Ikzf1 regulates embryonic T lymphopoiesis via Ccr9 and Irf4 in zebrafish

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Ikzf1 is a Krüppel-like zinc-finger transcription factor that plays indispensable roles in T and B cell development. Although the function of Ikzf1 has been studied extensively, the molecular mechanism underlying T lymphopoiesis remains incompletely defined during the embryonic stage. Here we report that the genetic ablation of ikzf1 in mutant zebrafish resulted in impaired thymic migration, proliferation, and differentiation of hematopoietic stem/progenitor cells (HSPCs). Ccr9a and Irf4a, two indispensable factors in T lymphopoiesis, were absent in the ikzf1 mutants. Genetic deletion of either ccr9a or irf4a in the corresponding mutant embryos led to obvious T cell development deficiency, which was mainly caused by disrupted thymic migration of HSPCs. Restoration of ccr9a in ikzf1 mutants obviously promoted HSPC thymus homing. However, the HSPCs then failed to differentiate into T cells. Additional replenishment of irf4a efficiently induced HSPC proliferation and T cell differentiation. Our findings further demonstrate that Ikzf1 regulates embryonic T lymphopoiesis via Ccr9 and Irf4 and provide new insight into the genetic network of T lymphocyte development.

As a key cellular component in adaptive immunity, T lymphocytes are considered to originate from hematopoietic stem cells (HSCs) through a tightly controlled hierarchy (1, 2). Although common lymphoid progenitors are regarded as the major sources of T cells (3), the identification of lymphoid-primed multipotent progenitors, which are endowed with both myeloid and lymphoid potential, has suggested that myeloid and lymphoid lineages share a common precursor (4). The seeding of the thymus by these hematopoietic progenitor cells is required for the commitment, proliferation, differentiation, and maturation of T lymphocytes (5). Although the processes of T cell differentiation and maturation have been investigated extensively, the molecular mechanisms governing the migration of hematopoietic progenitor cells to the thymus and their differentiation during embryonic stages are poorly understood.

Zebrafish (Danio rerio) are widely used as a model system for studying hematopoiesis because they present several unique characteristics, including extraterrene development and optical transparency. Zebrafish T lymphoid cells result from the definitive hematopoiesis that occurs in the ventral wall of the dorsal aorta (VDA), where hematopoietic stem/progenitor cells (HSPCs) emerge from the aortic endothelium through endothelial hematopoietic transition (6–8). These newly formed HSPCs subsequently migrate to the caudal hematopoietic tissue (CHT), thymus, and kidney, where they proliferate and differentiate to produce distinct blood lineages (9–12). At ∼60 h post-fertilization (hpf), VDA-born hematopoietic precursors appear to start colonizing the developing thymus (13), where they undergo rapid proliferation and differentiation and give rise to mature T cells (13).

The IKAROS family zinc finger 1 (Ikzf1) transcription factor, also known as Ikaros, was initially detected in mouse liver rudiment (14). The Ikzf1 gene generated various spliced isoforms that translated into a complex group of proteins characterized by a DNA-binding domain and protein–protein interaction domain in the N and C terminus, respectively (15, 16). Ikzf1 proteins are detected in various subsets of hematopoietic cells and are especially enriched in lymphocytes (15, 17, 18). Mice carrying distinct Ikzf1 mutant alleles display similar lymphopoiesis defects and other hematopoietic phenotypes (19–26). These studies indicate that Ikzf1 plays critical roles in multiple steps of hematopoietic cell growth, especially in T cell development. Ikzf1 targets the nucleosome–remodeling–deacetylase complex (NuRD) to promote lymphoid priming of HSPCs (27). Ikzf1 cooperates with other members of the IKAROS family (28, 29) to regulate several key molecules in T cell differentiation.
are directly regulated by Ikzf1 and are functionally indispensable in T lymphopoiesis. We further reveal that loss of Ikzf1 function in zebrafish impairs thymus migration, proliferation, and differentiation of HSPCs, which results in failure of embryonic hematopoiesis, especially T lymphopoiesis, remain largely undefined.

In this study, two ikzf1 mutant alleles that affect the functional domains were generated. We show that loss of Ikzf1 function in zebrafish impairs thymus migration, proliferation, and differentiation of HSPCs, which results in failure of embryonic hematopoiesis, especially T lymphopoiesis, remain largely undefined.

Results

Embryonic T lymphopoiesis is abolished in ikzf1 mutants

To explore the function of ikzf1 in early hematopoiesis, including T lymphopoiesis, ikzf1 expression patterns and levels in different embryonic blood cells were examined in zebrafish. Ikzf1 clearly appeared in runx1-GFP(+) cells and cmyb-GFP(+) (33) cells (Fig. S1A) in the VDA regions at 36 hpf. Ikzf1 was then detected in the larval hematopoietic organs (CHT, thymus, and kidney; Fig. S1B) (11). Ikzf1 transcript levels were highest in rag2-DsRed(+) lymphoid cells (34), followed by CD41-GFP(+) HSPCs (10), mpx-GFP(+) myeloid cells (35), and gata1-DsRed(+) erythroid cells (36). In CD41-GFP(+) thrombocytes (37), a limited amount of ikzf1 transcript was detected (Fig. S1C). The hematopoietic expression of ikzf1 suggested its critical role during hematopoiesis, especially in T cell development. Therefore, the ikzf1 gene was edited (Fig. 1A). Two types of ikzf1 mutants were identified. One mutant allele was 1 bp short in exon 3 (deletion of 4 bp but addition of another 3 bp), and the other showed a 1-bp insertion in exon 9 (Fig. S1, D–F). Both mutations led to a premature stop codon in their coding sequences (Fig. 1B and Fig. S1F), which did not cause an obvious reduction in expression but resulted in synthesis of truncated Ikzf1 proteins in both mutant alleles (Fig. 1B and Fig. S1G and H). This suggested loss of function in both alleles. Similar hematopoietic phenotypes were seen in both mutants (Fig. S2, A and B). Therefore, the ikzf1 mutant was selected for intensive investigation.

