

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

Yonglong Wei^{1,2}, Jin Xu³, Wenqing Zhang⁴, Zilong Wen³ and Feng Liu^{1,2,*}

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 (Warner, 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; Crpc – 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 Rpc9 forms a heterodimer with another Pol III subunit Rpc25, which is the paralogue 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, Rpc9 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 G α 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

¹State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China. ²University of Chinese Academy of Sciences, Beijing 100049, China. ³State Key Laboratory of Molecular Neuroscience, Center of Systems Biology and Human Health, Division of Life Science, Hong Kong University of Science and Technology, Kowloon, Hong Kong, China. ⁴Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases of Guangdong Higher Education Institutes, Department of Cell Biology, Southern Medical University, Guangzhou 510515, China.

*Author for correspondence (liuf@ioz.ac.cn)

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hematopoietic defects can be rescued by downregulation of *p53* (*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 *rag1*, the T cell-deficient mutant line 116 was isolated. According to whole-mount *in situ* hybridization (WISH), expression of HSPC markers [*cmyb* and *ikaros* (*ikzf1* – Zebrafish Information Network)], early T cell markers (before β/δ selection; *rag1*, *rag2* and *bcl11b*), chemokine receptor markers (*ccr9a* and *ccr9b*) and naive T cell markers (after β/δ selection; *icrb2* and *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 *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 *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

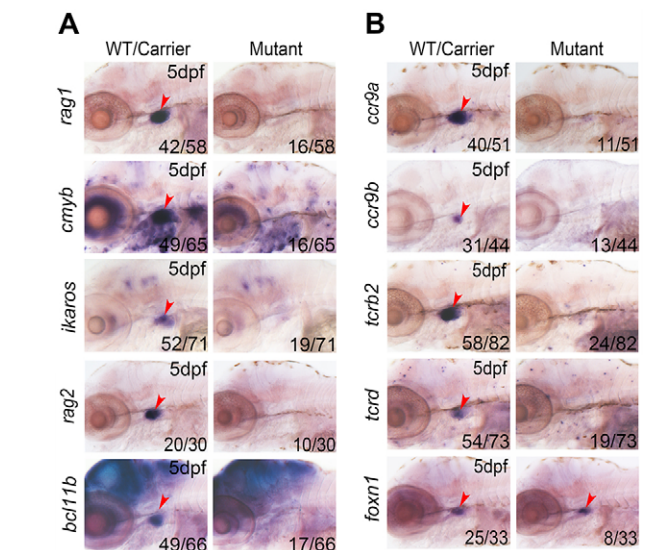


Fig. 1. T cells are absent in mutant line 116 embryos. (A,B) WISH showing that early T cell markers (*rag1*, *rag2* and *bcl11b*), HSPC markers (*cmyb* and *ikaros*) and T cell markers (*ccr9a*, *ccr9b*, *icrb2* and *tcrd*) are absent in the thymus of mutant line 116 at 5 dpf. By contrast, thymus epithelial cell marker *foxn1* was normal in the thymus of 116 mutants at 5 dpf. Red arrowheads indicate the thymus. Numbers at bottom right indicate the number of embryos with similar staining pattern among all embryos examined.

thymine was switched into an adenine in the second exon of the *rpc9* gene, which formed a premature stop codon in the coding region of *rpc9* mRNA (Fig. 2A,B). This mutation also generated a new restriction enzyme recognition site as TTAA (*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 *rpc9* (Fig. S1B). To confirm that the *rpc9* gene was responsible for the

