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

Short title for the running head: Distinct roles of CBFβ and RUNX1 in HSC production

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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 co-factor CBFβ.

Abstract

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 HSC remains unclear. Here we generated and characterized two 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 two 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.
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 (PLM) and form at later stages the intermediate cell mass (ICM) where erythroblasts are produced. In parallel, primitive myeloid progenitors originate from the anterior lateral mesoderm (ALM) and later differentiate into macrophages. The second wave is definitive hematopoiesis with the generation of HSCs, which can differentiate to all definitive blood lineages. Starting at 30 hours post fertilization (hpf) HSCs emerge from the hemogenic endothelium of the ventral wall of the dorsal aorta (DA) in the zebrafish equivalent of the AGM region. High-resolution imaging revealed a stereotyped cell behavior during which endothelial cells from the ventral DA bend into the sub-aortic space and trans-differentiate into HSCs. This dynamic process has been termed endothelial hematopoietic transition (EHT). The HSCs in the sub-aortic 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 erythro-myeloid progenitors (EMPs) originates within the posterior blood island (PBI) 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, 2 or 3, 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 Chfb show essentially identical phenotypes with complete lack of definitive hematopoiesis and lethality between
embryonic days 11.5 and 13.5\textsuperscript{12-15}. The observations suggest that RUNX1 and CBFβ function together \textit{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\textsuperscript{16,17}. The absence of all definitive hematopoietic lineages in both $\text{Runx1}^{-/-}$ and $\text{Cbfb}^{-/-}$ embryos also suggests that both genes are required at the stage of HSC specification. Subsequent studies demonstrated that $\text{Runx1}$ is required for the emergence of HSCs from the hemogenic endothelium within the AGM region in the mouse\textsuperscript{18,19}. Our group previously generated a zebrafish $\text{runx1}$ mutant with a nonsense mutation (W84X) within the RUNT domain, resulting in a prematurely truncated RUNX1 protein. Homozygous $\text{runx1}^{W84X/W84X}$ mutants lack expression of the HSC marker $\text{c-myb}$ and do not develop definitive blood lineages in the CHT and thymus\textsuperscript{20,21}. Further studies from other groups demonstrated that, in zebrafish, $\text{runx1}$ is also required for the emergence of HSCs from the hemogenic endothelium in the AGM\textsuperscript{6}.

On the other hand, relatively little is known about the exact role of $\text{Cbfb}$ during the early stages of HSC development in the mouse\textsuperscript{22}, although it is assumed that $\text{Cbfb}$ plays a similar role as $\text{Runx1}$ does. Even though a highly conserved $\text{cbfb}$ gene (the encoded protein is 87\% identical to the mammalian CBFβ proteins) has been identified in the zebrafish\textsuperscript{23}, the role of the zebrafish $\text{cbfb}$ during HSC production in definitive hematopoiesis remains to be investigated.

In this study we generated and characterized two independent zebrafish $\text{cbfb}$ knockout mutant lines ($\text{cbfb}^{-/-}$), which revealed a previously unknown role of $\text{cbfb}$ 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\(^2^4\). The following strains were used: wild type EK (Ekkwill), runx1\(^{W84X}\)\(^^{2^1}\), tg(c-myb:eGFP)\(^^{2^5}\), tg(cd41:GFP)\(^^{2^6}\), tg(flk1:moesin1-eGFP)\(^^{2^7}\). The mind bomb\(^{a52b}\) mutant line\(^^{2^8}\), the transgenic line tg(uas:NICD) and tg(hsp70:gal4)\(^^{2^9}\) were kindly provided by Ajay Chitnis.

