RNA polymerase III component Rpc9 regulates hematopoietic stem and progenitor cell maintenance in zebrafish

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ABSTRACT
Hematopoietic stem and progenitor cells (HSPCs) are capable of self-renewal and replenishing all lineages of blood cells throughout life and are thus crucial for tissue homeostasis. However, the mechanism regulating HSPC development is still incompletely understood. Here, we isolate a zebrafish mutant with defective T lymphopoiesis and positional cloning identifies that Rpc9, a component of DNA-directed RNA polymerase III (Pol III) complex, is responsible for the mutant phenotype. Further analysis shows that rpc9 deficiency leads to the impairment of HSPCs and their derivatives in zebrafish embryos. Excessive apoptosis is observed in the caudal hematopoietic tissue (CHT; the equivalent of fetal liver in mammals) of rpc9−/− embryos and the hematopoietic defects in these embryos can be fully rescued by suppression of p53. Thus, our work illustrates that Rpc9, a component of Pol III, plays an important tissue-specific role in HSPC maintenance during zebrafish embryogenesis and might be conserved across vertebrates, including mammals.

KEY WORDS: RNA polymerase III, Rpc9, P53, Hematopoietic stem and progenitor cells, Zebrafish

INTRODUCTION
In vertebrates, hematopoiesis occurs in two successive waves designated as primitive and definitive hematopoiesis. Primitive hematopoiesis generates erythroid and myeloid precursors to fulfill the oxygen and immunoprotection demand in early embryogenesis, whereas definitive hematopoiesis gives rise to hematopoietic stem and progenitor cells (HSPCs) capable of self-renewal and differentiation into all lineages of blood cells that sustain physiological homeostasis throughout the lifetime (Costa et al., 2012). HSPCs are derived from endothelial cells in the ventral wall of dorsal aorta via an endothelial to hematopoietic transition (EHT) process that can be traced in vivo with the help of fluorescent protein transgenic animals (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). In zebrafish, newly specified HSPCs at 30 hours post fertilization (hpf) will migrate into the caudal hematopoietic tissue (CHT, the equivalent of fetal liver in mammals), and transiently reside and rapidly expand there until 48 hpf. Subsequently, HSPCs colonize the kidney marrow, which is the functional equivalent of bone marrow in mammals and initiate adult hematopoiesis from 4 days post fertilization (dpf) (Paik and Zon, 2010). The balance among self-renewal, proliferation and differentiation is essential for HSPC maintenance in these specialized niches that are delicately orchestrated by cell intrinsic networks and environment cues (Mendelson and Frenette, 2014).

DNA-directed RNA polymerase III complex (Pol III) is a specialized enzyme responsible for transcription of small noncoding RNAs (snRNAs) including 5S rRNA, tRNAs and 7SL RNA (Dieci et al., 2007). It is the most complex RNA polymerase, comprising 17 subunits with a total molecular mass of about 700 kDa (Schramm and Hernandez, 2002). The major snRNAs transcribed by Pol III are implicated in protein synthesis. As a component of the large subunit of ribosomes, 5S rRNA binds the ribosome proteins RPL5 and RPL11 to initiate assembly of ribosome precursor complex (Donati et al., 2013). tRNAs, which are amino acid transporters indispensable for protein synthesis, are the other major transcripts of Pol III. Notably, the synthesis of rRNA and tRNAs consumes 70-80% of transcriptional capacity (White, 1997) and ribosomal gene transcription accounts for as much as 50% of the Pol II transcriptional workload in yeast (Warnier, 1999), suggesting that Pol III activity should also be tightly regulated during cell growth (Geiduschek and Kassavetis, 2001) in various environments, including nutrition availability (Boguta and Graczyk, 2011; Marshall et al., 2012). In fact, the activity of Pol III is well coupled with cell cycle regulation (Hu et al., 2004) and abnormal Pol III activity is closely correlated with cancers (Marshall and White, 2008) or developmental anomalies (Borck et al., 2015), but the role of Pol III in developmental hematopoiesis has not been reported.