The enrichment of the ikzf1 transcript in rag2-DsRed(+) cells drew our attention to the T cell phenotype. As expected, T cell formation, as indicated by the expression of ccr9a, ccrb, lck, and rag1, was severely compromised in ikzf1(3A/3A + 3) mutants (Fig. 1C). We confirmed that this T cell deficiency phenotype in ikzf1(3A/3A + 3) mutants was caused by the ikzf1 mutation; forced expression of ikzf1 in hematopoietic cells, driven by the hematopoiesis-specific corola promoter and overexpressed in hematopoietic progenitors (Fig. S1I) and leukocytes (38, 39), was sufficient for restoring T cell development in mutants (Fig. 1C). The expression of ccl25a, a chemokine gene expressed in thymic epithelial cells (13), was not detectably altered (Fig. S1J). Collectively, these data demonstrate that Ikzf1 is required for T cell development, in accordance with previous findings (30).
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Failed thymic migration of HSPCs in ikzf1^{3A4} + 3/4A4 + 3 mutants

The drastically compromised T lymphopoiesis in ikzf1 mutant zebrafish (Fig. 1C and Fig. S2A) (30, 31) and mice (19–21, 25) prompted a dissection of the molecular mechanisms involved. Zebrafish embryonic T lymphopoiesis initiated from HSPCs. However, the signature of cmyb^{+} cells showed no obvious change at 36 hpf and 48 hpf in the VDA and CHT regions between ikzf1 mutants and nonmutant siblings (Fig. S2, B–D), suggesting that ikzf1 is dispensable in HSPC production. At ~60 hpf, HSPCs first appeared in the thymus (Fig. 2A) (13). However, almost no cmyb^{+} cells were detected in the thymus of ikzf1^{3A4} + 3/4A4 + 3 mutants compared with WT siblings at this stage, whereas a comparable number of cmyb^{+} cells was seen in the CHT at the same time point (Fig. 2, A and B). This result suggested that the initial thymic migration of progenitor cells was disrupted by ikzf1 deficiency. To test this hypothesis, we used time-lapse imaging to monitor the thymus homing of hematopoietic progenitors, from 60 hpf onwards, in ikzf1^{3A4} + 3/4A4 + 3, Tg(coro1a:DsRed;lyz:GFP) transgenic mutants. To exclude the influence of myeloid cells, we focused on coro1a-Dsred^{+}/lyz-GFP^{−} cells (40), located around the thymus, that featured a spherical morphology (presumably thymocytes and their progenitors). In control embryos, ~0.7 spherical coro1a-DsRed^{+}/lyz-GFP^{−} cell per hour entered the thymus between 60 and 72 hpf (Fig. 2, C and D, and Movie S1). In comparison, limited spherical coro1a-DsRed^{+}/lyz-GFP^{−} cells were located around and entered the ikzf1^{3A4} + 3/4A4 + 3 thymus during the same period (Fig. 2, C and D, and Movie S2). These results indicated that movement to the thymus in hematopoietic progenitors was abrogated in ikzf1^{3A4} + 3/4A4 + 3 mutants. This conclusion was further validated by the results of another transient lineage-tracing assay, performed using the ikzf1^{3A4} + 3/4A4 + 3; Tg(coro1a:Kaede) and ikzf1^{3A4} + 3/4A4 + 3; Tg(kdrl:Dendra2) lines, in which specific hematopoietic progenitors in the VDA (somites 8–10) and CHT (somites 15–17) were labeled (coro1a-Kaede^{+} or kdrl-Dendra2^{+} cells) and then followed after UV irradiation (41, 42) (Fig. S2, E and F). In WT siblings, the labeled coro1a-Kaede^{+} and kdrl-Dendra2^{+} cells were clearly detected in the thymus and kidney at 1–2 days post-UV irradiation (Fig. 2, E–I). In contrast, no red coro1a-Kaede^{+} or kdrl-Dendra2^{+} cells were detected in the thymus of ikzf1^{3A4} + 3/4A4 + 3 mutants, although the cells were clearly observed in the CHT and kidney (albeit at reduced numbers).
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**Figure 3. Reduced proliferation and differentiation of HSPCs in ikzf1Δ4 + 3/Δ4 + 3 mutant embryos.** A and B, WISH of cmyb in the CHT (A) and glomerulus (B) of ikzf1Δ4 + 3/Δ4 + 3 mutant embryos and their siblings. C, double staining of CD41-GFP and EdU in the CHT of ikzf1Δ4 + 3/Δ4 + 3 mutant embryos and their siblings. Scale bar = 50 μm. D, the percentage of EdU+/CD41-GFP+ cells in C (mean ± S.D.; Sib: 26.55 ± 3.80, n = 16; ikzf1Δ4 + 3/Δ4 + 3: 16.73 ± 5.42, n = 14). E, double staining of runx1-GFP and EdU in the CHT of ikzf1Δ4 + 3/Δ4 + 3 mutant embryos and their siblings. Scale bar = 50 μm. F, the percentage of EdU+/runx1-GFP+ cells in E (mean ± S.D.; Sib: 29.53 ± 7.49, n = 23; ikzf1Δ4 + 3/Δ4 + 3: 15.08 ± 4.12, n = 13). The blue arrowheads point to the WISH signals in A. The blue arrows, purple arrows, and white arrowheads in C and E indicate the green, red, and double positive signals, respectively. The values in the bottom right corner in A and B indicate counts with a typical appearance as presented (first number) in the total number of examined samples (last number). ****, p < 0.001; g, glomerulus.