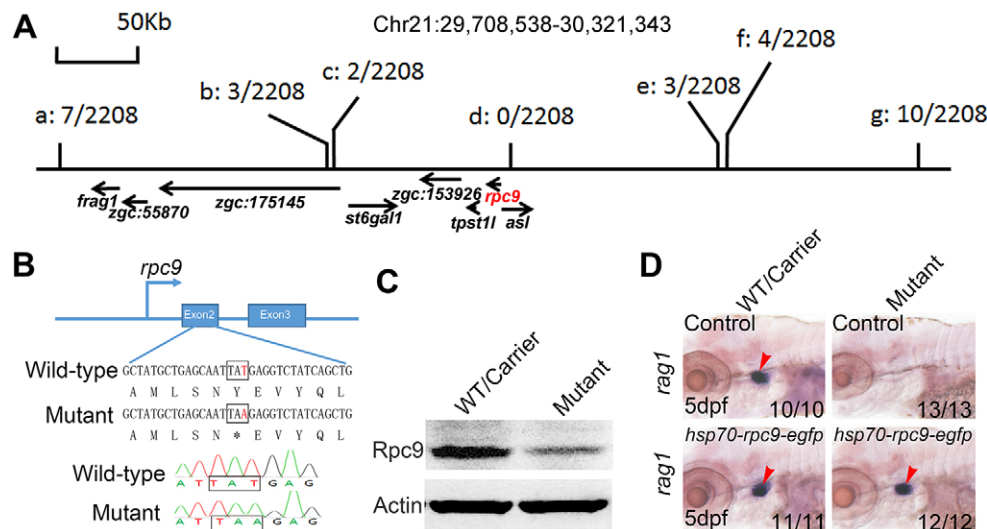


Fig. 2. Lack of Rpc9 is responsible for the phenotype of mutant line 116 embryos. (A) Schematic of positional cloning. a-g indicate the locations of polymorphic primers distributed between 29,708,538 to 30,321,343 bp of chromosome 21 and the ratios of recombination out of total 2208 embryos in each position. Genes scattered in this region are marked and *rpc9* (in red) is adjacent to the polymorphic marker d. Note that the chromosomal locations and sequences of these primers are listed in Table S3. (B) Gene sequencing identified a transversion of T>A in the second exon (exon 2) of *rpc9*, which formed a pre-mature stop code in the coding region of *rpc9* in mutant line 116 embryos. (C) Western blot showed that the protein level of Rpc9 was greatly decreased in mutants at 4 dpf. (D) Injection of the *rpc9* overexpression plasmid driven by *hsp70* promoter (*hsp70-rpc9-egfp*) exerted a full rescue effect of *rag1*. Red arrowheads in D indicate the thymus.

phenotypes observed, we applied two approaches. First, we designed an antisense morpholino (MO) to block the translation of Rpc9. The efficiency of this MO was validated by co-injection with an EGFP plasmid reporter and by western blot (Fig. S1C). When Rpc9 was knocked down by injection of *rpc9* MO, expression of *rag1* but not *foxn1* was dramatically decreased, which was in line with results in the mutant embryos (Fig. S1D). Second, we tried an mRNA rescue approach. The full rescue effect exerted by overexpression of *rpc9* driven by the *hsp70* promoter in mutant line 116 embryos was observed (Fig. S1E and Fig. 2D). Altogether, these data strongly demonstrated that *rpc9* deficiency was responsible for the hematopoietic phenotypes in the T-cell deficient mutant line 116.

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* (*spi1b*), *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 *icrb2* and *trcd* was dramatically decreased at 4 dpf (Fig. 3A), suggesting that a subset of early thymic progenitors (ETPs) could enter the thymus and develop through β/δ -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* (*tal1*), *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.

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, $P=0.0447$) 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

Since Rpc9 may 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)