Generation of cbfb mutants and genotyping

Sixteen pairs of CompoZr 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 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\(^3^0\). For each experiment cbfb-Del4 and cbfb-Ins4 were genotyped by fluorescent PCR using a mixture of M13F-tailed (5’-TGCTCGGGCTGGCTTTCT-3’) cbfb-specific forward primer (5’-ATGCTCGGGCCTGGCTTTCT-3’), 6-FAM labeled M13F primer and PIG-tailed (5’-GTGCTTT-3’) cbfb-specific reverse primer (5’-AGGGGCGTGAGTTAGAGT-3’) in the PCR mix\(^3^0\). Genotyping of fixed samples has been performed as above but using a different PCR mix (Sigma RED Extract-N-Amp™ PCR Ready Mix R4775).
Whole-mount in situ hybridization (WISH), o-dianisidine staining and imaging

WISH were carried out essentially as described by Thisse\textsuperscript{31}. The *cbfb* antisense mRNA probe has been generated as described by Blake et al\textsuperscript{23}. The following DIG-labeled antisense mRNA probes were generated by using UTP-digoxigenin (Roche): *cbfb*, *gata1*, *ae1-globin* (*hbai*), *l-plastin*, *mpx*, *ikaros*, *rag1*, *runx1* and *c-myb*. Zebrafish embryos were stained in *o-dianisidine* staining solution for 15 min in the dark, as previously described\textsuperscript{1}. The embryos were observed with a Leica MZ16F stereo-microscope, 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*\textsuperscript{+/-del}/*tg(c-myb):eGFP*) incrosses were anesthetized with tricaine, mounted in 0.8% low melting agarose and imaged from 48-63 hpf. Z-stacks were collected every 5 minutes for 15 hours. The embryos were then recovered and genotyped. Tg(*flk1:moesin1*-eGFP) were treated with DMSO (0.1%) or Ro5-3335 at 5 \(\mu\)M 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 \(\mu\)M. Treated embryos were imaged every 5 minutes for 10 hours. Details about the imaging systems used are available in the Supplemental Material.

Significance and standard deviation between samples were calculated using Excel (Microsoft).

Ro5-3335 treatment

Two zebrafish embryos at 22 hpf were placed into each well of 96-well plates (Costar #3635). These embryos were then incubated with Ro5-3335 at 5 \(\mu\)M, 2.5 \(\mu\)M, 0.5 \(\mu\)M.
and 0.25 μM in E3 embryo medium (32-48 embryos per treatment in a final volume of 300 μl per well). As a control embryos were treated with 0.1% DMSO in E3 embryo medium. Embryos were treated from 24 hpf to 36 hpf, or from 24 hpf to 3 days post fertilization (dpf), then fixed with 4% PFA and processed for in situ hybridization for c-myb or runx1 expression.

**Heat shock treatment of NICD transgenic embryos**

The transgenic line tg(uas:NICD) was crossed to tg(hsp70:gal4). Embryos between 8-12-somite stages were collected in 50-ml Falcon tubes and were heat shocked for 30 minutes in a 37°C water bath. After the heat shock, the embryos were placed in Petri dishes, allowed to develop until 36-40 hpf and then fixed in 4% PFA. Fixed embryos were then processed for in situ hybridization for c-myb, runx1 or cbfb expression.
Results

Generation of zebrafish cbfb\(^{-}\) mutants

To determine the role of CBFβ in the formation of HSCs we generated two independent cbfb mutant lines by zinc-finger nuclease (ZFN) - mediated targeted mutagenesis\(^ {32,33}\). The selected CompoZr ZFN pair targeted a specific region within cbfb exon 3 (Fig. 1A)\(^ {30}\).

Among nine mutations identified\(^ {30}\) from six germline transmitting founders, we selected two mutations predicted to cause frameshifts with premature terminations, cbfb\(^{hg10}\) (c.215delACCT, p.N72IfsX25) and cbfb\(^{hg11}\) (c.215insACCT, p.H74PfsX43), denoted here as cbfb\(^{del4}\) and cbfb\(^{ins4}\) (Fig. 1B). In order to test whether the mutations lead to loss of cbfb expression we evaluated the presence of cbfb transcripts in cbfb\(^{del4/del4}\) and cbfb\(^{ins4/ins4}\) mutants by WISH. At 36 hpf, cbfb expression in the ventral DA was detectable in wild type (WT) embryos (Fig. 1C) but not in cbfb\(^{del4/del4}\) (Fig. 1D) or cbfb\(^{ins4/ins4}\) (Fig. 1E) embryos, suggesting that the mutant mRNA was degraded in both cbfb\(^{del4/del4}\) and cbfb\(^{ins4/ins4}\) mutants.