RNA polymerase III component 9 (Rpc9; Crcp – Zebrafish Information Network) is identified as a component of Pol III in yeast and human (Ferri et al., 2000; Hu et al., 2002; Siaut et al., 2003). Structure analysis revealed that Rpe9 forms a heterodimer with another Pol III subunit Rpe25, which is the parologue of Rpa43 of RNA Pol I or Rpb7 of RNA Pol II (Zaros and Thuriaux, 2005), and binds to nucleic acids and interacts with transcription factors to promote transcription initiation and may also facilitate RNA exit (Jasiak et al., 2006). Interestingly, Rpe9 is also known as calcitonin gene-related peptide-receptor component protein (CGRP-RCP), indicating this protein may be a bi-functional factor. The CGRP receptor complex comprises calcitonin receptor-like receptor (Crlr; Calcr1a – Zebrafish Information Network), receptor activity-modifying protein 1 (Ramp1) and Rpc9, which facilitates coupling of Gα receptors. When the CGRP receptor complex is activated, CGRP-mediated signal is transduced through activating cyclic adenosine monophosphate (cAMP) signaling pathway in mouse and human (Prado et al., 2002; Russo, 2015). However, whether Rpc9 is involved in developmental hematopoiesis during embryogenesis is still unknown.

Here, we show that, in zebrafish, a genetic mutation of rpc9 leads to the impairment of HSPC survival in the CHT, and these
hematopoietic defects can be rescued by downregulation of p53 \textit{(tp53 - Zebrafish Information Network)}. Therefore, these findings further our understanding on the role of Rpc9 as a component of RNA polymerase III in developmental hematopoiesis via regulation of P53 signaling in zebrafish.

**RESULTS**

**T cells are absent in mutant line 116**

To determine the genetic network involved in definitive hematopoiesis in vertebrates, an ENU-based forward genetic screening in zebrafish was performed to isolate mutants with definitive hematopoietic defects (Du et al., 2011). By examining expression of the T cell marker \textit{rag1}, the T cell-deficient mutant line 116 was isolated. According to whole-mount \textit{in situ} hybridization (WISH), expression of HSPC markers \textit{cmyb} and \textit{ikaros} (\textit{ikzf1 - Zebrafish Information Network}), early T cell markers (before \(\beta/\delta\) selection; \textit{rag1}, \textit{rag2} and \textit{bcl11a}), chemokine receptor markers (\textit{ccr9a} and \textit{ccr9b}) and naïve T cell markers (after \(\beta/\delta\) selection; \textit{tcrb2} and \textit{tcrd}) was absent in the thymus of mutant embryos at 5 dpf (Fig. 1A,B), indicating that T cells were severely attenuated. By contrast, expression of the thymus epithelial cell (TEC) marker \textit{foxn1} was normal (Fig. 1B), suggesting that the TEC microenvironment was intact. Notably, we were unable to distinguish morphologically homozygous mutant embryos from wild-type or heterozygous embryos, further indicating that the mutation in line 116 led to a specific defect in T cells.

**The mutation in the T cell-deficient mutant lies in \textit{rpc9}**

To identify the gene responsible for the mutant phenotype, we performed a positional cloning assay. Heterozygous mutant line 116 fish (AB strain) were crossed with wild-type WIK strain fish and the embryos were raised to adult fish as F1 generation. The carriers (F1 generation) were identified and in-crossed to get F2 embryos, which were subsequently subjected to bulk segregant analyses (BSAs). Chromosomal mapping and the following sequence analysis identified a point mutation located on chromosome 21 where a thymine was switched into an adenine in the second exon of the \textit{rpc9} gene, which formed a premature stop codon in the coding region of \textit{rpc9} mRNA (Fig. 2A,B). This mutation also generated a new restriction enzyme recognition site as TTAA (\textit{Mse I}), which could be used, in addition to polymorphic primers, for genotyping (Fig. S1A).