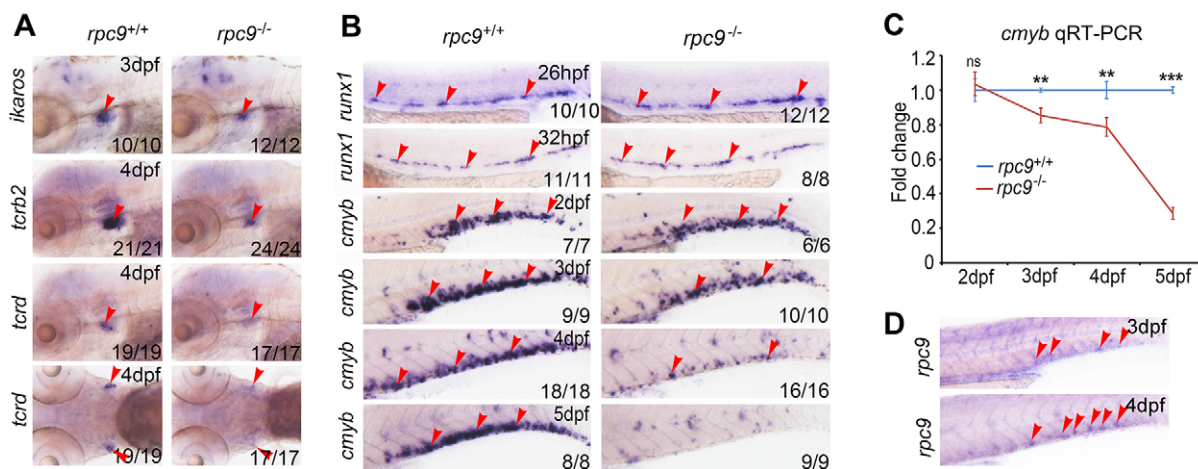


Fig. 3. HSPCs are impaired in *rpc9*^{-/-} embryos. (A) WISH showing that *ikaros*, at 3 dpf, and *tcrd* and *tcrb2*, at 4 dpf, were decreased in the thymus of *rpc9*^{-/-} embryos. (B) WISH demonstrates that *runx1* expression is not decreased in the AGM region of *rpc9*^{-/-} embryos at 32 h post-fertilization (hpf) and *cmyb* clearly begins to decrease from 3 dpf in the CHT region of *rpc9*^{-/-} embryos. (C) qRT-PCR with the cDNA reverse transcribed from total RNA of posterior trunk region reveals that *cmyb* expression is significantly decreased in *rpc9*^{-/-} embryos from 3 to 5 dpf. β -actin was used as internal control (mean±s.d., $n=3$, ** $P<0.01$, *** $P<0.001$). (D) Expression pattern of *rpc9* in the CHT region at 3 and 4 dpf. Note that arrowheads in A mark the thymus, whereas arrowheads in B,D mark the CHT. Values are mean±s.e.m.; ** $P<0.01$, *** $P<0.001$, Student's *t*-test. ns, not significant.