These data support the conclusion that the thymus-homing of hematopoietic progenitors during early zebrafish development is compromised in ikzf1Δ4 + 3/Δ4 + 3 mutants.

**Compromised HSPC expansion in ikzf1 mutants**

The reduction of corola-Kaede+ cells in ikzf1Δ4 + 3/Δ4 + 3 mutant kidneys (Fig. 2G) suggested HSPC pool shrinkage at later stages. Indeed, the cmyb+ population in ikzf1 mutants was significantly decreased from 4 dpf compared with those of siblings (Fig. 3, A and B, and Fig. S2B). Consistently, the confocal microscopic images and statistical analysis indicated that the CD41-GFP+ (10) and runx1-GFP+ (32) cells were significantly reduced in ikzf1Δ4 + 3/Δ4 + 3 mutants compared with WT siblings (Fig. S3, A–C). The ratio of EdU+ cells and expression levels of several cell cycle–related genes in the CD41-GFP+ and runx1-GFP+ population were clearly decreased in ikzf1Δ4 + 3/Δ4 + 3 mutants compared with WT siblings (Fig. 3, C–F, and Fig. S3, D and E). However, TUNEL+ cells remained unchanged (Fig. S3, F and G). These results indicated that the reduction of HSPCs in ikzf1Δ4 + 3/Δ4 + 3 mutants was caused by impaired cellular proliferation rather than increased cell apoptosis. Collectively, ikzf1 was essential for thymic migration and proliferation of HSPCs at embryonic stages, which play indispensable roles in T cell development.

**Ccr9a was a key factor mediating thymus homing in HSPCs**

The downstream factors accounting for HSPC thymus homing were investigated by focusing on the chemokine (C-C motif) receptor Ccr9, a critical factor involved in HSPC recruitment to the thymus (43–45). Two orthologues, ccr9a and ccr9b, exist in zebrafish. Both molecules were expressed in the thymic T lymphoid cells from 4 dpf onward in WT siblings, but neither was detected in the ikzf1 mutants (Fig. 1C and Fig. S2A). However, the ccr9a transcript was seen in the WT CHT much earlier than ccr9b (2.5–3 dpf) and was barely observed in ikzf1Δ4 + 3/Δ4 + 3 mutants (Fig. 4A). Genomic sequence analysis of the ccr9a promoter indicated that there were elements directly targeted by Ikzf1 (Fig. 4B). Notably, the results of Chip-qPCR (46) demonstrated that the DNA fragment containing the binding site was enriched in the anti-HA immunoprecipitation group by using Tg(coro1a:HA-ikzf1) (Fig. 4C and Fig. S4, A–D). Furthermore, Dual-Luciferase reporter analysis (47) revealed that deletion of the Ikzf1 binding sites markedly reduced the activity of the ccr9a promoter (Fig. 4D), suggesting that ccr9a might be a critical mediator of ikzf1 in the early migration of HSPCs. Therefore, a ccr9a mutant allele with a 10-bp genomic fragment deletion in exon 3 was generated, which resulted in synthesis of a truncated protein (Fig. S5, A–D). The populations of cmyb+ and irf4a+ cells in the thymus were notably reduced but not totally lost in the ccr9aΔ10/Δ10 mutants compared with WT siblings (Fig. 4, E and F, and Fig. S5E). This phenomenon was reversed by restoration of ccr9a in corola+ hematopoietic cells (Fig. 4E). In contrast, cmyb+ and irf4a+ cells were unaltered in the CHT and kidney of ccr9aΔ10/Δ10 mutants compared with siblings (Fig. 4F and Fig. S5, F and G). Thus, ccr9a function deficiency might impair the thymic trafficking of HSPCs. To prove this, similar time-lapse imaging and transient lineage-tracing assays utilized to study ikzf1 mutants were performed.

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Time-lapse imaging showed that the frequency of Kaede<sup>+</sup> cells entering the thymus was strikingly decreased in ccr9a<sup>Δ10/Δ10</sup> mutants (Fig. 4, G and H, and Movies S3 and S4), and the numbers of red-Kaede<sup>+</sup> and red-Dendra2<sup>+</sup> cells were obviously decreased in the ccr9a<sup>Δ10/Δ10</sup> mutant thymus compared with siblings (Fig. 4, I and J, and Fig. S5, H and I). These data suggest defective homing of hematopoietic progenitors in ccr9a<sup>Δ10/Δ10</sup> mutants.