Embryos heterozygous for the mutations in cbfb were indistinguishable to their wild type clutchmates and presented normal hematopoiesis (data not shown). cbfb\(^{del4/del4}\) and cbfb\(^{ins4/ins4}\) embryos did not show any obvious morphological or developmental defects and were indistinguishable in appearance from their wild type or heterozygous clutchmates. cbfb\(^{del4/del4}\) and cbfb\(^{ins4/ins4}\) embryos presented identical hematopoietic phenotypes and died around 14 dpf; therefore they are frequently referred collectively as cbfb\(^{-}\) 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 (PLM), where primitive hematopoietic progenitors are formed, and in the intermediate cell mass (ICM) where primitive erythroid cells arise. To determine if cbfb—mutants had any primitive hematopoietic defects we tested the expression of several hematopoietic markers in cbfb—mutants and WT siblings by WISH. The expression of the erythroid marker gata1 appeared unaltered in cbfbdel4/del4 and cbfbins4/ins4 mutants at 16-somites and 24 hpf (Supplemental Fig. 1A-F), suggesting that the development of primitive erythroid cells was unaffected in cbfb—mutants. Primitive erythroblasts differentiated into erythrocytes in cbfbdel4/del4 and cbfbins4/ins4 embryos as whole embryo o-dianisidine staining appeared normal at 48 hpf (Supplemental Fig. 1J-L), and the expression of the hemoglobin gene ae1-globin (hbae1) was unaffected (Supplemental Fig. 1M-O). The expression of the myeloid marker l-plastin was also maintained in both cbfb null mutants at 24 hpf (Supplemental Fig. 1G-I), suggesting that primitive myeloid cells were unaffected. In addition, cbfb null embryos had normal expression of gata1, l-plastin and mpx in the PBI at 36 hpf, indicating that the EMP progenitors were correctly specified (Supplemental Fig. 2A-I).

The zebrafish cbfb—embryos lack definitive hematopoiesis

It is known that loss of either Runx1 or Chfb abolishes the onset and the development of definitive hematopoietic cells in mouse embryos. Since the zebrafish runx1W84X/W84X mutant embryos also lack definitive hematopoiesis, we evaluated the presence of definitive blood lineages in the cbfb—mutants (Fig. 2 and Supplemental Fig. 3). At 3 dpf, the expression of l-plastin in definitive myeloid progenitors within the CHT was almost
undetectable in \(cbfb_{\text{del4/del4}}\) (Fig. 2B) and \(cbfb_{\text{ins4/ins4}}\) (Supplemental Fig. 3B) mutants when compared to WT siblings (Fig. 2A and Supplemental Fig. 3A). The expression of \(hbae1\) in erythroid precursors within the CHT was also abrogated in \(cbfb_{\text{del4/del4}}\) (Fig. 2C and D) and \(cbfb_{\text{ins4/ins4}}\) (Supplemental Fig. 3C and D) embryos at 6 dpf. The lymphoid markers \(ikaros\) and \(rag1\) in the developing thymus were absent in \(cbfb_{\text{del4/del4}}\) (Fig. 2E-H) and \(cbfb_{\text{ins4/ins4}}\) (Supplemental Fig. 3E-H) mutants at 5 dpf as well. Moreover, circulating thrombocytes were almost undetectable in \(cbfb_{\text{del4/del4}}^{\text{tg}(\text{cd41}:\text{GFP})}\) embryos, in which the expression of GFP is driven by the promoter of a thrombocyte-specific gene, \(\text{cd}41\) (Supplementary movies 1 and 2). Therefore, our results are consistent with a complete failure of definitive blood lineages in the \(cbfb^{\text{+/-}}\) zebrafish mutants.

