that runx1 and cbfb are required at different steps during the early formation of HSCs. Indeed, the emergence of the nascent, runx1+/c-myb+ HSCs from the hemogenic endothelium along the ventral wall of the DA was unaffected in the cbfb−/− mutants. Further support for this finding comes from our data with pharmacologic inhibition of the RUNX1-CBFβ interaction in WT zebrafish embryos with a specific inhibitor, Ro5-3335.37 Similar to the cbfb−/− mutants, the emergence of nascent HSCs within the ventral DA was not affected by Ro5-3335 treatments, even at relatively high doses. Moreover, neither cbfb+/−/del4 nor runx1+/+sh4 embryos showed a reduction in c-myb expression within the DA after Ro5-3335 treatment. The presence of nascent runx1+/c-myb+ HSCs does not appear to be due to compensatory mechanism driven by other runx family members, as their expression in cbfb−/− mutants was normal. We can also exclude any contribution...
from maternal cbfb mRNA as cbfb expression is only zygotic.23 Overall, the emergence of nascent HSCs from the hemogenic endothelium in the absence of cbfb or a functional CBF complex indicates that CBFβ is not necessary for the EHT and strongly suggests that the function of RUNX1 and CBFβ during HSC development can be uncoupled. In the future, the temporal requirement of CBFβ during HSC development can be defined more precisely by treating the embryos with Ro5-3335 within different time windows.

Interestingly, we found that c-myb expression in the HSCs was progressively lost and no c-myb+ cell colonizes the CHT region of the cbfb−/− embryos at 3 dpf. Similarly, treatments with WT embryos with Ro5-3335 resulted in a dose-dependent reduction of c-myb expression in the CHT region. We confirmed that this phenotype resulted from the specific inhibition of the RUNX1-CBFβ interaction by showing that cbfb+/del4 mutants were more sensitive to Ro5-3335 treatment than WT embryos.

The role of function of cbfb did not affect the expression of the early HSC marker runx1. Strikingly, runx1+ cells persisted in the AGM of cbfb homozygous embryos and never translocated to the CHT region. However, this phenotype did not appear to be related to any circulatory defect, as blood circulation and heart development in the cbfb−/− mutants were normal. Similar to the phenotype reported for the cmyb(hk) mutants,39 quantitative time-lapse observations of cbfb+/del4 mutants treated with the eGFP (eGFP) embryos demonstrated a strong impairment in the intravasation of c-myb:eGFP+ cells to the arterial vein from the saorotic mesenchyme. The same phenotype was also recapitulated in tg(fkl:moesin1-eGFP) embryos treated with the RUNX1-CBFβ inhibitor Ro5-3335. Our study, therefore, demonstrates a novel function of cbfb in the release of HSCs from the AGM region during definitive hematopoiesis.

In order to gain insight into the genetic mechanisms that regulate cbfb expression, we investigated the Notch pathway, because the Notch signaling mutant mind bomb, cbfb expression in hematopoietic regions was abrogated. Thus, our results suggest that cbfb is also downstream of the Notch pathway during hematopoiesis.

Overall, our results indicate that a functional CBF complex is important for the onset of definitive hematopoiesis, but runx1 and cbfb functions appear to be required at different steps during HSC development. Our study strongly suggests a novel role for CBFβ and the CBFβ-RUNX1 heterodimer in the release of HSCs from the AGM during early definitive hematopoiesis. The presence of nascent, runx1−/−/cbfb−/− HSCs in cbfb−/− embryos indicates that cbfb is dispensable for the emergence of HSCs but also implies that RUNX1 is able to drive HSC formation in the absence of its known obligate cofactor CBFβ. The mechanism for this functional separation of RUNX1 and CBFβ during early definitive hematopoiesis is unclear. It is possible, however, that a certain level of RUNX1 is adequate to turn on hematopoietic markers, but a higher functional level, achieved by increased binding in the presence of CBFβ, is necessary to get through the later process.

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Authorship

Contribution: E.B., B.C., S.W., M.P.J., and A.V.G. designed and performed the experiments and analyzed the data; B.M.W., R.S., and P.P.L. designed and organized the experiments and analyzed the data; and E.B. and P.P.L. wrote the manuscript.

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CBFβ and RUNX1 are required at 2 different steps during the development of hematopoietic stem cells in zebrafish

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