Restoration of Ccr9a rescued the thymus-settlement of HSPCs in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants

The importance of ccr9a in HSPC thymus-homing and their disappearance in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants prompted supply of this factor and an exploration of T cell phenotypes. To this end, the coro<sub>1</sub>Lac<sup>crt9a</sup> transgenic line was generated (Fig. S6, A and B). Overexpression of ccr9a had no detectable effects on hematopoietic development, as shown by the comparable expression

Figure 4. Defective thymus migration of hematopoietic progenitors in ccr9a mutant embryos. A, WISH of ccr9a in the CHT of ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutant embryos and their siblings. B and C, ChIP-qPCR analysis (C) (mean ± S.D.; ccr9a-1F/1R, IgG: 1.00 ± 0.03, anti-HA: 1.12 ± 0.08) of the binding sites (green) in the ccr9a promoter for ikzf1 (B). CF, control forward primers; CR, control reverse primers. D, luciferase assay showing the activities of ccr9a promoter (with the binding site (BS) and mutant binding site (MBS)) when regulated by the ikzf1 protein (full-length CDS) (mean ± S.D.; ccr9a promoter: 1.00 ± 0.06, n = 3; ccr9a promoter + ikzf1 CDS: 3.07 ± 0.29, n = 3; ccr9a promoter (MBS1) + ikzf1 CDS: 2.17 ± 0.04, n = 3; ccr9a promoter (MBS2) + ikzf1 CDS: 2.51 ± 0.34, n = 3; ccr9a promoter (MBS1 + MBS2) + ikzf1 CDS: 1.79 ± 0.04, n = 3). EV, pCS2 empty vector. E and F, WISH of tcrb and rag1 (E), and cmyb (F) in ccr9a<sup>Δ10/Δ10</sup> mutant larvae and their siblings. H, calculation of spherical coro1a-Kaede<sup>+</sup> cells entering the thymus per hour (from 60–72 hpf; mean ± S.D.; Sib: 6.03 ± 0.15, n = 6; ccr9a<sup>Δ10/Δ10</sup>: 0.09 ± 0.06, n = 5). See also Movies S3 and S4. I, fluorescence images indicating labeled coro1a-Kaede<sup>+</sup> cells in thymi of ccr9a<sup>Δ10/Δ10</sup> mutant embryos and their siblings. J, calculation results of I (mean ± S.D.; Sib: 11.27 ± 5.06, n = 26; ccr9a<sup>Δ10/Δ10</sup>: 3.56 ± 2.04, n = 18). The blue arrowheads indicate the WISH signals (A and F). The red (E and F) and white (G and I) circles represent the thymus. The values in the bottom right corner in A, E, F, and I indicate counts with a typical appearance as presented (first number) in the total number of examined samples (last number). OV, otic vesicle. Scale bars = 50 μm. ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 4. (A–J) Defective thymus migration of hematopoietic progenitors in ccr9a mutant embryos.
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A. fluorescence images indicating CD41-GFP+ cells in thymi of ikzf1Δ4 + 3/Δ4 + 3, Tg(coro1a:ccr9a) mutant embryos and their siblings. B. calculation results of A (mean ± S.D; Sib: 21.57 ± 5.97, n = 14; ikzf1Δ4 + 3/Δ4 + 3, 0, n = 10; Tg(coro1a:ccr9a): 22.29 ± 6.75, n = 17; ikzf1Δ4 + 3/Δ4 + 3, Tg(coro1a:ccr9a): 10.5 ± 3.1, n = 16). C–E, WISH of cmyb (C), rag1 (D), and tcrb (E) in thymi of ikzf1Δ4 + 3/Δ4 + 3 mutant animals and their siblings upon replenishment of ccr9a. The white (A) and red (C–E) circles indicate the thymi. The values in the bottom right corner in A and C–E indicate counts with a typical appearance as presented (first number) in the total number of examined samples (last number). Scale bars = 50 μm, ns, not significant; *** p < 0.001.

of various hematopoietic lineage markers (ikzf1, irf4a, mpx, gata1, and mpl) in Tg(coro1a:ccr9a) (Fig. S6C). Restoration of ccr9a in ikzf1Δ4 + 3/Δ4 + 3 mutant hematopoietic cells efficiently promoted colonization of CD41-GFP+, even lyz-DsRed+, cells in the thymic anlage (Fig. 5, A and B, and Fig. S6 and Movies S5–S7). These recolonizing CD41-GFP+ cells should act as hematopoietic progenitors, as cmyb+ and lyz+ but not mpl+ signals were detected in the same situation (Fig. 5C and Fig. S6, D and E). However, they failed to differentiate into rag1+ and tcrb+ T cells in the thymus of ikzf1Δ4 + 3/Δ4 + 3, Tg(coro1a:ccr9a) mutants (Fig. 5, D and E). Thus, ccr9a was critical in the initial settlement of HSPCs into the thymus but played a limited role in further differentiation, suggesting the requirement for additional factors.