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

To investigate whether loss of \(cbfb\) affected the onset of definitive hematopoiesis we analyzed HSC development by testing the expression of \(runx1\) and \(c-myb\) by WISH. Nascent \(runx1^{+/+}/c-myb^{+/+}\) HSCs emerge from the hemogenic endothelium in the ventral wall of the DA around 30 hpf\(^{34,35}\). In \(runx1^{W84X/W84X}\) mutants \(c-myb^{+/+}\) HSCs were absent\(^{21}\).

However, at 36 hpf \(c-myb\) expression was observed along the ventral wall of the DA in both \(cbfb_{\text{del4/del4}}\) (Fig. 3B, B’) and \(cbfb_{\text{ins4/ins4}}\) (Fig. 3C, C’) embryos at similar levels to their WT clutchmates (Fig. 3A, A’). Similarly, expression of the early HSC marker, \(runx1\), was intact or even slightly increased in the ventral DA region of the \(cbfb_{\text{del4/del4}}\) (Fig. 3E, E’) and \(cbfb_{\text{ins4/ins4}}\) (Fig. 3F, F’) embryos at 36 hpf, as compared to the controls (Fig. 3D, D’). Given the normal expression of \(c-myb\) and the strong expression of \(runx1\) in \(cbfb^{\text{+/-}}\) mutants at 36 hpf, we evaluated the presence of a compensatory mechanism involving other \(runx\) family members. However, in \(cbfb^{\text{+/-}}\) embryos between 36 hpf and
3dpf the expression pattern and level of runx2a, runx2b and runx3 were normal (in the pharyngeal arches and cartilage) and no ectopic expression was detectable, especially not in the hematopoietic tissues (data not shown).

Overall, the presence of runx1+ and c-myb+ cells within the hemogenic endothelium of cbfb<sup>del4/del4</sup> and cbfb<sup>ins4/ins4</sup> mutants indicates that the emergence of nascent HSCs does occur in cbfb<sup>-/-</sup> embryos.

**HSCs do not reach CHT and kidney in the cbfb<sup>-/-</sup> embryos**

Starting from 30 hpf HSCs asynchronously egress from the ventral DA into the sub-aortic space and intravasate into the axial vein to seed the CHT<sup>7</sup>. Consistent with the translocation of HSCs, at 2 dpf c-myb expression is observed in both AGM and CHT regions of wild type embryos (Fig. 4A and A’). However, c-myb expression in cbfb<sup>-/-</sup> mutants appeared strongly reduced and was detectable only in the AGM (Fig. 4B, B’, C, C’). At 3 dpf, when c-myb+ cells were found only in the CHT of wild type embryos (Fig. 4D and D’), no c-myb expression was detected in any hematopoietic region of cbfb<sup>del4/del4</sup> (Fig. 4E and E’) and cbfb<sup>ins4/ins4</sup> (Fig. 4F and F’) mutants. At 5 dpf c-myb expression in CHT and kidney was detectable in WT (Fig. 4G, G’, G’’) but not in cbfb<sup>-/-</sup> (Fig. 4H, H’, H’’, I, I’, I’’) embryos.

Apoptosis was not likely the reason for the reduction in c-myb expression, since TUNEL staining was not increased in cbfb<sup>-/-</sup> embryos between 36 and 48 hpf (Supplemental Fig. 4A). Moreover, the expression of the proliferating cell nuclear antigen (PCNA)<sup>36</sup> in hematopoietic progenitors within the AGM of cbfb<sup>-/-</sup> mutants between 36-48 hpf appeared comparable to wild type controls (Supplemental Fig. 4B). Similarly, anti-phospho-histone H3 staining of cbfb<sup>del4/del4</sup>tg(c-myb:eGFP) embryos and wild type
siblings at 36 and 48 hpf did not show differences in proliferation of the hematopoietic progenitors (eGFP+ cells) within the AGM (Supplemental Fig. 4C-F).