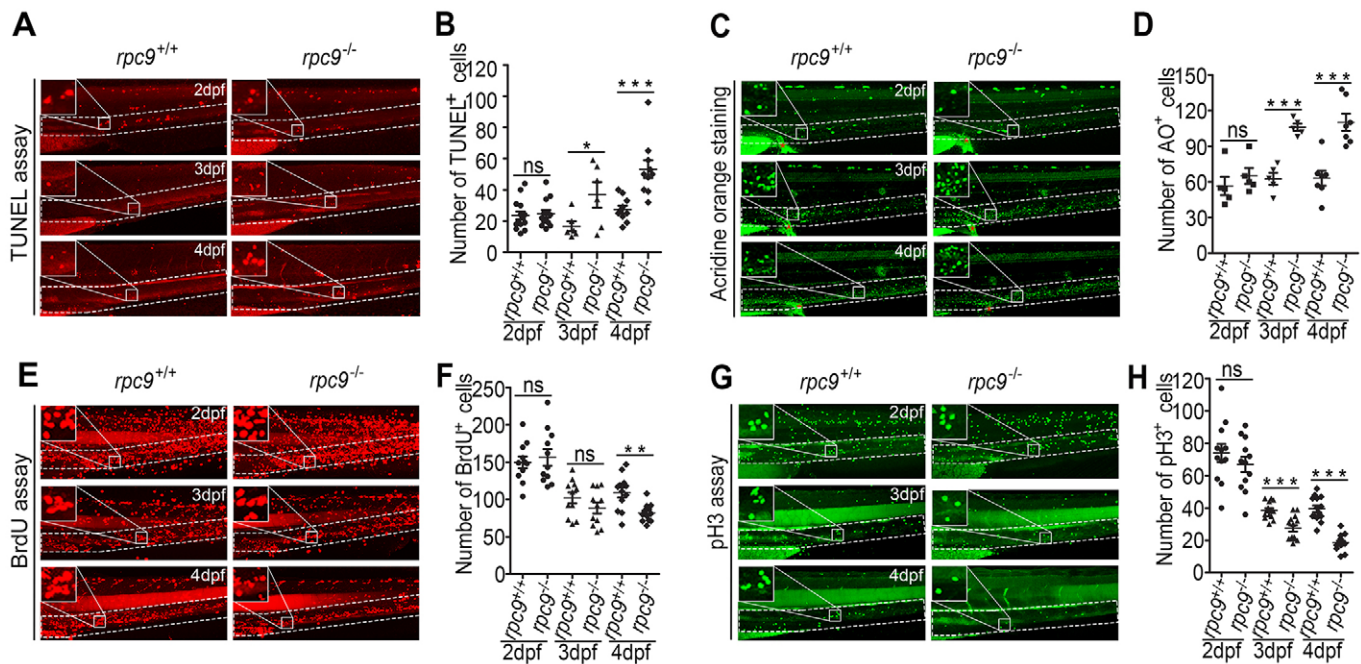


Fig. 4. Enhanced apoptosis and reduced proliferation in the CHT of *rpc9*^{-/-} mutants. (A,B) TUNEL assay shows apoptosis signals in the CHT region of *rpc9*^{+/+} and *rpc9*^{-/-} embryos. TUNEL signals are significantly enhanced in *rpc9*^{-/-} embryos at 3 and 4 dpf, but not at 2 dpf. (C,D) Acridine 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 *rpc9*^{-/-} embryos at 4 dpf compared with *rpc9*^{+/+} embryos. (G,H) pH3 assay demonstrates the pH3 proliferation signals are significantly reduced in *rpc9*^{-/-} embryos at 3 and 4 dpf compared with *rpc9*^{+/+} embryos. Note that white dashed boxes in A,C,E and G enclose the CHT region where the signal spots (indicated by white arrowheads) were counted. A 3× magnified image is shown in the top left corner of each panel. Values are mean±s.e.m.; **P*<0.05, ***P*<0.01, ****P*<0.001, Student's *t*-test. ns, not significant.

at a gradient of doses around the previously reported functional 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-tRNA^{Ile}, pre-tRNA^{Leu}, 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* (*kdrl*) 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*^{-/-} embryos 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 down-regulated. 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,

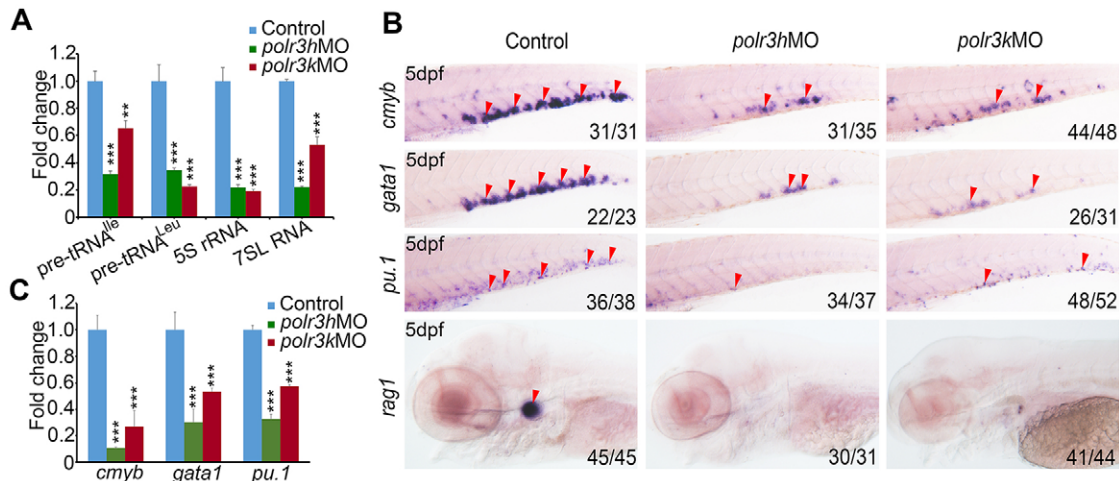


Fig. 5. Knockdown of Pol III components *polr3h* and *polr3k* leads to HSPC defects. (A) qRT-PCR result shows that, compared with control embryos, products of Pol III (pre-tRNA^{Ile}, pre-tRNA^{Leu}, 5S rRNA and 7SL RNA) are all significantly decreased in both *polr3h* and *polr3k* morphants (5 dpf). 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 \pm s.d. ** P <0.01, *** P <0.001, Student's t -test.

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 \pm 1.56 vs 11.33 \pm 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 5S 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;

