Runx1 Regulates Embryonic Myeloid Fate Choice in Zebrafish through a
Negative Feedback Loop that Confines Pu.1 Expression

Running head: Regulation of Zebrafish Embryonic Myelopoiesis

Key words: Zebrafish, Myelopoiesis, Cell fate, Macrophages, Neutrophils

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Abstract

Proper cell fate choice in myelopoiesis is essential for generating correct numbers of distinct myeloid subsets manifesting a wide spectrum of subset-specific activities during development and adulthood. Studies have suggested that myeloid fate choice is primarily regulated by transcription factors; however, new intrinsic regulators and their underlying mechanisms remain to be elucidated. Zebrafish embryonic myelopoiesis gives rise to neutrophils and macrophages and represents a promising system to derive new regulatory mechanisms for myeloid fate decision in vertebrates. Here we present an in vivo study of cell fate specification during zebrafish embryonic myelopoiesis through characterization of the embryos with altered Pu.1, Runx1 activity alone or their combinations. Genetic analysis shows that low and high Pu.1 activities determine embryonic neutrophilic granulocyte and macrophage fate, respectively. Inactivation and overexpression of Runx1 in zebrafish uncovers Runx1 as a key embryonic myeloid fate determinant that favors neutrophil over macrophage fate. Runx1 is induced by high Pu.1 level and in turn transrepresses *pu.1* expression, thus constituting a negative feedback loop that fashions a favorable *pu.1* level required for balanced fate commitment to neutrophils versus macrophages. Our findings define a Pu.1-Runx1 regulatory loop that governs the equilibrium between distinct myeloid fates by assuring an appropriate Pu.1 dosage.
Introduction

Myeloid cells are a collection of morphologically, phenotypically, and functionally distinct blood cells that conventionally consist of two separated lineages, granulocytes (neutrophils, basophils, and eosinophils) and monocytes/macrophages. The importance of this class of cells is exemplified by their wide engagement in diverse physiological processes including organogenesis, tissue homeostasis as well as immune defense. Perturbation in the formation, differentiation as well as function of these cells will incur devastating consequences like leukemia. Hence, comprehensive understanding of how functional myeloid system is established will render novel therapeutic opportunities for curing myeloid malformation associated diseases.

During ontogeny, there exist multiple waves of myeloid cell production, termed myelopoiesis. In the adult myelopoiesis, all granulocytes and monocytes/macrophages are derived from the hierarchical transformation of multi-potential hematopoietic stem cells (HSCs) into lineage restricted progenitors and subsequent maturation of unilineage restricted progenitors. In the developing embryos where HSCs are not formed, myeloid cells are transitorily supplied by uni-potent or bi-potent progenitors which appear to arise directly from mesoderm without transiting through multi-potent HSCs-like progenitors.

A key indispensable step shared by different waves of myelopoiesis is the specification of granulocyte or monocyte/macrophage fate from initially equivalent pool of myeloid progenitors. Resolving granulocyte versus monocyte/macrophage
fate is thought to be primarily dictated by the interplay among various transcription
factors and their abundances at appropriate developmental timepoints.\textsuperscript{2} Although a
number of transcription factors have been shown to be required for the general or
certain specific aspect of myeloid development, few transcription factors are
demonstrated to have a primary role in regulating granulocyte versus
monocyte/macrophage fate choices.\textsuperscript{2} PU.1, also known as Spi-1, is a member of Ets-
family transcription factors. Apart from the earlier obligatory role of PU.1 in
generating myeloid progenitors,\textsuperscript{3,4} PU.1 activity level appears to be critical for fate
decision of myeloid progenitors. In vitro reconstitution of PU.1 expression in PU.1\textsuperscript{-/-}
progenitors showed that granulocytes developed from progenitors supplied with low
PU.1 expression whereas macrophages developed from progenitors supplied with
high PU.1 expression.\textsuperscript{5,6} However, there is still controversy over whether different
PU.1 dosages cause distinct myeloid fate\textsuperscript{7} partly because the consequence of altering
PU.1 expression on myeloid fate choice has not been directly tested in vivo. The
transcriptional regulation of \textit{Pu.1} expression has been studied over years, which leads
to the identification of an upstream cis-regulatory element important for proper
expression of \textit{Pu.1} in mice.\textsuperscript{8} However, such regulation mediated by this cis-regulatory
element has not been causatively linked to the control of PU.1 dosage in the context
of myeloid fate decision. Thus, the mechanism by which appropriate PU.1 level is
attained in vivo to ensure balanced commitment towards different myeloid subsets
remains elusive.
Zebrafish, as a genetically tractable system, is increasingly employed to dissect vertebrate specific developmental mechanisms. Embryonic myelopoiesis in zebrafish occurs in anteriorly located rostral blood island (RBI)\(^9\)\(^-\)\(^11\) where cephalic mesoderm derived myeloid progenitors differentiate to produce either macrophages or neutrophils.\(^10\)\(^,\)\(^12\) Embryonic myeloid cells might also arise from a transient population of erythromyeloid progenitors (EMPs),\(^13\) yet the relative contribution of this source to the total embryonic myeloid pool is not clear. Nevertheless, the derivation of zebrafish embryonic myeloid cells without transiting through multi-potential HSCs allows for bypassing the potential epistatic role of transcription factors in HSCs and directly accessing their roles in the myeloid development per se.\(^14\) The utility of zebrafish embryonic myelopoiesis as a model to study the common mechanisms of vertebrate myelopoiesis is supported by numerous reports showing the conserved expression and function of genes for both myeloid fate determination and terminal differentiation.\(^14\)\(^,\)\(^15\) Like in the adult mouse myelopoiesis, the early undifferentiated embryonic myeloid cells in the RBI express transcription factor \textit{pu.1}, and maximal knockdown of \textit{pu.1} disrupted the formation of both macrophages and neutrophils.\(^16\)\(^,\)\(^17\) However, how embryonic macrophages and neutrophils in zebrafish establish their respective fates is still unclear.

Here we describe a previously unidentified role of transcription factor, \textit{runx1}, in regulating zebrafish embryonic myeloid fate choices through directly suppressing \textit{pu.1} expression in a negative feedback loop. Loss and gain-of-function studies
established a differential requirement of Pu.1 activity in vivo for the formation of RBI originated neutrophil versus macrophage fates with low Pu.1 activity permissive for neutrophil fate while high Pu.1 activity instructive for macrophage fate. Using a candidate gene approach along with loss and gain of function study, runx1 is shown to regulate embryonic myeloid fate decision by favoring neutrophil over macrophage fates. Expression analyses followed by the compound mutant studies indicate that runx1 allows for neutrophil commitment by feedback repressing pu.1 expression. Further biochemical analyses suggest that runx1 binds to pu.1 promoter and directly suppresses pu.1 expression. Together, we propose that runx1 acting as a direct feedback repressor to constrain pu.1 expression is an important mechanism that determines fate choice between different embryonic subsets.