On the other hand, the expression of the early HSC marker, runx1, was maintained in the AGM of cbfb−/− mutants (Fig. 5B, B’, C, C’) at 48 hpf as compared to the wild type embryos (Fig. 5A, A’). In WT embryos at 3 dpf HSCs were located in the CHT and runx1 expression was no longer detectable in the AGM (Fig. 5D and D’). At the same stage of development, however, strong runx1 expression was still detectable in the AGM region in cbfbdel4/del4 and cbfbins4/ins4 mutants (Fig. 5E, E’, F, F’). Since cbfbdel4/del4 and cbfbins4/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 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 AGM into circulation by performing time lapse imaging analysis of multiple cbfbdel4/del4/tg(c-myb:eGFP) and wild type tg(c-myb:eGFP) siblings between 48-63 hpf (Fig. 5G-I and Supplemental movies 6 and 7). We observed an average of 12 eGFP+ cells leaving the AGM and entering the circulation through the axial vein per tg(c-myb:eGFP) embryo (n=5) during the recorded period (15 hrs; Fig. 5J). On the other hand, on average we observed only 1 eGFP+ cell leaving AGM per cbfbdel4/del4/tg(c-myb:eGFP) embryo (n=5) in the same recorded period (Fig. 5J), which was significantly lower than the control (p<0.001).

Thus, unlike runx1W84X/W84X mutants where HSC formation was completely abrogated, nascent HSCs were formed in the cbfb−/− 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.

**Pharmacological inhibition of RUNX1-CBFβ interaction phenocopies the hematopoietic defects in cbfb−/− embryos**

Recently we identified a specific inhibitor of the RUNX1-CBFβ interaction, Ro5-3335\(^{37}\). 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 one allele of the runx1 truncation mutation (runx1\(^{+/W84X}\)) are more sensitive to Ro5-3335 treatment (for developing hematopoietic defects) than WT transgenic embryos\(^{37}\). Since 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−/− 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 \(\mu\)M, 2.5 \(\mu\)M and 0.25 \(\mu\)M from 24 hpf to 36 hpf showed normal expression of c-myb and runx1 within the ventral DA (Fig. 6A and Supplemental Fig. 5A-D). At a higher concentration (5 \(\mu\)M) we observed only a slight reduction in c-myb expression in 12% of the embryos when compared to their DMSO controls (Fig. 6A). Neither cbfb\(^{+/del4}\) nor runx1\(^{+/W84X}\) embryos showed more reduction in c-myb expression than wild type 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−/− embryos (Fig. 6B). In addition, cbfb+/del4 mutants were more sensitive to Ro5-3335 treatment than WT embryos for the absence of c-myb expression in CHT, as more cbfb+/del4 embryos developed the phenotype than WT embryos at a given concentration (compare Fig. 6B with Supplemental Fig. 5E).

To confirm that Ro5-3335 fully recapitulated the cbfb−/− 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 48-58 hpf with time lapse imaging (Fig. 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 hrs; p<0.001; Fig.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.

**cbfb acts downstream of the Notch pathway**

The Notch-Runx1 pathway is critical for the initial specification of HSCs during definitive hematopoiesis. 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. Conversely, runx1 expression in HSCs is abrogated in the mind bomb mutant, where an E3 ubiquitin ligase essential for Notch signaling is
mutated\textsuperscript{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 (Fig. 7A, A’, B, 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 12 somites. We confirmed that the expression of c-myb and runx1 was expanded in the heat-shocked embryos (Fig. 7C-F’). We observed that cbfb expression was also expanded in the aorta and ectopically expressed in the vein (Fig. 7G, G’, H, H’), similar to both c-myb and runx1 (Fig. 7C-F’). These results suggest that cbfb expression is regulated by Notch activity.
Discussion

The *Cbfb* gene has been demonstrated as a key regulator of definitive hematopoiesis during embryogenesis in mice\(^\text{12,15}\). *Cbfb\(^{-/-}\)* embryos lacked definitive hematopoiesis, while some EMPs remained\(^\text{15}\). In a recent study, lineage specific expression of a *Cbfb* transgene in *Cbfb* knockout mice showed that EMPs and HSCs differentiate from distinct populations of hemogenic endothelial cells\(^\text{22}\). However, there have been no reported studies on the exact roles of *Cbfb* for the emergence of HSCs from hemogenic endothelium.