Western blot showed the protein level of Rpc9 was markedly decreased in mutant line 116 embryos at 4 dpf (Fig. 2C) and the residual Rpc9 protein might result from maternal expression of \textit{rpc9} (Fig. S1B). To confirm that the \textit{rpc9} gene was responsible for the
RPC9 deficiency leads to excessive apoptosis and abated proliferation in the CHT region

To determine how these HSPCs were disturbed, we first examined apoptosis by TUNEL assay and observed that apoptotic signals in the CHT region of mutant embryos were much more intensive than that of wild-type embryos at 3 dpf (36.83±8.20 vs 16.67±3.18, \( P<0.0047 \)) and 4 dpf (53.20±5.65 vs 27.50±2.44, \( P=0.0006 \)) (Fig. 4A,B). Consistently, apoptosis spots in the CHT region of rpc9\(^{-/-}\) embryos stained with Acridine Orange were much prevalent compared with rpc9\(^{-/-}\) embryos at 3 dpf (106.00±2.98 vs 62.60±5.15, \( P<0.0001 \)) and 4 dpf (110.14±7.19 vs 63.14±6.39, \( P=0.0004 \)) (Fig. 4C,D). BrDU assay showed that proliferation signals in the CHT region of the mutant were greatly decreased compared with that in the wild-type at 4 dpf (109.25±6.82 vs 81.75±3.42, \( P=0.0016 \)) (Fig. 4E,F). pH3 assay revealed that proliferation signals in rpc9\(^{-/-}\) embryos were significantly reduced at both 3 dpf (38.57±1.38 vs 27.43±1.99, \( P<0.0001 \)) and 4 dpf (39.50±1.99 vs 18.69±1.15, \( P<0.0001 \)) (Fig. 4G,H). Given that the pH3 but not the BrDU signal was significantly reduced in rpc9\(^{-/-}\) embryos at 3 dpf, we reasoned that some mitotic cells, although initiating DNA replication at S phase, might fail to acquire phosphorylation of histone H3 at G2 phase and accomplish the cell cycle.

Perturbation of Pol III leads to HSPC defects

SinceRpc9may act as a bi-functional factor, to determine whether hematopoietic defects in rpc9\(^{-/-}\) mutants resulted from the disruption of its function as a CGRP receptor or a component of the Pol III complex, the full-length coding sequence (CDS) of rpc9 was fused with enhanced green fluorescent protein (egfp) and zebrafish embryos injected with rpc9-egfp mRNA were examined. Confocal imaging demonstrated that Rpc9-EGFP was highly enriched in the nucleus (Fig. S3A) and this localization was confirmed by western blot (Fig. S3B), suggesting that Rpc9 mainly acts as a nuclear factor rather than a membrane receptor component in zebrafish cells. Moreover, we only observed a slight decrease of cAMP level in rpc9\(^{-/-}\) embryos (Fig. S3C) and the hematopoietic defects in rpc9\(^{-/-}\) mutants could not be rescued by treatment with Forskolin (a cAMP activator) or 8-bromo-cAMP (a cAMP analog)