Materials and Methods

Zebrafish husbandry

Zebrafish were raised, bred, and staged according to standard protocols.18 The following strains were utilized: AB, Tg(mpx:eGFP)i114,19 Tg(lyz:Dsred2)n50,20 Tg(lyz:eGFP)n117,20 Tgt(-5.3pu.1:eGFP),21 csf1ra1e1,22 runx1n84s,23,24 pu.1G242D, Tg(hsp70:myc-runx1)hkz02t and Tg(hsp70:myc-pu.1)hkz03t.

Lineage tracing

Lineage tracking using DMNB caged fluorescein was performed essentially as described previously.25
Targeted induced local lesions in genomes (TILLING) and isolation of \( pu.I^{G242D} \)

Tilling isolation of \( pu.I^{G242D} \) was performed essentially as described previously.\(^\text{26}\) The mutation was identified by sequencing the genomic DNA of \( pu.I \) locus.

**In vitro synthesis of antisense RNA probe and mRNA**

Antisense RNA probes were synthesized by in vitro transcription reaction according to standard protocols.\(^\text{18}\) The probes used are listed in the Supplemental Material and Methods.

**Histology**

Single color whole mount in situ hybridization using NBT/BCIP as a staining substrate was performed based on standard protocol.\(^\text{18}\) Single color antibody staining and two color fluorescence in situ hybridization were carried out as previously described.\(^\text{27}\) Conventional Sudan Black (SB) staining and combined detection of SB staining and protein targets were essentially performed according to previous report.\(^\text{12}\) The antibodies used are listed in the Supplemental Materials and Methods.

**Scoring myeloid cells expressing specific lineage markers**

The number of positive cells in embryos having been stained for a particular lineage marker was manually counted under Nikon AZ100 microscope. Each embryo was scored twice for all the identifiable positive cells and the average count is calculated. After scoring, individual embryo was transferred into 96-well plates for genotyping. Individual counts were eventually sorted based on genotype for comparison among different genotype groups.
MO injection

*pup.1* sp3 MO (5’-AATAACTGATACAAACTCACCGTTC-3’) targeting exon2 intron2 boundary was designed and synthesized by Gene Tools. Control MO used was a standard control morpholino purchased from Gene Tools. MO was administrated at 16 ng per embryo.

Quantitative and semi-quantitative RT-PCR

The RNA preparation, cDNA synthesis, qRT-PCR were performed as described (Supplemental Materials and Methods).

Pu.1 promoter reporter assay

The activities of various *pup.1* promoter fragment in wild-type or *runx1* background were analyzed by either injecting DNA constructs (-9.0*pup.1*:eGFP; -9.0*pup.1*ΔR:eGFP) into one-cell stage embryos or using a stable transgenic line *Tg(-5.3pup.1*:eGFP). The expression levels of GFP were determined by living monitoring and quantified by qRT-PCR at 17.5 hpf (Supplemental Materials and Methods).

Chromatin immunoprecipitation (ChIP)

For ChIP analysis, 450 embryos derived from *Tg(hsp70:myc-runx1)hkh02t* +/- crossed with AB wild-type were heat shocked at 14 hpf stage for 45mins and harvested at 20 hpf for brief fixation. Crosslinked chromatin was immunoprecipitated with anti-myc antibody or anti-Brdu antibody (negative control) according to the procedure described. The resultant immunoprecipitated samples were subjected to semi-quantitative PCR using primer pairs: *runx1* I: 5’-cagcgtgtattaataaaga-3’/5’-cagcgtgtattaataaaga-3’/5’-
acattatatgcattcatt-3'; runx1 II: 5’-agtaacacagtgaacacatt-3’/5’- atgaatctttactgtca-3’; mespa: 5’-aagagtaagctggtggagaaaaact-3’/5’-ctcttctcaagtctgactgaatc-3’.

Results

RBI gives rise to two distinct embryonic myeloid lineages, neutrophils and macrophages, which are distinguishable by distinct markers

During zebrafish embryonic myelopoiesis, two alternative myeloid lineages, neutrophils and macrophages, have been reported to arise.9,10,17,31,32 It has been suggested that both embryonic neutrophils and macrophages were originated from the RBI in zebrafish.10,12,33 To validate this observation, we employed a more rigorous assay in which we photolabeled the RBI myeloid cells with fluorescein (flu+) at 14–16 hours post-fertilization (hpf) and subsequently identified their fates based on the cellular and morphological characteristics of flu+ progeny in 36–48 hpf living embryos (supplemental Figure 1A). Very often in the same embryo, certain portion of the flu+ progeny were found to be macrophages because these cells were loaded with engulfed material (supplemental Figure 1B) such as apoptotic cells while other flu+ progeny were determined as neutrophils as they bore segmented nucleus and actively moving granules (supplemental Figure 1B). Thus it confirms the notion that both embryonic neutrophils and macrophages derive from the RBI. We then sought to define the segregation of these two embryonic myeloid lineages in the RBI and their subsequent differentiation with molecular markers. CCAAT/enhancer binding protein 1 (cebp1) is a presumptive functional orthologue of C/ebpε, a transcription factor
indispensable for neutrophil maturation in mammals\textsuperscript{34-36} and \textit{lysozyme C (lyz)} encodes a primary and secondary granule protein in granulocytes.\textsuperscript{37,38} We found \textit{cebp1} and \textit{lyz} extensively overlapped with \textit{myeloperoxidase (mpx)},\textsuperscript{9,31} a canonical neutrophil specific marker in zebrafish but not with macrophage specific markers, such as interferon regulatory factor 8 (\textit{irf8})\textsuperscript{28} and macrophage colony-stimulating factor receptor (\textit{csf1ra})\textsuperscript{33} (supplemental Figure 2A-B, data not shown).\textsuperscript{17,32} In addition, most of \textit{lyz}:Dsred\textsuperscript{+} cells\textsuperscript{20} coexpressed \textit{mpx}:GFP\textsuperscript{19} in the double transgenic line \textit{Tg(lyz:Dsred; mpx:eGFP)} and \textit{lyz}:GFP\textsuperscript{+} cells exhibited defining neutrophil characteristics\textsuperscript{12} such as motile SB\textsuperscript{+} granules and poor phagocytotic activity (supplemental Figure 2C-D). Thus, embryonic neutrophils and macrophages preferentially express \textit{cebp1/mpx/lyz/SB} and \textit{irf8/csf1ra}, respectively. Such discrimination of embryonic myeloid subsets is supported by the expanded expression of \textit{cebp1/mpx/lyz/SB} but the diminished expression of \textit{csf1ra} in \textit{irf8} knocked-down embryos which have a skewed output towards neutrophils.\textsuperscript{28}