In this study we generated two 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 endothelial-hematopoietic transition (EHT) of the hemogenic endothelium into HSC during the early phases of definitive hematopoiesis\(^\text{6,18,19}\). Therefore, since CBF\(\beta\) is considered the obligate partner of RUNX1, the impairment of all definitive hematopoietic lineages in both *Runx1\(^{-/-}\)* and *Cbfb\(^{-/-}\)* 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 pharmacological inhibition of the RUNX1-CBF\(\beta\) interaction in WT zebrafish embryos with a specific inhibitor, Ro5-3335\(^\text{37}\). 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^{+/W84X} embryos showed a reduction in c-myb expression within the DA after Ro5-3335 treatment. The presence of nascent runxl^{+}/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 indicate that CBFB is not necessary for the EHT and strongly suggests that the function of RUNX1 and CBFB during HSC development can be uncoupled. In the future, the temporal requirement of CBFB 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 of wild type 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-CBFB interaction by showing that cbfb^{+/del4} mutants were more sensitive to Ro5-3335 treatment than WT embryos.

The loss of function of cbfb did not affect the expression of the early HSC marker runx1. Strikingly, runxl^{+} 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^{hkc3} mutants^{39}, quantitative time-lapse observations of cbfb^{del4/del4}tg(c-myb:eGFP) embryos demonstrated a strong
impairment in the intravasation of c-myb:eGFP+ cells to the axial vein from the sub-aortic mesenchyme. The same phenotype was also recapitulated in tg(flk1: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, since the Notch-Runx1 pathway is critical for the initial specification of HSCs during definitive hematopoiesis34. We found that transient Notch activation enhanced cbfb expression and expanded it ectopically. On the other hand, in 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 two different steps during HSCs 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+/c-myb+ HSCs in cbfb−/− embryos indicate that cbfb is dispensable for the emergence of HSCs but also imply that RUNX1 is able to drive HSC formation in the absence of its known obligate co-factor 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: EB, BC, SW, MPJ, and AVG designed and performed the experiments, and analyzed the data; BMW, RS, and PPL designed and organized the experiments, and analyzed the data; EB and PPL wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Figure Legends

Figure 1. Zinc-finger nuclease-mediated targeted mutagenesis of cbfb

(A) A schematic of genomic organization of cbfb with numbered boxes depicting exons, connecting lines depicting introns and red bars marking the location of ZFN pair used to generate knockout mutants. (B) Alignment of nucleotide sequences from nt198-231 of cbfb open reading frame to show the ZFN target site (grey highlight in WT), spacer sequence (red letters) and exact sequences of the mutant alleles, del4 and ins4, which are characterized in this study. Yellow highlighted area marks the deleted or inserted nucleotides. (C) At 36 hpf expression of cbfb in WT sibling was detectable in the ventral dorsal aorta (DA), where HSCs originate (black arrowhead). cbfb expression in the ventral DA was abrogated in both cbfb<sup>del4/del4</sup> (D) and cbfb<sup>ins4/ins4</sup> mutants (E).

Figure 2. Definitive blood lineages are absent in cbfb<sup>del4/del4</sup> embryos

Expression of markers for definitive hematopoietic lineages in WT control siblings (A, C, E, G) and cbfb<sup>del4/del4</sup> embryos (B, D, F, H) by WISH. At 3 dpf the myeloid marker <i;l-plastin</i> was expressed in the caudal hematopoietic tissue (CHT) of wild type embryos (A, black arrow) but not in cbfb<sup>del4/del4</sup> embryos (B). <i>hbae1</i> expression in the erythroid progenitors within the CHT (C, black arrow) was completely abrogated in cbfb<sup>del4/del4</sup> embryos (D) at 6 dpf. Expression of the T-lymphocyte markers <i>ikaros</i> and <i>rag1</i> within the thymus (E, G, black arrowheads) was also abrogated in cbfb<sup>del4/del4</sup> embryos (F, H) at 5 dpf.