Homing of hematopoietic progenitors was disrupted in irf4aΔ18/Δ18 mutants

Ir4a, a member of the interferon regulatory factor family that is essential in zebrafish T lymphoid determination (40), was then examined. Similar to ccr9a, the expression of irf4a was barely detected in ikzf1Δ4 + 3/Δ4 + 3 mutants (Fig. 6A). The data from ChIP and Dual-Luciferase reporter experiments showed that irf4a was directly regulated by Ikzf1 (Fig. 6, B–E), suggesting that irf4a may be the candidate factor. To this end, an irf4aΔ18/Δ18 mutant line (harbor ing an 18-bp deletion) was created (Fig. S7, A and B). This mutation resulted in removal of six amino acids from the conserved DNA-binding domain of the protein (48) and, consequently, in a drastic reduction in Irf4a protein level (Fig. S7, C and D). Neither cmyb+, ikzf1+, or ccr9a+ cells in the CHT (Fig. S7, E and F) nor thymic structure/chemokines were affected by the irf4a mutation (Fig. S7G). However, most of the irf4aΔ18/Δ18 larvae exhibited complete loss of ccr9a− and ikzf1− cells in the thymus at 3 dpf, and the remaining larvae only showed a trace amount of these cells, which suggests that early T cell development was interrupted (Fig. 6, F and G, and Fig. S7H). Intriguingly, the T cell number partially recovered in irf4aΔ18/Δ18 mutants as the embryos grew. At 6 dpf, only a small group of irf4aΔ18/Δ18 larvae showed complete lack of T cells, whereas a large proportion of the mutants displayed relatively smaller but detectable T cell foci in the thymus (Fig. 6, F and G, and Fig. S7H). The normal population of hematopoietic progenitors and the gradual recovery of T cells in the irf4aΔ18/Δ18 mutants suggest that the T cell deficiency was probably caused by inefficient homing but was not due to the cell fate alteration (40) (Fig. S7I), as found in the ikzf1Δ4 + 3/Δ4 + 3 and ccr9a mutants. Indeed, time-lapse imaging revealed the presence of only a limited number of spherical coro1a-DsRed+lyz-GFP− cells in the irf4aΔ18/Δ18 thymus, in contrast with that of the WT control (Fig. 6, F and J, and Movies S1 and S8). Furthermore, transient tracing of the coro1a-Kaede+ cells in the CHT (somites 15–17) showed that the marked cells presented notably compromised movement to the thymus of irf4aΔ18/Δ18 mutants relative to that in the siblings (Fig. 6, J and K), recapitulating what occurred in the ikzf1 and ccr9a mutants. Collectively, these data suggested that the progenitors in the irf4aΔ18/Δ18 mutants, as with those in the ikzf1 and ccr9a mutants, were compromised by a thymus homing blockade.

Supplementation of irf4a drastically promoted HSPC proliferation and T cell differentiation in ikzf1Δ4 + 3/Δ4 + 3, Tg(coro1a:ccr9a) mutants

The irf4a mutant data prompted us to continue irf4a supplementation in ikzf1 mutants. The Tg(coro1a:irf4a) transgenic line (Fig. S8, A–C) partially promoted the production of rag1+ and lck+ cells (~36%) in ikzf1Δ4 + 3/Δ4 + 3 mutants (Fig. 7, A–D). We detected an increase in cmyb+ cells, which were achieved by the recovered cell proliferation, as indicated by the EdU incorporation assay (Fig. S8, F and G), in the CHT of ikzf1Δ4 + 3/Δ4 + 3, Tg(coro1a:irf4a) compared with ikzf1Δ4 + 3/Δ4 + 3 mutants (Fig. S8D). These rescued cmyb+ cells had lymphoid potential. They expressed high levels of the lymphoid (ccr9a) gene but limited myeloid (lyz) marker (Fig. S8E). However, achieving irf4a function in T lymphopoiesis partially required ccr9a-mediated thymic migration of HSPCs, as the rescue efficiency obviously reduced (below 20%) when ccr9a was mutated in ikzf1Δ4 + 3/Δ4 + 3; Tg(coro1a:irf4a) animals (Fig. 7, A and B). These data highlighted the roles of ccr9 in HSPC...
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**Figure 6.** Impaired thymus homing of the hematopoietic progenitors in irf4a<sup>−/−</sup><sup>Δ18/Δ18</sup> mutant larvae. A, WISH of irf4a in the CHT of ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutant embryos and their siblings. B-D, ChIP-PCR (C) and ChIP-qPCR (D) (mean ± S.D.; irf4a-1F/1R; IgG: 1.00 ± 0.05, anti-HA: 23.34 ± 1.68; irf4a-CF/CR; IgG: 1.00 ± 0.30, anti-HA: 0.57 ± 0.13) analysis of the binding site (green) in the irf4a promoter (B) for Ikzf1. CF, control forward primers; CR, control reverse primers. E, luciferase assay showing irf4a promoter (with the BS and MBS) activity when regulated by the Ikzf1 protein (full-length CDS) (mean ± S.D.; Ikzf1-BS: ikzf1 mRNA, 2.55 ± 1.35, n = 12; ctrl: 1.00 ± 0.19; Ikzf1-MBS: ikzf1 mRNA, 1.00 ± 0.38, n = 11; ctrl: 1.00 ± 0.22, n = 10). See also Movies S1 and S8.

**migration and the crucial function of irf4a in HSPC proliferation** and T cell differentiation. Supporting this assumption, supplementation with both irf4a and ccr9a in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants effectively recovered great numbers of rag1<sup>+</sup> and lck<sup>+</sup> cell foci (~65%) in the thymus compared with irf4a alone (~36%) (Fig. 7C and D). As a previous study suggested that ccr9a was directly regulated by Irf4a (40), Ikzf1 and Irf4a probably functioned together to regulate ccr9a activity. This prediction was validated by the results from the coimmunoprecipitation and Dual-Luciferase reporter experiments, which indicated that GFP-Ikzf1 or Ikzf1-GFP interacted with HA-Irf4a (Fig. 7E) and that cotransfection of both factors elevated the transcriptional activity of ccr9a more obviously than transfection with either one (although Ikzf1 played a major role) (Fig. 7F). Taken together, Ikzf1 regulated the thymic migration, proliferation, and differentiation of HSPCs via ccr9a and irf4a. These factors worked closely together to accomplish T lymphopoiesis in embryonic zebrafish (Fig. 7G).