We next utilized these lineage specific markers to define the developmental sequence of embryonic neutrophils and macrophages. Consistent with the transcriptional activation of \textit{lyz} by \textit{cebp1},\textsuperscript{39} we found that the expression of \textit{cebp1} preceded that of \textit{lyz} and \textit{mpx} with \textit{cebp1} expression detectable as early as 18 hpf when that of \textit{lyz} and \textit{mpx} was barely seen (supplemental Figure 2E). The early \textit{cebp1}\textsuperscript{+} population was presumably early neutrophil progenitors that later transited to differentiated neutrophils because by 24 hpf, a subset of \textit{cebp1}\textsuperscript{+} cells turned on
mpx/lyz expression but none of them expressed csf1ra (supplemental Figure 2A-B). Mpx+/lyz+ cells proceeded to be weak SB+ at about 32 hpf and strong SB+ from 36 hpf onwards (data not shown, supplemental Figure 2D). Hence, we postulate a defined sequence for embryonic neutrophil development through which cebp1+ early neutrophilic progenitors progressively mature into cebp1+/mpx+/lyz+ intermediate progenitors and SB+ granule containing maturing neutrophils. On the other hand, irf8 expression was detected at 16 hpf preceding csf1ra expression which initiated at 21.5 hpf, while phagocytic macrophages and apoeb+ microglia emerged at a later stage, 24 hpf and 55 hpf, respectively (data not shown).\textsuperscript{10,28,33} Correlated with the temporal appearance of these cell populations, suppression of irf8 abolished the development of csf1ra+ macrophages, phagocytic macrophages and apoeb+ microglia\textsuperscript{28} whereas only phagocytic macrophages and microglia were affected by csf1ra mutation (supplemental Figure 3 and data not shown). Thus, parallel to neutrophil development, embryonic macrophages differentiate along the pathway whereby irf8+ macrophage progenitors, csf1ra+ young macrophages, phagocytic and apoeb+ resident macrophages sequentially arise.

The development of embryonic macrophages but not neutrophils requires full Pu.1 activity

To test whether the formation of embryonic macrophage and neutrophil fates requires different pu.1 levels, we generated a partial loss of function or hypomorphic pu.1
zebrafish allele $pu.1^{G242D}$ through targeted induced local lesion in genome (TILLING) approach. The G242D allele harbored a missense mutation which resulted in a conserved residue Gly (Gly 242) replaced by Asp (supplemental Figure 4). We observed that, although $pu.1$ transcript expressed comparably between $pu.1^{G242D}$ mutants and siblings (supplemental Figure 5A-B), $Pu.1$ protein expression was markedly reduced (supplemental Figure 5C-E), indicating that $pu.1^{G242D}$ mutation destabilized $Pu.1$ protein in vivo and thereby weakened overall $Pu.1$ activity. Assessment of embryonic myeloid development revealed that the initiation of the RBI myelopoietic program and subsequent commitment towards neutrophils in $pu.1^{G242D}$ mutants was essentially maintained as evidenced by normal expression of the markers of early undifferentiated myeloid progenitors ($cebp\alpha$ and $pu.1$) at 14 hpf (data not shown, supplemental Figure 5A-B) and neutrophils of various differentiated stages ($cebp1$, $lyz$, $mpx$ and SB) (Figure 1A-B, E-F, and data not shown). In contrast, the development of embryonic macrophage lineage was completely interrupted in $pu.1^{G242D}$ mutants as shown by the loss of the macrophage markers at various differentiation stages (Figure 1C-D, G-H, and data not shown). In accordance with previous study, only maximal knockdown of $Pu.1$ expression by a high dose of $pu.1$ antisense morpholino oligonucleotides (MO) abolished the initiation of embryonic myelopoietic program in the RBI and subsequent neutrophil development (Figure 1K-L (n=29/29), I-J (n=35/38), M-N (n=45/48)). Based on these data, we conclude that similar to studies in mice, full $Pu.1$ activity is required for
embryonic macrophage specification whereas low Pu.1 activity suffices for embryonic myeloid initiation and neutrophil specification in zebrafish.

**Overexpression of Pu.1 promotes embryonic macrophage fate at the expense of neutrophil fate**

To investigate whether high Pu.1 activity is permissive to macrophage development or instructively influenced macrophage versus neutrophil fate choice, we elevated pu.1 level utilizing an inducible pu.1 overexpression line Tg(hsp70:myc-pu.1)hkz03t in which pu.1 expression was controlled by the heat shock protein (hsp) 70 promoter. When raised at 28°C, Tg(hsp70:myc-pu.1)hkz03t embryos did not produce detectable exogenous Myc-pu.1 expression and had a normal composition of embryonic macrophages and neutrophils (supplemental Figure 6A). Heat-shock treatment of Tg(hsp70:myc-pu.1)hkz03t embryos triggered high level of Myc-pu.1 expression (supplemental Figure 6A-B). As a result, heat-shock treated Tg(hsp70:myc-pu.1)hkz03t embryos had 50% increase in both irf8+ macrophage progenitors (Figure 2A-B, I) and csf1ra+ young macrophages (Figure 2C-D, I) but 50% reduction in cebp1+ neutrophil progenitors (Figure 2E-F, I), 30% reduction in mpx+ (Figure 2I) and SB+ maturing neutrophils (Figure 2G-H, I). Thus in the presence of uniformly high Pu.1 activity, myeloid commitment shifted towards macrophage lineage, suggesting an instructive role of high Pu.1 activity in promoting macrophage over neutrophil fate. Besides, it can also be inferred from these loss-of-function and gain-of-function studies that to ensure balanced commitment toward macrophage
versus neutrophil lineage, endogenous *pu.1* level must be appropriately tuned to a proper range.

**Runx1 represses *pu.1* expression in a negative feedback loop**

To interrogate how appropriate *pu.1* level is attained in vivo, we hypothesized that it could be achieved by transcription factors tuning *pu.1* expression at the transcription level and therefore analysis of *pu.1* promoter might provide a handle. Prompted by this hypothesis, we performed in silico analysis to search for transcription factor binding sites within *pu.1* upstream regulatory region. Among some other putative transcription factor binding sites, 8 putative *runx1* recognition motifs were identified in the 9 kb *pu.1* promoter region. In light of the presence of putative *runx1* motifs in the *pu.1* promoter and the expression of *runx1* in embryonic myeloid cells in the RBI (Figure 3E, G), we set out to address the potential regulation of *pu.1* expression by *runx1* in subsequent study.

To test the role of *runx1* in controlling *pu.1* expression, we investigated the expression of *pu.1* in the *runx1* loss-of-function mutants (*runx1*<sup>w84x</sup>) which harbor a premature stop codon in the essential functional domain, the runt domain. The initial *pu.1* RNA expression at 12 hpf was found to be unaffected by *runx1*<sup>w84x</sup> mutation (data not shown). However, per-cell *pu.1* expression at 17.5 hpf, was markedly intensified in the *runx1*<sup>w84x</sup> mutants, especially in the midline population, which normally expressed less *pu.1* than those emigrating to the yolk sac (Figure 3A-B). A similarly marked enhancement (per cell basis) was detected at protein level
using an anti-Pu.1 antibody (Figure 3C-D). These expression analyses indicate that runxl limits the pu.1 expression after the onset of embryonic myelopoiesis in the RBI.