HSPCs are impaired in rpc9\(^{-/-}\) embryos

To determine whether myeloid fate is acquired when T lymphoid fate is blocked in the absence of rpc9 (Wada et al., 2008), the myeloid markers pu.1, lyz and mfap4 were examined. However, no difference was observed in expression of these markers in the thymus of rpc9\(^{+/+}\) and rpc9\(^{-/-}\) embryos at 4.5 dpf (Fig. S2A). Interestingly, at 5 dpf, expression of myeloid [pu.1, l-plastin (lcp1)] and erythroid (gata1) markers in the CHT region was greatly decreased in rpc9\(^{-/-}\) embryos (Fig. S2B,C). At earlier stages, expression of ikaros in the thymus of rpc9\(^{-/-}\) embryos was slightly decreased at 3 dpf, whereas expression of tcrb2 and tcrd was dramatically decreased at 4 dpf (Fig. 3A), suggesting that a subset of early thymic progenitors (ETPs) could enter the thymus and develop through βδ-selection while gradually vanished afterwards. Since ETPs are derived from HSPCs, we analyzed the expression of HSPC markers runx1 and cmyb and observed that HSPCs were only affected from 3 dpf in the CHT regions but not before in the aorta-gonad-mesonephros (AGM) region (Fig. 3B), which was confirmed by qRT-PCR (Fig. 3C). expression of scl (tail1), gata1 and pu.1 in rpc9\(^{-/-}\) embryos at 10 somite stage, 24 hpf and 36 hpf was not altered (Fig. S2D,E). Taken together, loss of rpc9 led to the defect in HSPC maintenance and finally, the impairment of all hematopoietic lineages. Intriguingly, expression of rpc9 was detected in the CHT region (Fig. 3D) and this may partly explain the specific defect of HSPCs in rpc9\(^{-/-}\) embryos.
Fig. 4. Enhanced apoptosis and reduced proliferation in the CHT of rpc9−/− mutants. (A,B) TUNEL assay shows apoptosis signals in the CHT region of rpc9−/− and rpc9−/+ embryos. TUNEL signals are significantly enhanced in rpc9−/− embryos at 3 and 4 dpf, but not at 2 dpf. (C,D) Acridine Orange (AO) staining shows that rpc9−/− embryos display more intensive apoptosis signals in the CHT region than rpc9−/+ embryos at 3 and 4 dpf. Note that asterisk indicates the cloaca where unspecific spots were not taken into account when quantifying the apoptosis signals. (E,F) BrdU assay reveals that proliferation is affected in hematopoietic defects (Fig. S3F,G). These results together suggest that the concentration in zebrafish (Kumai et al., 2014) (Fig. S3D,E). In addition, administration of the CGRP receptor antagonist MK-3207 caused a significant decrease of cAMP level without any discernible hematopoietic defects (Fig. S3F,G). These results together suggest that hematopoietic defects in rpc9−/− mutants might be independent of the CGRP signaling pathway.

As a component of Pol III, Rpc9 deficiency may disrupt the integrity of Pol III. In order to demonstrate the function of Pol III upon the loss of Rpc9, pre-tRNAIle, pre-tRNALeu, 5S rRNA and 7SL RNA were examined by qRT-PCR and they were all found to be decreased in rpc9−/− embryos (Fig. S4A). To further explore the role of Pol III in HSPC development, we designed MOs against polr3h encoding Rpc25 which forms a heterodimer with Rpc9, and polr3k encoding Rpc11 which is a core component of Pol III. Intriguingly, polr3h and polr3k were also expressed in the CHT region (Fig. S4B). The efficiency of both MOs was validated by fluorescence assay (Fig. S4C) and confirmed by the observation that expression of all Pol III products was significantly decreased (Fig. 5A). Notably, knockdown of polr3h and polr3k led to a remarkable diminished expression of hematopoietic markers (cmyb, gata1, pu.1 and rag1) (Fig. 5B,C). By contrast, expression of the endothelial cell marker flk1 (kdr) and the thymus epithelial cell marker foxn1 was not obviously altered (Fig. S4D), suggesting that loss of Pol III components caused specific hematopoietic defects. Moreover, we treated zebrafish embryos with 1.32 nM ML-60218 (Pol III inhibitor) from 1 to 5 dpf. The effectiveness of ML-60218 was validated, as all the examined products of Pol III were significantly decreased after ML-60218 administration (Fig. S5A).

In line with that in polr3h and polr3k morphants, expression of hematopoietic markers (cmyb, gata1, pu.1 and rag1), but not of niche cell markers (flk1 and foxn1) was obviously attenuated (Fig. S5B,C). Taken together, rpc9 deficiency might disrupt the function of Pol III and lead to HSPC impairment.