Figure 3. HSCs emerge from hemogenic endothelium in cbfb<sup>+/−</sup> embryos

Expression of HSC markers <i>c-myb</i> and <i>runx1</i> in cbfb<sup>+/−</sup> and WT embryos at 36 hpf by WISH. Compared to WT siblings (A, A’), the HSC marker <i>c-myb</i> was normally expressed in the hemogenic endothelium
of the ventral DA of $cbbb^{del4/del4}$ (B, B’) and $cbbb^{ins4/ins4}$ (C, C’) embryos at 36 hpf. Similarly, the expression of runx1, another HSC marker, was also unaffected in $cbbb^{del4/del4}$ (E, E’) and $cbbb^{ins4/ins4}$ (F, F’) embryos, as compared to WT embryos (D, D’). The panels A’-F’ depict the boxed regions in panels A-F.

**Figure 4. HSCs do not translocate from AGM to CHT and Kidney in $cbbb^{-/-}$ embryos**

Expression of HSC marker c-myb in 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’), while they did not in $cbbb^{del4/del4}$ (B, B’) or $cbbb^{ins4/ins4}$ (C, C’) embryos. At 3 dpf, c-myb+ hematopoietic cells could readily be detected in the CHT in WT embryos (D, D’), while no c-myb expression was detectable in the CHT in $cbbb^{del4/del4}$ (E, E’) or $cbbb^{ins4/ins4}$ (F, F’) embryos. At 5 dpf c-myb expression in CHT and kidney was detectable in WT (G, G’, G’’) but not in $cbbb^{del4/del4}$ (H, H’, H’’) or $cbbb^{ins4/ins4}$ (I, I’, I’’) embryos. Panels A’-F’ depict the boxed regions in panels A-F. Panels G’, H’, I’ depict the regions in the left boxes in panels G, H, and I. Panels G’’, H’’, and I’’ depict the regions in the right boxes in panels G, H, and I.

**Figure 5. HSCs are not released from the AGM in $cbbb^{-/-}$ embryos**

(A-F’) Expression of HSC marker runx1 in embryos at 48 hpf and 3 dpf, detected by WISH. At 48 hpf the expression of runx1 was maintained in the AGM of $cbbb^{del4/del4}$ (B, B’) and $cbbb^{ins4/ins4}$ (C, C’) embryos than in the WT embryos (A, A’). runx1 expression in hematopoietic regions, including both the AGM and the CHT regions, was downregulated in WT embryos at 3 dpf (D, D’). In $cbbb^{del4/del4}$ and $cbbb^{ins4/ins4}$ embryos, however, runx1 remained strongly expressed in the AGM (E, E’, F, F’). Panels A’-F’ depict boxed regions in panels A-F. (G-J) Time lapse imaging analysis of wild type tg(c-myb:eGFP) and $cbbb^{del4/del4}/tg(c-myb:eGFP)$ embryos between 48-63 hpf (5-minute intervals for 15 hours) to record the numbers of eGFP+ HSCs released from the AGM. Panel G is a lateral view of a 2 dpf embryo, with
the boxed area indicating the region that was imaged by time lapse. Panels H and I show merged video captures of fluorescence and bright field images (Z=21) of the same region at different time points, displaying the egression of an eGFP+ cell (white arrowhead) in a wild type tg(c-myb:eGFP) embryo (present in H but disappeared in I). DA: dorsal aorta. AV: axial vein. (J) Bar graphs depicting average numbers of eGFP+ HSCs leaving AGM in 5 embryos of each genotype. On average 12 eGFP+ cells per embryo left the AGM and entered the circulation through the axial vein in wild type tg(c-myb:eGFP) embryos during the recording period. In cbfb^{del4/del4}/tg(c-myb:eGFP) embryos an average of 1 cell per embryo was released into circulation during the same recording period. *** p<0.001 vs wild type.