To further explore the hierarchical regulation between pu.1 and runxl, we evaluated the effect of pu.1 deficiency on runxl expression. We found that runxl expression was nearly absent when pu.1 was maximally depleted with a pu.1 MO and was markedly reduced when pu.1 activity was partially removed by the pu.1G242D allele (Figure 3E-F (n=47/48), G-H). As the count of pu.1+ cells in pu.1G242D mutants were comparable to that in wild-type siblings (supplemental Figure 5A-B) and runxl+ cells co-expressed pu.1 in the RBI, the decreased expression of runxl in pu.1G242D mutants thus indicated that the expression of runxl rather than the frequency of runxl+ cells was regulated by high pu.1 level. Taken together, the observations that runxl expression is suppressed upon pu.1 deficiency and only late phase of pu.1 expression is elevated in runxlw84x mutants suggest a negative feedback loop whereby pu.1 initiates runxl expression and runxl in turn represses pu.1 expression.

**Suppressed embryonic neutrophil but enhanced macrophage development in runxlw84x mutants**

As pu.1 overexpression alone was able to shift the fate of embryonic neutrophil to that of macrophage, we thus speculated that similar fate switching might also occur in runxlw84x mutants, given the upregulated expression of pu.1 in runxlw84x mutants. To test this possibility, we firstly scrutinized embryonic myeloid development in
runx1<sup>w84x</sup> mutants with a panel of markers that discern embryonic myeloid subsets at different development stages. We observed that the expression of pu.1 at 12 hpf was essentially unaffected in runx1<sup>w84x</sup> mutants, indicating the uncommitted embryonic myeloid progenitors was normally formed in runx1<sup>w84x</sup> mutants (data not shown). By contrast, the expression of cebp1, a marker of early neutrophil progenitor, was markedly decreased in runx1<sup>w84x</sup> mutants from the time when this marker became first detectable in the RBI of the embryos and quantification showed that the number of cebp1<sup>+</sup> cells at 22 hpf in runx1<sup>w84x</sup> mutants was only about one-third of that of siblings (Figure 4A-B, G). Accordingly, the expression of more differentiated neutrophil markers including mpx at 22 hpf (Figure 4C-D), mpx/lyz at 32 hpf (Figure 4E-F and supplemental Figure 7A-B) and SB at 36 hpf (supplemental Figure 7C-D) was also significantly decreased in runx1<sup>w84x</sup> mutants, when compared to siblings. Quantification showed that the total number of mpx<sup>+</sup> cells at 32 hpf in runx1<sup>w84x</sup> mutants was about one-tenth of that of siblings (Figure 4H). Neutrophil scoring with in vivo video enhanced DIC microscopy further confirmed that living runx1<sup>w84x</sup> mutants at 36-48 hpf indeed contained far less number of mature neutrophils (supplemental Figure 7E, F). Brdu pulse-chase experiments and Hoechst DNA content analysis indicated that decreased embryonic neutrophil development was not due to the inhibited cell proliferation (supplemental Figure 8). Altogether, diminished neutrophil progenitors in the RBI but intact uncommitted progenitors and unchanged cell division suggest that the diminution of neutrophil number in runx1<sup>w84x</sup> mutants
results from a blockage of neutrophil specification in the RBI. As embryonic neutrophils were also implicated to be derived from erythromyeloid progenitors (EMPs) residing in the posterior blood island (PBI), we thus inferred the impact of runx1w84x mutation on EMP derived neutrophils by comparing the number of neutrophils in the PBI to the total neutrophil number. It showed that in the runx1w84x mutants, the extent of decrease in the neutrophil number in the PBI and in the total neutrophil number was comparable (data not shown), indicating neutrophil arising from EMPs might also be affected by runx1w84x in a way similar to their RBI counterparts.

We next examined whether reduced specification of embryonic neutrophils in runx1w84x mutants was coupled with a skew towards the alternative lineage, macrophage. Contrary to the profound decrease of neutrophils, we noted an expanded macrophage compartment including irf8+ macrophage progenitors, csf1ra+ young macrophages and apoeb+ microglia (Figure 4I-N). Cell counting revealed that both csf1ra+ macrophages and apoeb+ microglia in runx1w84x mutants increased by 40% in comparison to siblings (Figure 4O-P). The enhanced macrophage but concomitantly reduced neutrophil formation in runx1w84x mutants indicates that akin to pu.1 overexpression, runx1 deficiency biases myeloid output to macrophage fate.

**Overexpression of Runx1 promotes embryonic neutrophil fate at the expense of macrophage fate**
The opposing effect of *runx1* loss-of-function mutation (*runx1<sup>*w84x*</sup>) on neutrophil versus macrophage formation implies that *runx1* might regulate embryonic myeloid fate choices by favoring neutrophil fate over macrophage fate. To further support this notion, we examined the consequence of *runx1* overexpression by creating a heat inducible *runx1* overexpression line *Tg(hsp70:myc-runx1)hkz02t*. *Tg(hsp70:myc-runx1)hkz02t* embryos did not yield detectable exogenous myc-*runx1* and displayed normal embryonic myelopoiesis, if grown at normal temperature 28°C (supplemental Figure 6C). Upon heat-shock treatment (at 39.5°C), a high level of myc-*runx1* expression was induced in *Tg(hsp70:myc-runx1)hkz02t* embryos (supplemental Figure 6C-D). Heat-shock induction of myc-*runx1* expression resulted in an enhanced neutrophil development, indicated by 30% increase of cellp1<sup>+</sup> early neutrophil progenitors and 40% increase of SB<sup>+</sup> neutrophils, and a suppressed macrophage development, as evidenced by 30% decrease of both irf8<sup>+</sup> macrophage progenitors and csf1ra<sup>+</sup> macrophages (Figure 4Q). Thus overexpression of *runx1* promotes neutrophil formation but inhibits that of macrophages. Collectively, our loss-of-function and gain-of-function studies demonstrate that *runx1* critically regulates embryonic myeloid cell fate choices through promoting neutrophil fate over that of macrophage.
Reducing pu.1 level in the runx1w84x mutant rescues its phenotype