P53 mediates the regulation of Rpc9 in HSPC survival
To test whether the observed excessive apoptosis in rpc9−/− mutants was attributable to P53 signaling, we then examined the expression of p53. Interestingly, WISH result revealed that, compared with the controls, p53 expression was specifically increased in the CHT region of rpc9−/− mutants (Fig. 6A). Activation of P53 was also confirmed by qRT-PCR and western blot (Fig. S6A, Fig. 6B,C). To determine whether upregulation of p53 was responsible for the observed hematopoietic defects in rpc9−/− mutants, we applied two approaches: creation of a double mutant by outcrossing rpc9−/− with p53−/− fish, and knockdown of p53 in rpc9−/− embryos. WISH results showed that expression of cmyb in the CHT region and rag1 in the thymus was fully rescued in rpc9−/− p53−/− embryos (Fig. 6D,E), which was confirmed by qRT-PCR (Fig. 6F) and also by WISH with rpc9−/− injected with p53 MO (Fig. S6B). These results demonstrate that p53 is involved in HSPC maintenance regulated by Rpc9.

The impaired survival and proliferation in the CHT region of rpc9−/− embryos and the full rescue effect of HSPCs exerted by alteration of P53 in rpc9−/− embryos made it intriguing to explore the cell cycle and apoptosis in rpc9−/− embryos when P53 is downregulated. Apoptosis signals were obviously decreased in both rpc9−/+ and rpc9−/− embryos upon p53 knockdown, and the difference of apoptosis signals between rpc9−/− embryos injected with control or p53 MO was significant (35.92±3.33 vs 9.91±1.44, Student’s t-test. ns, not significant.)
P<0.0001) (Fig. S6C,D). By contrast, there was no significant difference in proliferation between rpc9−/− embryos injected with control or p53 MO (14.75±1.56 vs 11.33±2.15, P=0.1993) (Fig. S6E,F). These observations suggest that alleviation of apoptosis may be the main cause of the HSPC rescue effect exerted by downregulation of p53 in rpc9−/− embryos.

**DISCUSSION**

Our work characterizes a T cell-deficient mutant (116) that lacks expression of T cell markers. Positional cloning identifies that rpc9 is responsible for the hematopoietic phenotype. Further experiments demonstrate that rpc9 deficiency specifically causes the impairment of HSPCs and affects the development of all hematopoietic lineages. Rpc9 functions mainly as a component of RNA polymerase III complex and its deficiency leads to the inefficiency of SS rRNA and tRNA synthesis, which will thus affect ribosome biogenesis and protein synthesis. Excessive apoptosis and abated proliferation were clearly detected in the CHT region of rpc9−/− embryos. Mechanistically, p53 is specifically induced in the CHT region of rpc9−/− embryos and HSPC defects in the mutant can be rescued by P53 suppression.

In early embryogenesis, HSPCs undergo rapid expansion while retaining their stemness in the fetal liver (CHT in zebrafish) to establish the HSPC pool (Copley and Eaves, 2013). In fact, cell cycle analysis and reconstitution studies revealed that almost all fetal liver HSPCs are in the mitotic phase (Trumpp et al., 2010; Fig. 5. Knockdown of Pol III components polr3h and polr3k leads to HSPC defects. (A) qRT-PCR result shows that, compared with control embryos, products of Pol III (pre-rRNAα, pre-rRNAβ, 5S rRNA and 7SL RNA) are all significantly decreased in both polr3h and polr3k morphants (5 dpf). 18S rRNA was used as internal control. (B) WISH result showing that HSPC marker (cmyb) and differentiated hematopoietic cell lineage markers (gata1, pu.1 and rag1) are all decreased in polr3h and polr3k morphants at 5 dpf. (C) qRT-PCR result reveals that cmyb, gata1 and pu.1 are significantly decreased in polr3h and polr3k morphants at 5 dpf. β-actin was used as internal control. Note that arrowheads in B mark the CHT or the thymus. Values are mean±s.d. **P<0.01, ***P<0.001, Student’s t-test.