**T lymphopoiesis was impaired in adult ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> zebrafish.**

The survival of ikzf1<sup>Δ4+4958</sup> (30) led us to examine whether ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants survive to adulthood. The homozy-
in a reported zebrafish mutant (30), the newly created \textit{ikzf1} null alleles in this study presented a drastic reduction of T cells in the adult stage as well as diminished hematopoietic progenitors with impaired proliferation. The difference was ascribed to the more significant impairment of Ikzf1 function in mutants that lacked functional domains compared with previous mutants (30, 31) that carried a point mutation in the sequence encoding the C-terminal protein interaction domain. The mutants were not considered a dominant-negative mutation because no overt hematopoietic phenotype was detected in heterozygous mutant embryos.

T cells were completely absent in \textit{ikzf1} mutant zebrafish at the embryonic stage as a result of impaired HSPC thymus homing, proliferation, and differentiation. However, it is challenging to define whether the compromised homing of HSPCs was the primary cause of T cell loss or whether this simply reflected a defect in the specification or commitment of lymphoid cells in \textit{ikzf1} mutants. Although \textit{ikzf1} has been recognized previously

**Discussion**

In addition to the compromised embryonic T lymphopoiesis that recovered at later stages and persisted for over 17 months in \textit{ikzf1} mutants in the background of \textit{Tg\(\text{coro1a:DsRed}\)} (39) was selected for rearing based on a lack of \textit{coro1a-DsRed} cells in the thymus. The \textit{ikzf1} mutant presented higher mortality from 9 dpf onward compared with the controls (Fig. S9A). Approximately 45% of the \textit{ikzf1} mutants survived to 2.5 months, and no \textit{ikzf1} mutants lived beyond 6 months (Fig. S9A). The \textit{ikzf1} mutants that survived were considerably smaller than their siblings (Fig. S9B) and were less fertile. As in the larval stage, fewer T cells were detected in the thymus of adult \textit{ikzf1} mutants and the thymus was substantially smaller in the mutants than in their siblings (Fig. S9C). Therefore, T cell numbers could recover but were still reduced in adult \textit{ikzf1} mutants.
as a lymphoid progenitor marker (13), our study indicated that ikzf1 was also expressed in hematopoietic progenitors for multiple lineages and plays a critical role in their proliferation. Consistently, ikzf1 was seen to be highly expressed in mouse KTLS + long-term HSCs, and ikzf1<sup>Plastic/Plastic</sup> mutant mice initially generated long-term HSCs but quickly failed to maintain and expand this pool at the embryonic stage (49). Similar HSPC phenotypes were observed in our ikzf1 mutant zebrafish, suggesting a functionally conserved role of Ikzf1 as a self-renewing regulator in HSC maintenance. However, it is difficult to exactly identify which HSPC populations were affected in ikzf1 mutant zebrafish because of the shortage of specific markers for different subtypes in zebrafish HSPCs.

Ccr9 was essential in the thymic seeding of hematopoietic progenitors in mice (43–45). One orthologue of ccr9, ccr9a, was absent in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutant larvae. The obvious impairment of hematopoietic progenitor migration was seen in ccr9a mutants. Restoration of ccr9a in ikzf1 mutants efficiently promoted thymic migration of HSPCs but resulted in failed differentiation, implying that thymic migration of HSPCs was necessary but not sufficient for T cell development. Irf4a was another critical factor downstream of ikzf1 in T lymphopoiesis. Irf4a has been reported to regulate lymphoid versus myeloid determination in zebrafish (40). In this study, irf4a expression was lost, and continual replenishment of irf4a only partially rescued the T cell deficiency in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants by promoting both CHT expansion and ccr9a-mediated thymic seeding of HSPCs, implying a requirement for additional factors and more complicated regulatory networks in this process.

Recently, IRF4 was reported to cooperate with Ikzf1 and serve as a transcriptional complex to target the zinc finger–IRF composite elements (ZICEs) that appeared in the promoters of genes that functioned essentially during plasma cell differentiation in mice (50). This report suggested the possibility that irf4a, when turned on by Ikzf1 in HSPCs with lymphoid potential, functioned together with Ikzf1 to further guide lymphoid differentiation in zebrafish. However, unlike the suppressive roles of the Ikzf1–IRF4 complex on the ZICE motif in mice (50), zebrafish Ikzf1 interacted with Irf4a and more effectively promoted ccr9a expression working together than singly. This difference was probably caused by the fact that there was no ZICE element in the ccr9a promoter. A previous study revealed the Ets-IRF and AP-1–IRF composite elements in the ccr9a promoter (40), which were located adjacent to the ikzf1 binding sites. The highest transcriptional activity of Ikzf1/Irf4a in the ccr9a promoter, like the Ikzf1–IRF4 complex, acted as a transcriptional activator in the presence of E26 transformation–specific family members such as PU.1 (50), suggesting the involvement of other factors. This process should be a focus of future investigation.