To directly demonstrate that lineage skewing and the resultant diminished neutrophil lineage development in the runx1w84x mutants were indeed due to unconstrained Pu.1 activity, we tested whether reducing pu.1 level in runx1w84x mutants could reverse the runx1w84x phenotype by crossing hypomorphic pu.1G242D line and runx1w84x fish together. Introduction of one allele of pu.1G242D mutation into runx1w84x/w84x mutants partially restored the number of cebp1+ neutrophil progenitors (Table 1 and supplemental Figure 9A-D, O; comparing runx1w84x/w84xpu.1G242D/+ to runx1w84x/w84xpu.1+/*). Biallelic pu.1G242D mutation in the runx1w84x/w84x background fully revert the number of cebp1+ neutrophil progenitors to the level comparable to those in wild-type and pu.1 single homozygous mutants (Figure 5A-D, I, and Table 1; comparing runx1w84x/w84xpu.1G242D/G242D with runx1w84x/w84xpu.1+/* and with runx1+/*pu.1+/* and runx1+/*pu.1G242D/G242D). Furthermore, both the numbers of lyz+ intermediate cells and SB positive mature cells in runx1w84x/w84x mutants were also significantly increased by combining runx1w84x/w84x with one or two alleles of pu.1G242D (Figure 5E-H, J, Table 1, and supplemental Figure 9E-N, P-Q; comparing runx1w84x/w84xpu.1G242D/+ and runx1w84x/w84xpu.1G242D/G242D with runx1w84x/w84xpu.1+/*).

Thus reducing Pu.1 activity fully corrected embryonic neutrophil specification defects and partially rescued subsequent neutrophil differentiation in runx1w84x/w84x mutants. For macrophage development, akin to pu.1 single homozygous mutants, pu.1 and runx1 double homozygous mutants completely lacked macrophage cells as shown by
WISH assay of multiple macrophage specific markers, underscoring the indispensable requirement for high Pu.1 level in driving macrophage fate (supplemental Figure 10). Collectively, the rescue of runxl<sup>w84x/w84x</sup> mutants by pu.1<sup>G242D</sup>, together with the upregulated pu.1 expression in runxl<sup>w84x/w84x</sup> mutants, demonstrate that runxl regulates bifurcate fate choice between neutrophils and macrophages via confining endogenous pu.1 expression.

**Runx1 directly represses pu.1 promoter**

The presence of putative runxl recognition motifs in the 9.0 kb pu.1 promoter raises the possibility that runxl might directly suppress pu.1 promoter. As these 8 putative runxl recognition motifs were dispersed throughout the 9.0 kb pu.1 promoter, we firstly conducted promoter deletion analysis to assess the contribution of identified runxl sites to transcription repression by runxl. We took the advantage of two available pu.1 reporters, Tg(-5.3pu.1:eGFP) and Tg(-9.0pu.1:eGFP),<sup>21,42</sup> and assayed GFP expression driven by these two regulatory fragment in runxl<sup>w84x</sup> mutants and sibling controls. We found that the level of GFP expressed from Tg(-5.3pu.1:eGFP) was comparable between runxl<sup>w84x</sup> mutants and clutchmates (supplemental Figure 11A) measured by quantitative RT-PCR. In contrast, the level of GFP expressed from injected Tg(-9.0pu.1:eGFP) DNA construct was substantially higher in runxl<sup>w84x</sup> mutants compared to clutchmates (supplemental Figure 11B). The finding that GFP driven by 9.0 kb but not 5.3 kb pu.1 upstream regulatory region exhibited higher
expression in runx1<sup>1<sup>w84c</sup></sup> mutants than in clutchmateds indicates that runx1 binding sites in the 3.7 kb distal region (-9.0- -5.3 kb) might be candidate cis-elements mediating the suppressive effect by runx1. Two adjoining runx1 recognition motifs of high similarity score were present in the 3.7 kb distal promoter region (Figure 6A).

To demonstrate the occupancy of these sites by Runx1, we performed chromatin immunoprecipitation (ChIP) experiment with extracts from heat-shocked Tg(hsp70:myc-runx1)<sub>hkz02t</sub> embryos overexpressing a myc tagged version of runx1. We immunoprecipitated recombinant Runx1 protein and its associated chromatin with an anti-myc antibody and then subjected the immunoprecipitated DNA to semi-quantitative PCR analysis using primer pairs that tiled the 3.7 kb pu.1 distal promoter region (Figure 6D). Using this assay, we demonstrate the specific binding of Runx1 protein to the region harboring the two putative runx1 sites. To further determine whether runx1 acts through these two runx1 motifs to repress pu.1 expression in vivo, we generated a reporter construct (-9.0pu.1<sup>ΔR</sup>:eGFP) harboring GFP driven by the 9.0 kb pu.1 upstream regulatory region specifically lacking the two Runx1-binding motifs. GFP expressed from transiently injected -9.0pu.1<sup>ΔR</sup>:eGFP construct maintained the same spatial expression pattern as that from the intact -9.0pu.1:eGFP construct, suggesting removal of Runx1-binding motifs does not affect the tissue specificity of pu.1 promoter. However, eGFP expression in the RBI of the embryos receiving -9.0pu.1<sup>ΔR</sup>:eGFP construct was evidently increased compared to that in the embryos receiving -9.0pu.1:eGFP construct (Figure 6B). Quantitatively, deletion of
Runx1-binding motifs resulted in a 3.5 fold increase of GFP when measured in a transient transgenic assay (Figure 6C). Together, these data suggest that runx1 feedback represses pu.1 expression via directly acting on pu.1 promoter.

Discussion

In the present study, we exploited the strength of this highly tractable zebrafish system to establish a transcriptional regulatory hierarchy required for the homeostasis of embryonic macrophage and neutrophil specification. The core of this regulatory circuit is a Pu.1-Runx1 negative feedback loop wherein Pu.1 activates the expression of a transcriptional repressor, Runx1, to limit its own activity (Figure 6E). Unlike positive feedback circuit which leads to rapid spiral change of activities, negative feedback loop attains an equilibrium state of the output concentration and renders resistance to perturbation. Given the central role of Pu.1 dosage in driving alternative myeloid fates suggested by the in vitro5,6 and our current in vivo studies, the Pu.1-Runx1 negative feedback loop uncovered here would thus ensure the stabilization of Pu.1 concentration within a range favorable for balanced macrophage and neutrophil commitment.

Several possibilities can explain how Pu.1-Runx1 loop contribute to the regulation of embryonic myeloid fates. One possibility is that this loop may actively specify neutrophil fate by facilitating the expression of unknown factors. An alternative possibility is that this loop may be utilized to maintain cell competence for
responding to neutrophil inducing factors by preventing the dominant macrophage program from being overactive. Candidate dominant macrophage fate promoting factor emerging might include interferon regulatory factor 8 (\textit{irf8})\textsuperscript{28,43} and miR-146a\textsuperscript{44} as enforced expression of both factors were shown to drive macrophage development. In particular, \textit{irf8} expression was completely lost in \textit{pu.1}\textsuperscript{G242D} mutants (supplemental Figure 10B) but augmented in \textit{runx1}\textsuperscript{w84x} mutants (Figure 4I-J). Additionally, while overexpression of \textit{irf8} in wild-type embryos shifted fate towards macrophages\textsuperscript{28}, knocking-down of \textit{irf8} rescue the phenotype of \textit{runx1}\textsuperscript{w84x} mutants. Elucidating the function relationship between \textit{pu.1-runx1} loop and these macrophage promoting molecules will thus aid comprehension of the regulatory circuits governing macrophage versus neutrophil fate choice.