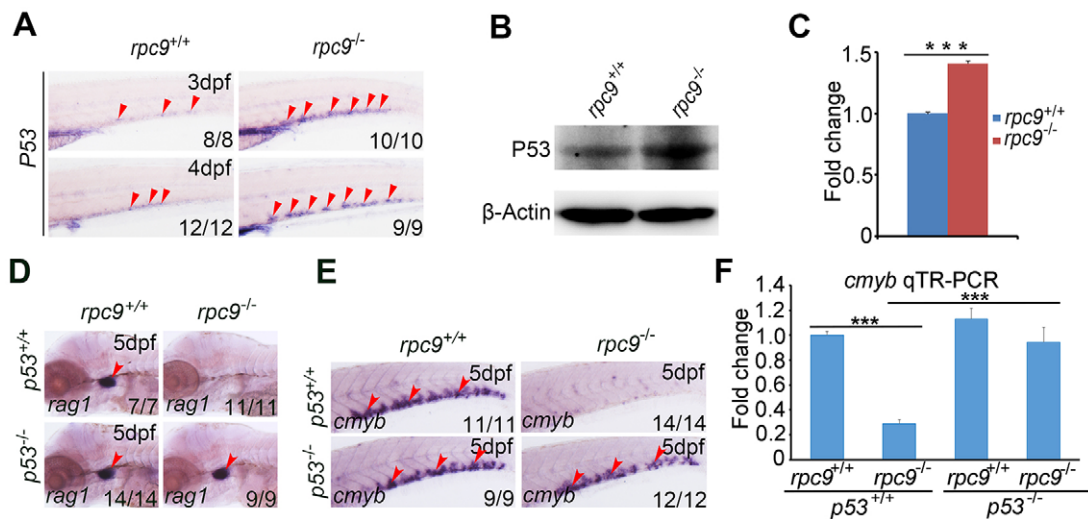


Fig. 6. P53 mediates the regulation of *Rpc9* in HSPC survival. (A) WISH result reveals that *p53* is specifically increased in the CHT region of *rpc9*^{-/-} embryos at 3 and 4 dpf. (B) Western blot shows that, compared with *rpc9*^{+/+} embryos, the protein level of P53 was greatly increased in *rpc9*^{-/-} 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 *p53* deficiency. (F) qRT-PCR result reveals that the decrease 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 \pm 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 (Harzenetter et al., 2002). However, in that report, only Rpc9, not other CGRP receptor components, was expressed in granulocytes (Harzenetter et al., 2002), implying that Rpc9 may function beyond the CGRP receptor complex. Crlr, 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 *rpc9*^{-/-} mutants. Based on the data that Rpc9 was mainly localized in the nucleus and because modulating the CGRP receptor signaling activities exerted no hematopoietic effects on *rpc9*^{-/-} or wild-type embryos, we reasoned that Rpc9 may regulate HSPC maintenance independent of the CGRP receptor signaling pathway.

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 *rp11* (encoding ribosome protein Rpl11), *nop10* (18S RNA processing) and *kri11* (18S RNA maturation) mutants in zebrafish. In humans, however, most of the ribosomal gene mutations observed in 5q-myelodysplastic 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 TopBP1 (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). Although apoptosis signals in the CHT region were decreased upon *p53* deficiency, the proliferation signals between *rpc9*^{-/-} embryos with or without intact *p53* signaling showed no significant difference, suggesting that increased apoptosis but not decreased proliferation was responsible for HSPC impairment in *rpc9* mutants.

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), *p53*^{M214K} (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*, *gatal*, *lyz*, *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 (F1 generation) were screened by multiple random incrosses and WISH assay with their offspring (F2 generation). Then the genomic DNAs from embryos of F2 generation were extracted after WISH and subjected to PCR with polymorphic primers for positional cloning (Zhou and Zon, 2011). Upon positional cloning, the mutant could be easily distinguished from the wild-type by PCR with two pairs of polymorphic primers flanking the mutated site, or with a pair of primers embracing the mutation locus and a restriction enzyme *MseI*, recognizing the mutated site (Table S3). The genotyping of *p53*^{M214K} was performed by PCR with reported primers (Johnson et al., 2011).

Morpholinos and *rpc9* overexpression

All morpholinos (MOs) were ordered from GeneTools and dissolved in ultrapure water. The MO sequences are listed in Table S1. The injection dose for *rpc9*, *p53*, *polr3h* and *polr3k* MO was 6, 8, 1.5 and 0.5 ng per embryo, respectively. For morpholino evaluation, partial cDNA of a gene containing the morpholino target was cloned into the pEGFP-N1 vector (with *HindIII* and *SacII*) and fused to *egfp* in-frame (primers are listed in Table S2), which can be used as a reporter. Then the vector was injected alone or co-injected with a corresponding atgMO. EGFP signal can be detected at the shield stage or later. The efficiency of the atgMO was measured by the extent of inhibition of EGFP. For temporal-controlled overexpression of *rpc9*, the

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 pDestTol2pA 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: 'YQLLDLKEKR', 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 for 2 h. 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 AIR⁺ 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 proteinase 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, AIR⁺).

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 nM 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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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.126797/-/DC1>

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