Fig. 6. P53 mediates the regulation of Rpc9 in HSPC survival. (A) WISH result reveals that p53 is specifically increased in the CHT region of rpc9−/− embryos at 3 and 4 dpf. (B) Western blot shows that, compared with rpc9+/+ embryos, the protein level of P53 in P53−/− embryos at 4 dpf. (C) The quantitative result of the western blot in B. (D) The absence of rag1 in the thymus of rpc9−/− embryos, at 5 dpf, can be rescued by loss of p53. (E) The absence of cmyb in the CHT region of rpc9−/− embryos at 5 dpf can be rescued by loss of p53. Note that arrowheads in A,E mark the CHT, whereas arrowheads in D mark the thymus. Values are mean±s.e.m. ***P<0.001, Student’s t-test.
Pietras et al., 2011). These dividing cells require rapid biological macromolecule synthesis, including RNA transcription and protein synthesis and this is implicated in elevated Pol III activity (Goodfellow and White, 2007; Signer et al., 2014), which is tightly regulated and vulnerable to alteration of intrinsic network or environment cues (Goodfellow and White, 2007; Boguta and Graczyk, 2011; Acker et al., 2014). Notably, tissues with a high proliferation rate, including central nervous and hematopoietic systems, display hypersensitivity to adverse genetic mutations (Zaros and Thuriaux, 2005; Li et al., 2012; Ramirez et al., 2012; Belle et al., 2015), which is in line with the impairment of survival and proliferation of HSPCs observed in rpc9−/− embryos.

The CGRP receptor complex, which includes Rpc9, has previously been shown to be expressed on human CD34+ cells and is required for granulopoiesis (Harzannettet et al., 2002). However, in that report, only Rpc9, not other CGRP receptor components, was expressed in granulocytes (Harzannettet et al., 2002), implying that Rpc9 may function beyond the CGRP receptor complex. Cfr, another component of this CGRP receptor complex, was reported to be required for zebrafish arterial patterning (Nicoli et al., 2008; Wilkinson et al., 2012). However, we observed no obvious alteration of expression of arterial markers in rpc9−/− embryos (Fig. S6G) and this was in line with the result that HSPCs were not affected at the emergence stage in embryos (Fig. S6G) and this was in line with the result that HSPCs and proliferation of HSPCs observed in Belle et al., 2015), which is in line with the impairment of survival (Zaros and Thuriaux, 2005; Li et al., 2012; Ramirez et al., 2012; Belle et al., 2015), which is in line with the impairment of survival and proliferation of HSPCs observed in rpc9−/− embryos.

As an integral subunit of RNA polymerase III, the major role of Rpc9 is in protein synthesis (Dieci et al., 2007). Interestingly, the observation of neural cell-specific snRNAs transcribed by Pol III (Dieci et al., 2007), the discovery of tissue-specific tRNAs in Bombyx mori (Underwood et al., 1988; Taneja et al., 1992) and humans (Dittmar et al., 2006), and the finding of cell type-specific 5S RNA (Barciszewska et al., 2000) implied that, beyond the basic housekeeping role in most cells, the activity of Pol III is dynamically regulated in a cell context-dependent manner to fit with requirements of diverse tissue development and homeostasis. Accordingly, the specific expression of rpc9 in the CHT region may indicate that the rapidly expanding HSPCs require many more Pol III transcripts. In fact, in the absence of Pol III components, recent reports demonstrated neuron-specific dysfunction in humans (Bernard et al., 2011; Saito et al., 2011; Wong et al., 2011) and digestive organ-specific defects in zebrafish (Yee et al., 2007). Intriguingly, the rpc9 heterozygous mutant embryos have no hematopoietic defects and can develop into fertile adults, similar to rpl11 (encoding ribosome protein Rpl11), nop10 (18S RNA processing) and kril1 (18S RNA maturation) mutants in zebrafish. In humans, however, most of the ribosomal gene mutations observed in 5q-myeolodysplastic syndromes (MDS) or Blackfan–Diamond anemia (BDA) patients were heterozygous (Komrokji et al., 2013; Nakhoul et al., 2014). Therefore, the discrepancy of hematopoietic phenotype between zebrafish and human heterozygous mutants might be due to the following possibilities: mutation type, genetic background, developmental stage (human adult patients versus zebrafish embryos) and the more complex gene regulation system in human but not in zebrafish.