Our study shows that Runx1 plays critical roles in regulating embryonic myeloid fate choice through promoting neutrophil fate over that of macrophage. However, whether this myeloid lineage selection role of Runx1 is relevant to adult phase of myelopoiesis has not been investigated. Blood profiling of adult kidney marrow of viable \textit{runx1}\textsuperscript{w84x} mutants revealed that the extent of decrease in neutrophil counts significantly exceeds that in macrophage counts (unpublished data), thus favoring a similar role of Runx1 in adult myelopoiesis. It has occurred to us that adult ablation of Runx1 in mice instead causes either no observable myeloid phenotype\textsuperscript{45} or mild myeloid expansion with a basis of increased granulocyte-macrophage progenitors.\textsuperscript{46} Although such discrepancy might be attributed to species difference or
hematopoiesis phase difference, alternative explanation might lie in the difference of these two systems used to study these processes. The lineage specific role of Runx1 in mice was inferred by classical approach in which conditional alleles of Runx1 were removed in most adult blood cells by inducible Mx-Cre and the consequence of the deletion was assessed thereafter. In such assay, the effect of Runx1 depletion in myeloid development per se might be confounded by the loss of Runx1 in various upstream progenitors of myeloid potential and those blood cells capable of modulating myeloid differentiation, thus potentially masking the myeloid specific role of Runx1. Moreover, if a phenotype is discerned, it often suffers from the inadequacy to track down the exact underlying cellular mechanism. For instance, it often remains to show in many of these studies whether the alteration of a given population arising from a particular gene targeting is due to changes in upstream progenitors, or changes in cell fate, division or migration. By contrast, zebrafish embryonic myeloid cells arising without transiting from HSCs and with minimal influence from other blood cells (erythropoiesis occurs in an anatomically different site), allowing to directly access of the role of Runx1 in myelopoiesis. Additionally, the sequence of myeloid development in fish embryo could be defined by temporally ordered expression of molecular markers and many in vivo cell tracking tools including photoactivatable dye and photoswitchable protein are now available, making it possible to definitively pinpoint the underlying cellular defect. Hence, it can be anticipated that these merits combined with the genetic tractability of fish system would accelerate the
identification of new conserved players in myelopoiesis. In fact, the conserved role of
Runx1 in myeloid fate choices is supported by findings showing that lozenge, the
Drosophila orthologue of Runx genes, specifies crystal fate from undifferentiated
prohemocytes which otherwise give rise to plasmatocytes, a fly equivalent of
macrophage.49

Our current work does not deny any potential role of Runx1 in subsequent
maturation or differentiation of neutrophils after the fate of these cells is established.
Indeed, the expression of more advanced neutrophil markers such as mpx, lyz, and SB
was more severely disrupted compared to that of early neutrophil progenitor marker,
cebpl. In addition, we noted that introducing biallelic pu.1G242D mutation to runx1w84x
mutants was inadequate to fully restore the expression of more differentiated
neutrophil markers such as lyz, SB to the level in pu.1G242D single mutants and wild-
type, despite full recovery of cebp1+ neutrophil progenitors in pu.1G242Drunx1w84x
double mutants. These observations are consistent with studies showing Runx1
regulates the promoter of myeloid differentiation genes50 and point to a likely
additional role of Runx1 in subsequent neutrophil maturation or differentiation apart
from its early Pu.1 repressive role in fate decision. It will thus be of interest in the
future to pursue how Runx1 coordinates with other myeloid factors to fulfill its
differentiation role.
Acknowledgements

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Author Contributions

H.J. L.L., J.X., F.H.Z., and L.Z. designed the research, performed experiments and analyzed data. W.Q.Z. and Z.L.W. designed the research and analyzed data. M.J.Z. analyzed data. P.P.L. provided reagents.

Conflict-of-Interest Disclosure

The authors declare no competing financial interests.
References


Table 1 Counts of *cebp1*\(^+\), *lyz*\(^+\), *SB*\(^+\) neutrophil cells scored in various genetic combinations of *runx*\(^{w84x}\) and *pu.1*\(^{G242D}\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th><em>runx1</em>(^+/+)</th>
<th><em>runx1</em>(^+/+) <em>pu.1</em>(^{G242D}$/+)</th>
<th><em>runx1</em>(^+/+) <em>pu.1</em>(^{G242D}$/G242D)</th>
<th><em>runx1</em>(^{w84x}$/w84x) <em>pu.1</em>(^+/+)</th>
<th><em>runx1</em>(^{w84x}$/w84x) <em>pu.1</em>(^{G242D}$/+)</th>
<th><em>runx1</em>(^{w84x}$/w84x) <em>pu.1</em>(^{G242D}$/G242D)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cebp1</em>(^+)</td>
<td>50.3/3.1/13</td>
<td>54.2/3.2/23</td>
<td>51.2/3.7/12</td>
<td>11/1.3/10</td>
<td>24.0/2.0/13</td>
<td>53.2/4.3/15</td>
</tr>
<tr>
<td><em>lyz</em>(^+)</td>
<td>79.5/6.0/8</td>
<td>63.0/2.9/21</td>
<td>72.3/12.8/6</td>
<td>23.0/2.0/13</td>
<td>32.7/3.5/18</td>
<td>34.0/3.9/9</td>
</tr>
<tr>
<td><em>SB</em>(^+)</td>
<td>105.6/10.7/7</td>
<td>100.8/4.9/18</td>
<td>84.7/13.8/7</td>
<td>28.9/3.9/11</td>
<td>52.3/6.2/15</td>
<td>56.5/7.3/11</td>
</tr>
</tbody>
</table>

*: The stage of embryos scored for *cebp1*\(^+\), *lyz*\(^+\), *SB*\(^+\) cells were 23hpf, 30hpf, and 36hpf, respectively.
Figure Legends

Figure 1. High *pu.1* level supports the production of embryonic macrophages whereas low *pu.1* level supports embryonic neutrophil production.