Supplementation with both ccr9 and irf4 in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants restored thymic T cell phenotypes more efficiently than either one alone. Therefore, we propose that, during zebrafish embryonic T lymphopoiesis, ikzf1 activates irf4a expression to protect the progenitor pool and assure their lymphoid potential. Then ikzf1 works together with irf4a to produce sufficient amounts of ccr9a that efficiently accomplish thymic seeding of hematopoietic progenitors, where T cell differentiation is finally achieved. However, there was still approximately 30% T cell failure in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants supplied with both ccr9a and irf4a. One possibility for this phenomenon was that the ccr9a and irf4a levels, even in transgenic zebrafish lines, were insufficient for final thymic T cell formation. Concordant with this hypothesis, neither a transient supply of irf4a and ccr9a mRNA nor the weak transgenic Tg(coro1a:irf4a) allele rescued thymic T cells in ikzf1<sup>Δ4 + 3/Δ4 + 3</sup> mutants (data not shown). In addition, it remains possible that other factors were involved. Transcription factors such as Bcl11b, E2A, and Hes1 are crucial for T cell commitment (2). The P-selectin/PSGL-1 axis plays a role in recruiting progenitors to the thymus (2, 51, 52), and CCR9, CCR7, and CXCR4 chemokine receptors function together to support cells found in the thymus (43–45). Therefore, achieving thymic T cell development under physiological conditions depends on the coordinated actions of an ensemble of transcriptional factors, cytokines, adhesion molecules, and chemokine receptors. Overall, our findings extend the mechanistic understanding of embryonic T lymphopoiesis in zebrafish and are valuable for further elucidation of Ikzf1 function in hematopoiesis.

**Experimental procedures**

**Fish lines**

The AB, Tg(CD41:GFP) (37), Tg(runx1:GFP) (32), Tg(coro1a:DsRed)/Tg(coro1a:Kaede) (39), Tg(cmyb:GFP) (33), Tg(lz:GFP-P)nz117/Tg(lz:DsRed) (53), Tg(mpx-GFP)iil4 (35), Tg(rag2:DsRed) (34), Tg(gata1-DsRed) (36), and Tg(kdr1:Dendra2) strains were used. All zebrafish were maintained according to the guidelines of experimental animal welfare from the Ministry of Science and Technology of the People’s Republic of China (2006).

**Generation of mutants and transgenic lines**

The ikzf1 and ccr9a mutants were created by the CRISPR/Cas9 system (54). The in vitro synthesized guide RNA and hCas9 mRNA were injected into one-cell WT embryos. The irf4a mutants were obtained using transcription activator-like effector nucleases (55). Constructs targeting 5’–CCTGCTCTTGGACGACGTcagcttgttattttAtAGGGACATTACAGGG–3’ were created. The mutants were identified by sequencing or restriction enzymes. To generate Tg(coro1a:HA-ikzf1), Tg(coro1a: ccr9a), and Tg(coro1a:irf4a), their complementary DNA was amplified with specific primers (Table S1) and constructed into the p Tol2 vector with the coro1a promoter (39). To facilitate screening, a Cryaa-Cerulane-BGHPA element (56) was reversely inserted. The constructs were injected into one-cell WT embryos. The transgenic lines were identified based on both the fluorescent signals in the eyes and a similar ikzf1/ccr9a/irf4a expression pattern as that of coro1a.

**Real-time qPCR**

Approximately 250 GFP<sup>+</sup> and DsRed<sup>+</sup> cells in Tg(CD41:GFP), Tg(mpx-GFP), Tg(gata1:DsRed), and Tg(rag2:DsRed) were sorted out by using a MoFlo XDP fluorescence-activated cell sorter (Beckman) according to the standard protocol. The harvested cells were dissociated to extract RNA by using a Qia-
gen REPlI-g WTA Single Cell Kit (150063) according to the manufacturer’s protocols. Total RNA of the whole embryos was extracted using TRIzol reagent. The amplified mRNA was used for qPCR. Each sample was tested in triplicate. sep15 (selenoprotein F precursor 15) (57), β-actin, and ef1a (58) expression was measured and used to normalize signals for each queried transcript by using the ΔΔCt method. The primers of cell cycle–related genes (58) and other genes are listed in Table S2.

ChIP

For the ChIP assay, the tails of 300 Tg(coro1a:HA-ikzf1) embryos were collected at 3 dpf. After treatment with lysis buffer, the suspension was fragmented by 0.5 units of MNase (37 °C for 45 s) to generate 300–1000 fragments. Cross-linked chromatin was immunoprecipitated with anti-HA antibody (Abcam, ab91110) or anti-IgG antibody (Sigma, A6154, negative control), according to the procedure in Ref. 46. The resultant immunoprecipitation samples were subjected to semi-quantitative PCR or qPCR using primers (Table S1).