P53 is a crucial transcription factor involved in the quiescence, self-renewal, senescence and apoptosis of HSPCs (Nii et al., 2012; Pant et al., 2012). The constitutive activation of P53 is harmful for the stemness of HSPCs (Liu et al., 2010; Wang et al., 2011), whereas HSPCs in the p53 mutant are relatively normal (Lotem and Sachs, 1993). Here, we found that the transcript of p53 was specifically increased in the CHT region of rpc9 mutants, mediating HSPC impairments, which is similar to two works reporting that HSPC defects in zebrafish embryos deficient in TopBPI (topoisomerase II β binding protein 1, involved in DNA replication and DNA damage) or the ribosome protein Rpl11 could be rescued by knockdown of p53 (Danilova et al., 2011; Gao et al., 2015).

In summary, we reveal an unexpected role of Pol III during zebrafish definitive hematopoiesis and show that P53 signaling is involved in this process. To the best of our knowledge, this is the first demonstration that Rpc9, which is a component of the RNA polymerase III complex, plays an essential role in HSPC maintenance during embryogenesis in vertebrates.

**MATERIALS AND METHODS**

**Zebrafish lines**

Mutant line 116 (AB strain) was identified from a screening of mutants after ENU mutagenesis (Du et al., 2011). Wild-type (WIK or AB strain), p53M214K (Berghmans et al., 2005) and heterozygous mutant line 116 fish were raised and maintained at 28.5°C in system water and staged as previously described (Kimmel et al., 1995). Zebrafish embryos were acquired by natural spawning. This study was approved by the Ethical Review Committee of the Institute of Zoology, Chinese Academy of Sciences, China.

**Whole mount in situ hybridization and qRT-PCR**

WISH assay of zebrafish embryos was conducted as described previously (Liu and Patient, 2008) with probes against rag1, rag2, ikaros, bcl11b, tcrb2, tcrd, cmyb, runx1, ccr9a, ccr9b, foxn1, gata1, lyl, l-plastin, pu.1, mfap4, p53, polr3h and polr3k. Quantitative RT-PCR was performed with cDNA derived from dissected trunk regions of zebrafish embryos. Data are represented as mean±s.d. and Student’s t-test was used for comparison between control and experimental groups. P<0.05 indicates significant difference. The PCR primers used are listed in Table S2.

**Positional cloning and mutant genotyping**

Mutant line 116 carriers were crossed with wild-type WIK strain fish and the embryos were raised as the F1 generation. The carriers of 116/WIK mutant line 116 fish were raised and maintained at 28.5°C in system water and staged as previously described (Kimmel et al., 1995). Zebrafish embryos were acquired by natural spawning. This study was approved by the Ethical Review Committee of the Institute of Zoology, Chinese Academy of Sciences, China.
full-length CDS of zebrafish rpc9 was integrated into the pDONR221 vector by the ‘BP’ reaction, which can be used as donor vector to fuse a hsp70 promoter, an EGFP reporter and a pDestTo2PA destination vector by the ‘LR’ reaction and we finally obtained hsp70-rpc9-egfp (MultiSite Gateway Technology, Invitrogen). After injection of hsp70-rpc9-egfp together with tol2 mRNA, the embryos were heat-shocked at 42°C for 30 min at 2.5-5 dpf at intervals of 12 h. To examine the localization of Rpc9, the full-length CDS of zebrafish rpc9 was fused with egfp and inserted into the pCS2 plasmid. Then rpc9-egfp mRNA was synthesized according to the instruction manual of mMessage mMACHINE SP6 kit (Ambion) and injected into one cell stage zebrafish embryos. EGFP signal was examined at 9 hpf.