(A-B) WISH of *cebp1* expression in 23 hpf siblings (*sib*) (A, arrows) and *pu.1*<sup>G242D</sup> mutants (B, arrows). (C-D) WISH of *apoeb* expression in 3 dpf *sib* (C, arrows) and *pu.1*<sup>G242D</sup> mutants (D). Embryos in A-D are viewed dorsally with the anterior to the left. (E-F) WISH of *lyz* expression in 30 hpf *sib* (E, arrows) and *pu.1*<sup>G242D</sup> mutants (F, arrows). (G-H) WISH of *csf1ra* expression in 22 hpf *sib* (G) and *pu.1*<sup>G242D</sup> mutants (H). White arrows indicate *csf1ra* expression in macrophage while black arrowheads indicate its expression by neural crest cells. (I-J) WISH of *cebp1* expression in 23 hpf control (I, arrows) and *pu.1* morphants (J, n=35/38). (K-L) WISH of *apoeb* expression in 3 dpf control (K, arrows) and *pu.1* morphants (L, n=29/29). Embryos in I-L are viewed dorsally with the anterior to the left. (M-N) SB staining of 36 hpf control (M, arrows) and *pu.1* morphants (N, n=45/48).

Figure 2. Enforced *pu.1* expression promotes the formation of embryonic macrophages at the expense of embryonic neutrophils.

(A-B) WISH of *irf8* expression at 21 hpf in heat-shocked *Tg(hsp70:myc-pu.1)hkz03t* (Tg+, B, arrows) and non-transgenic sibling (Tg-, A, arrows). (C-D) WISH of *csf1ra* expression at 25 hpf in heat-shocked *Tg(hsp70:myc-pu.1)hkz03t* (Tg+, D, arrows) and non-transgenic sibling (Tg-, C, arrows). Arrowheads indicate *csf1ra* expression in neural crest cells. (E-F) WISH of *cebp1* expression at 21 hpf in heat-shocked
Tg(hsp70:myc-pu.1)hkz03t (Tg+, F, arrows) and non-transgenic sibling (Tg-, E, arrows). (G-H) SB staining at 36 hpf in heat-shocked Tg(hsp70:myc-pu.1)hkz03t (Tg+, H, arrows) and non-transgenic sibling (Tg-, G, arrows). (I) Summary of effects of transient pu.1 overexpression on the development of cebp1+, mpx+, SB+ neutrophils and irf8+, csf1ra+ macrophages. Embryos from Tg(hsp70:myc-pu.1)hkz03t +/- crossed with AB wild-type are heat shocked at 11 hpf and fixed at 21 hpf, 33 hpf, 36 hpf, 21 hpf, 25 hpf for WISH detection of cebp1 (E, F), mpx, SB (G, H), irf8 (A, B), csf1ra (C, D), respectively. Number (No.) of cells positive for these markers were counted and compared between Tg(hsp70:myc-pu.1)hkz03t (Tg+) and non-transgenic sibling (Tg-) embryos having undergone the same heat-shock and staining protocol. The asterisks indicate statistical difference (t-test, cebp1Tg- (mean/s.e./n)= 35.6/2.9/12, cebp1Tg+ =15.6/1.7/11; mpxTg- = 96.9/4.8/13; mpxTg+ = 74.4/5.8/10; SB Tg- = 134.4/5.6/18, SB Tg+ = 99.3/5.0/16; irf8Tg- = 41.8/7.6/4; irf8Tg+ = 66.3/5.2/12; csf1raTg- = 46.4/6.6/12; csf1raTg+ = 70.5/4.3/12; *: p<0.05, **: p<0.001).

Figure 3. Pu.1 initiates the expression of runx1 while Runx1 suppresses late-phase expression of pu.1.

(A-B) WISH of pu.1 expression in 17.5 hpf sib (A) and runx1<sup>W84x</sup> (B) embryos. Arrows indicate WISH signal of pu.1. (C-D) Whole mount antibody staining of Pu.1 protein in 17.5 hpf sib (C) and runx1<sup>W84x</sup> (D) embryos. Arrows indicate fluorescent antibody staining signal for Pu.1 protein. (E-F) WISH of runx1 expression in 17 hpf control (E) and pu.1 morphants (F, n=47/48). The arrow indicates WISH signal of
runx1 in control morphants (E), which is absent in pu.1 morphants (F). (G-H) WISH of runx1 expression in 17 hpf sib (G) and pu.1<sup>G242D</sup> mutants (H). Arrows indicate WISH signal of runx1, which is markedly reduced in pu.1<sup>G242D</sup> mutants (H) compared to sib (G).

**Figure 4. Runx1 regulates embryonic neutrophil versus macrophage fate choice.**

(A-B) WISH of cebp1 expression in 22 hpf siblings (sib) (A, arrows) and runx1<sup>w84x</sup> mutants (B, arrows). (C-D) WISH of mpx expression in 22 hpf sib (C, arrows) and runx1<sup>w84x</sup> mutants (D). (E-F) WISH of mpx expression in 32 hpf sib (E, arrows) and runx1<sup>w84x</sup> mutants (F, arrows). (G) Quantification of No. of cebp1<sup>+</sup> cells in 22 hpf sib and runx1<sup>w84x</sup> mutants. The asterisk indicates a statistics difference (t-test, cebp1<sub>sib</sub> (mean/s.e./n)=40.6/1.4/54, cebp1<sub>runx1w84x</sub> =14.2/1.3/17, *: p<0.001). (H) Quantification of the number (No.) of mpx<sup>+</sup> cells in 32 hpf sib and runx1<sup>w84x</sup> mutants. Asterisks indicate statistical difference (t-test, mpx<sub>sib</sub> (mean/s.e./n)=56.7/3.6/30, mpx<sub>runx1w84x</sub>=4.5/1.4/9; *: p<0.001). (I-J) WISH of irf8 expression in 18 hpf sib (I, arrow) and runx1<sup>w84x</sup> mutants (J, arrow). (K-L) WISH of csf1ra expression in 30 hpf sib (K) and runx1<sup>w84x</sup> mutants (L). Arrows indicate csf1ra expressed by macrophage while arrowheads indicate csf1ra expressed by neural crest cells. (M-N) WISH of apoeb expression in 3 dpf sib (M, arrows) and runx1<sup>w84x</sup> mutants (N, arrows). Embryos are viewed dorsally with the anterior to the left. (O) Quantification of No. of csf1ra<sup>+</sup> cells in the yolk sac of 30 hpf sib and runx1<sup>w84x</sup> mutants. The asterisk indicates a statistical difference (t-test, csf1ra<sub>sib</sub> (mean/s.e./n) =72.4/3.2/7,
csf1ra_{runx1w84x} = 101.5/4.5/11, *; p<0.001). (P) Quantification of No. of apoeb\textsuperscript{+} cells in the brain of 3 dpf sib and runx1\textsuperscript{w84x} mutants. The asterisk indicates a statistical difference (t-test, apoeb\textsubscript{sib}(mean/s.e./n)=24.0/2.1/24, apoeb\textsubscript{runx1w84x} = 33.0/1.7/33, *; p<0.01). (Q) Effects of transient runx1 overexpression on the development of cebp1\textsuperscript{+}, SB\textsuperscript{+} neutrophils and irf8\textsuperscript{+}, csf1ra\textsuperscript{+} macrophages. Embryos from Tg(hsp70:myc-runx1)hkz02\textsuperscript{+/} crossed with AB wild-type are heat shocked at 3s and fixed at 22 hpf, 36 hpf, 20 hpf, 24 hpf for WISH detection of cebp1, SB, irf8, csf1ra, respectively. No. of cells positive for these markers were counted and compared between Tg(hsp70:myc-runx1)hkz02\textsuperscript{Tg+} (Tg+) and non-transgenic sibling (Tg-) embryos having undergone the same heatshock and staining protocol. The asterisks indicate statistical difference (t-test, cebp1\textsubscript{Tg-}(mean/s.e./n)=33.4/2.4/27, cebp1\textsubscript{Tg+}=44.1/1.9/32; SB\textsubscript{Tg-}=90.3/6.8/17, SB\textsubscript{Tg+}=128/6.9/15; irf8\textsubscript{Tg-}=39.4/3.3/20; irf8\textsubscript{Tg+}=27.9/2.4/18; csf1ra\textsubscript{Tg-}=37/1.8/22, csf1ra\textsubscript{Tg+}=24.4/1.9/22; *; p<0.01).