**Figure 6. Treatments with Ro5-3335 phenocopy cbfb^{-/-} hematopoietic defects**

Bar graphs showing the effect of Ro5-3335 treatment on c-myb expression in wild type embryos from 24 hpf to 36 hpf (A) and from 24 hpf to 3 dpf (B). Percentages of embryos with unaffected (white bars), reduced (grey bars) and absence (black bars) of c-myb expression are depicted on the Y-axis. Right panels show representative images of different categories of c-myb expression (1: unaffected; 2: reduced; 3: absent). (C-F) Confocal time lapse imaging of the AGM region of tg(flk1:moesin1-eGFP) embryos between 48-58 hpf (5-minute intervals for 10 hours), which were treated with DMSO (0.1%) or Ro5-3335 (5 μM) from 24 hpf. Panel C shows bar graphs representing average numbers of eGFP+ HSCs leaving AGM in DMSO (n=3) and Ro5-3335 (n=5) treated embryos. *** p<0.001 vs DMSO. Panel D shows a lateral view of a 2 dpf embryo, the box indicates the region that was imaged by time lapse. Panels E and F show two representative video frames of fluorescence images (Z=6-8) of the same AGM region of a DMSO-treated tg(flk1:moesin1-eGFP) embryo at two time points (45 minutes apart). The red and the white dots in panel E mark two eGFP+ cells within the AGM. The eGFP+ cell marked in red in panel E is no longer present in panel F, indicating that it had been released into the axial vein. DA: dorsal aorta. AV: axial vein.
Figure 7. *cbfb* acts downstream of the Notch pathway.

*cbfb* expression in the hematopoietic progenitors in the ventral dorsal aorta was detectable in control siblings (A, A’) but not in the *mind bomb* mutant (B, B’) at 36 hpf. The expression of *c-myb* and *runx1* was expanded in the dorsal aorta and the axial vein of heat shocked *hsp70:gal4;uas:NICD* embryos at 36 hpf (C-F’). Similarly, *cbfb* expression was expanded in the heat shocked *hsp70:gal4;uas:NICD* embryos when compared to WT embryos (G, G’, H, H’) at 36 hpf. Panels A’-H’ depict the boxed regions in panels A-H.
Figure 1

A

1  2  3  4  5  6

B

WT: GCAGTTCTTTCCAGCAACCTTTCATGGGATTCAGCGCGCAG

cbfb Del4: GCAGTTCTTTCCAGCAACCTTTCATGGGATTCAGCGCGCAG

cbfb Ins4: GCAGTTCTTTCCAGCAACCTTTCATGGGATTCAGCGCGCAG

198  231

C cbfb

9/9 wild type

D cbfb<sup>del4/del4</sup>

7/7

E cbfb<sup>ins4/ins4</sup>

7/7
Figure 3

c-myb

Wild type

A 21/21

B 11/11

B’ 16/16

E 14/14

F 11/11

36 hpf

runx1

A’

D'

cbrfb del4/del4

C 9/9

C’

E'

F'

cbfb ins4/ins4

36 hpf
Figure 4

*c-myb*

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Counts:
- Wild type: 13/13, 7/7, 7/7
- cbfb del4/del4: 12/12, 10/10, 5/5
- cbfb ins4/ins4: 12/12, 11/11, 4/4
Figure 5

48 hpf

Wild type

runx1

15/15

runx1

8/8

runx1

10/10

3 dpf

runx1

20/20

runx1

19/19

runx1

9/9

J

WT  cbfb^{del4/del4}

n=5  n=5

tg(c-myb:eGFP)
Figure 6

A

WT

Unaffected (1) Reduced (2) Absent (3)

DMSO 0.25 μM 2.5 μM 5 μM Ro5-3335

B

WT

Unaffected (1) Reduced (2) Absent (3)

DMSO 0.25 μM 2.5 μM 5 μM Ro5-3335

C

# cells released from AGM

DMSO Ro5-3335

tg(flk1:moesin-GFP) 5 μM

n=3 n=5

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

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