Western blot
Zebrafish embryos were cut into two parts, the heads were used for genotyping and the trunks were homogenized with cell lysis buffer (protein inhibitor was added). To examine the localization of Rpc9, cytoplasmic and nuclear proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime), according to the manufacturer’s instructions. After quantification with Bradford protein assay, protein samples were resolved by SDS-PAGE and transferred into a nitrocellulose membrane. The membrane was then blocked with non-fat milk and incubated at 4°C overnight with a rabbit anti-Rpc9 polyclonal antibody (1:2000; peptide used to immunize rabbit: ‘YQLLTDLKEKR’, Abmax) or anti-P53 (1:500, GeneTex, GTX128135) antibody diluted in blocking buffer (5% nonfat dried milk in TBST; BD). Next, the membrane was washed with TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) and incubated with a secondary antibody conjugated with alkaline phosphatase (1:5000, Jackson ImmunoResearch Laboratories, 111-035-003) at room temperature with a rabbit anti-Rpc9 polyclonal antibody (1:2000, peptide used to immunize rabbit: ‘YQLLTDLKEKR’, Abmax) or anti-P53 (1:500, GeneTex, GTX128135) antibody diluted in blocking buffer (5% nonfat dried milk in TBST; BD). Finally, the membrane was washed and the signal was examined with a chemiluminescent HRP substrate (Millipore).

Acridine Orange staining
The embryos were incubated with 5 μg/ml Acridine Orange in system water for 30 min at 2 dpf, and for 60 min at 3 or 4 dpf. Then the embryos were washed with system water 8-10 times at intervals of 5 min and then viewed with a Nikon A1R* confocal laser microscope. After recording images, embryos were numbered and subjected to genome DNA extraction for genotyping.

pH3 assay
Zebrafish embryos were dissected into two parts, the anterior parts were used for genotyping and the posterior parts were fixed in 4% PFA at 4°C overnight and then dehydrated with methanol at −20°C for 30 min. After washing with PBST buffer (PBS with 0.1% Tween 20) four times (5 min each), the embryos were digested within 50 μM protease K for 1 h. The permeabilized embryos were re-fixed with 4% PFA for 20 min. After washing with PBST buffer three times on a shaker, the embryos were blocked with 1% blocking buffer (Roche) for 2 h at room temperature and then incubated with anti-pH3 antibody diluted in blocking buffer (1:500, Cell Signaling) overnight. On the next day, the embryos were washed three times (15 min) with PBST buffer and incubated with a secondary fluorescent antibody (Invitrogen) overnight. Finally, the embryos were washed three times (15 min) with PBST buffer and photographed by confocal microscopy (Nikon, A1R*).

TUNEL assay
Zebrafish were cut into two parts; the anterior parts were lysed for genome DNA extraction genotyping and the posterior parts were fixed in 4% PFA at 4°C overnight, dehydrated in methanol at −20°C for 30 min and then subjected to TUNEL assay according to the instructions of the TUNEL assay kit (Roche).

BrdU assay
Zebrafish embryos were injected with BrdU (10 mM, 1 nl per embryo) and 2 h later fixed in 4% PFA and left at 4°C overnight. On the next day, the embryos were cut into two parts, the anterior parts were used for genotyping and then the posterior parts were re-fixed in 4% PFA at 4°C overnight, dehydrated in methanol at −20°C for 30 min and then subjected to BrdU assay as previously described (Ma et al., 2012).

Chemical treatment
To determine the function of Pol III on hematopoiesis, zebrafish embryos were incubated with 1.32 mM ML-60218 (Pol III inhibitor, Millipore, 557404) from 1 to 5 dpf and harvested at 5 dpf. To explore the role of CGRP signaling pathway, zebrafish embryos were incubated with Forskolin (Selleck, S2449), 8-bromo-cAMP (Selleck, S7857) and MK-3207 (Selleck, S1542) from 2.5 to 5 dpf. Control fish were incubated in dimethylsulfoxide (DMSO), at a dilution in line with that of the examined chemicals.

Cyclic adenosine monophosphate assay
The level of cAMP was examined using a Monoclonal Anti-cAMP Antibody Based Direct cAMP ELISA Kit (Neweastbio), following the manufacturer’s instructions.

Statistical analysis
For statistical analysis, Student’s unpaired two-tailed t-test was used for all comparisons.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Y.W. performed the experiments and wrote the paper; J.X. and W.Z. provided reagents; Z.W. analyzed the data; F.L. conceived the project, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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