**Figure 5. Reducing pu.1 level rescues neutrophil deficit phenotype in runx1\textsuperscript{w84x} mutants.**

(A-D) WISH of cebp1 expression in 23 hpf runx1\textsuperscript{+/} pu.1\textsuperscript{+/} (A, arrows), runx1\textsuperscript{+/} pu.1\textsuperscript{G242D/G242D} (B, arrows), runx1\textsuperscript{w84x/w84x} pu.1\textsuperscript{+/} (C, arrow), runx1\textsuperscript{w84x/w84x} pu.1\textsuperscript{G242D/G242D} (D, arrows). (E-H) SB staining in 36 hpf runx1\textsuperscript{+/} pu.1\textsuperscript{+/} (E, arrows), runx1\textsuperscript{+/} pu.1\textsuperscript{G242D/G242D} (F, arrows), runx1\textsuperscript{w84x/w84x} pu.1\textsuperscript{+/} (G, arrows), runx1\textsuperscript{w84x/w84x} pu.1\textsuperscript{G242D/G242D} (H, arrows). (I) Quantification of cebp1\textsuperscript{+} cell numbers in 23 hpf runx1\textsuperscript{+/} pu.1\textsuperscript{+/}, runx1\textsuperscript{+/} pu.1\textsuperscript{G242D/G242D}, runx1\textsuperscript{w84x/w84x} pu.1\textsuperscript{+/}.
runx1<sup>w84x/w84x</sup><sup>pu.1<sup>G242D/G242D</sup></sup> embryos. The asterisk indicates a statistical difference (t-test, 
\[ \text{cebp1}_{runx1+/+\ pu.1+/+} \text{(mean/s.e./n)} = 50.3/3.1/13, \]
\[ \text{cebp1}_{runx1+/+\ pu.1G242D/G242D} = 51.2/3.7/12, \]
\[ \text{cebp1}_{runx1w84x/w84x\ pu.1+/+} = 11/1.3/10, \]
\[ \text{cebp1}_{runx1w84x/w84x\ pu.1G242D/G242D} = 53.2/4.3/15, \ast: p<0.001. \]

Figure 6. Runx1 represses *pu.1* expression through acting on *pu.1* promoter.

(A) Schematic diagram of *pu.1* promoter. Two adjoining putative *runx1* binding sites (red arrowheads) in the distal portion of *pu.1* promoter are predicted by promo 3.0 online software. Red bars indicate positions of primers designed to test for *runx1* binding. *runx1*-I amplifies the region containing two putative *runx1* sites whereas *runx1*-II amplifies the region devoid of *runx1* sites. (B) Representative fluorescent images (left panels) of transient eGFP expression at 17.5 hpf in the RBI of wild-type embryos injected with -9.0pu.1:eGFP (upper panels) and -9.0pu.1ΔR:eGFP (low panels) constructs. Right panels are overlays with bright field images. (C) Quantitative RT-PCR for GFP expression at 17.5 hpf in wt embryos injected with -9.0pu.1:eGFP and -9.0pu.1ΔR:eGFP. Units on y-axis represent the relative fold change of GFP expression in wild-type embryos injected with -9.0pu.1:eGFP and -
9.0pu.1ΔR:eGFP. Expression level was normalized with elf1α expression and the amount of injected DNA. Error bars, s.e. (D) semi-quantitative PCR analysis with chromatin before (input) and after immunoprecipitation with anti-Myc antibody or anti-Brdu antibody (negative control). Sequence of mespa gene promoter serves as a negative control. (E) A model for the regulatory network in controlling embryonic neutrophil and macrophage fate segregation. In this model, a graded Pu.1 level specifies embryonic neutrophil and macrophage fates with high Pu.1 activity required for macrophage fate formation and low Pu.1 supporting neutrophil fate formation. High Pu.1 activity might switch on the expression of its binding partner, Irf8, to establish the embryonic macrophage fate. High Pu.1 activity, on the other hand, turns on the expression of Runx1, which is a direct feedback repressor of pu.1 expression. This Pu.1-Runx1 negative feedback loop thus stabilizes a favorable Pu.1 level that is essential for the formation of neutrophil fate.
Figure 1.
Figure 2.
Figure 3.
Figure 5.

A. runx1+/+ pu.1+/+
B. runx1+/+ pu.1G242D/G242D
C. runx1-/- pu.1+/+
D. runx1-/- pu.1G242D/G242D
E. runx1+/+ pu.1+/+
F. runx1+/+ pu.1G242D/G242D
G. runx1-/- pu.1+/+
H. runx1-/- pu.1G242D/G242D
I. No. of 23 hpf cebp1+ cells
J. No. of 36 hpf SB+ cells
Figure 6.

A

\[ \text{GGCTTCTGTAGGCTCtgtggtTTGGACAGCTTGAATtgtggtGAAACTAAGCAAAAACAC} \]

\[ \text{Pu.1} \]

\[ \text{runx1 I} \]
\[ \text{runx1 II} \]

B

-9.0pu.1:eGFP

-9.0pu.1ΔR:eGFP

C

Relative Expression

\[ \text{Pu.1lo} \]
\[ \text{Pu.1hi} \]

E

\[ \text{Pu.1}^{lo} \]

Runx1

Irf8

neutrophil

macrophage

\[ \text{mespa} \]

\[ \text{runx1 I} \]
\[ \text{runx1 II} \]

\[ \text{α-brdu} \]
\[ \text{α-Myc} \]

Input

\[ \text{For personal use only.}\]
Runx1 regulates embryonic myeloid fate choice in zebrafish through a negative feedback loop that confines Pu.1 expression

Hao Jin, Li Li, Jin Xu, Fenghua Zhen, Lu Zhu, P. Paul Liu, Mingjie Zhang, Wenqing Zhang and Zilong Wen