Reporter assay

The luciferase assays were performed with theDual-Luciferase Reporter Assay System. The luciferase activity was measured with a GloMax® 20/20 luminometer (Promega) according to the manufacturer’s instructions. The full length of ccr9a promoter, 200 ng of pGL3-ccr9a according to the manufacturer’s instructions. To study the TGTTTAAG-3′/H11032 CACCATTG-3′/H11032 TGAGGAGGCACA-3′/H11032 CDS amplified with primers 5′-CCATCGATATGAACTTAGATGGGGACTG-3′/5′-GCTCTAGATCACTCTGTCAGGTGTTGTA-3′ were cloned into the pCS2 vector. The 2.8-kb ccr9a promoter was amplified with the primers (Table S1) and cloned into the pGL3-basic vector (Promega). The Ikzf1-binding sites were mutated by site-directed mutagenesis using the designed site-specific oligonucleotides primers 5′-GAAAGATCGGAAAAGACCTCTGATGACTGGTCCGACGAAG-3′/5′-CTT-GCTGGCACCCGAGTTCATGCGTCTTTTGGCAGATCCTTC-3′ for MBST (mutant Ikzf1 binding site 1) and 5′-CTTAAACAAAAACCCCACTCAACTCAACCTACTTCTGT-3′/5′-ACTAAGTAAAGGTGGTGATGTTGGGTGGTGTGGTGAAG-3′ for MBST2 (mutant Ikzf1 binding site 2) according to the manufacturer’s instructions. To study the ccr9a promoter regulated by Ikzf1, Irf4a or both, the plasmids (200 ng of pGL3-ccr9a promoter, 200 ng of pGL3-ccr9a promoter-MBS1, 200 ng of pGL3-ccr9a promoter-MBS2, 200 ng of pGL3-ccr9a promoter-MBS1-MBS2, 200 ng of pCS2-ikzf1 CDS, 200 ng of pCS2-irf4a CDS, 200 ng of pCS2-ikzf1-GFP CDS, and 10 ng of pRL-CMV) were transfected into HEK293T cells using Lipofectamine™ with different combinations. After 36 h, cells were harvested and proteins were purified using radioimmune precipitation assay buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, 0.5% NP40, and Roche protease inhibitor mixture). Then proteins were mixed with anti-GFP–agarose beads (Smart Life Sciences, SM03801) for 2–3 h at 4 °C. The beads were collected to perform Western blotting using goat anti-GFP antibody (Abcam, ab6658, 1:2000) and rabbit anti-HA (Santa Cruz Biotechnology, sc-805, 1:200). Detection was performed with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (31430 and 32260, Thermo, 1:5000) and ECL Plus reagent.

Histological analysis, WISH, immunostaining, double staining, TUNEL staining, and EdU incorporation

H&E staining was performed on 10-μm paraffin sections as described previously (60). After H&E staining, images were taken under an Axio Imager.Z2 Vario (Carl Zeiss). Antisense RNA probes and WISH were performed using a standard protocol (61). Single-color FISH and double staining were performed as described previously (38). TUNEL staining was performed according to the manufacturer’s instruction or as reported previously (62). For the EdU incorporation assay, the Click-iT® EdU Imaging Kit (Invitrogen, C10340) was used. Zebrfish larvae at different developmental stages were injected intravenously with 10 mM EdU and incubated for 2 h. The subsequent experiments followed the standard protocol. Goat anti-GFP antibody (Abcam, ab6658, 1:250, 4 °C, overnight) and
Alexa Fluor 488 donkey anti-goat secondary antibody (Invitrogen, 1:400, 4 °C, overnight) were used successively to visualize the signals. The WISH signals were observed under a SteREO Discovery.V20 microscope, and the fluorescence signals were imaged under an LSM700 confocal microscope (Carl Zeiss).

**Time-lapse live imaging and transient lineage tracing**

The fish embryos were anesthetized, mounted in 1% agarose, and subsequently imaged under an LSM700 confocal microscope (Carl Zeiss) with a ×20 objective. For live imaging, the targeted cells (DsRed−*, GFP+, or Kaede−* cells) located around the thymi were imaged and calculated. To achieve transient tracing, cells in the targeted regions of Tg(coro1a:Kaede) and Tg(kdrl:Dendra2) embryos (somites 8–10 in the VDA and 15–17 in the CHT) were selected and stimulated using the ROI mode via a 405-nm laser for around 15–20 s. The labeled cells were revealed and calculated 1–2 days later in the CHT, kidney, and thymus regions.

**Quantification, calculation, and statistical methods**

The positive signals in larval CHT were manually scored and double-confirmed blindly. All quantified data (mean ± S.D.) were analyzed by GraphPad Prism 6. Student’s t test (one-tailed) was used.

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**References**

1. De Obaldia, M. E., and Bhandoola, A. (2015) Transcriptional regulation of innate and adaptive lymphocyte lineages. Annu. Rev. Immunol. 33, 607–642 CrossRef Medline

2. Yang, Q., Jeremiah Bell, J., and Bhandoola, A. (2010) T-cell lineage determination. Immunol. Rev. 238, 12–22 CrossRef Medline

3. Kondo, M., Weissman, I. L., and Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91, 661–672 CrossRef Medline

4. Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O. J., Thoren, L. A., Anderson, K., Sittnicka, E., Sasaki, Y., Sigvardsson, M., and Jacobsen, S. E. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythroid-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121, 295–306 CrossRef Medline

5. Love, P. E., and Bhandoola, A. (2011) Signal integration and crosstalk during thymocyte migration and emigration. Nat. Rev. Immunol. 11, 469–477 CrossRef Medline

6. Bertrand, J. Y., Chi, N. C., Santos, B., Teng, S., Stainier, D. Y., and Traver, D. (2010) Haematopoietic stem cells derive directly from aortic endothelium during development. Nature 464, 108–111 CrossRef Medline

7. Kissa, K., and Herbomel, P. (2010) Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature 464, 112–115 CrossRef Medline

8. Lam, E. Y., Hall, C. J., Crosier, P. S., Crosier, K. E., and Flores, M. V. (2010) Live imaging of Runx1 expression in the dorsal aorta tracks the emergence of blood progenitors from endothelial cells. Blood 116, 909–914 CrossRef Medline

9. Jin, H., Xu, J., and Wen, Z. (2007) Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. Blood 109, 5208–5214 CrossRef Medline

10. Kissa, K., Murayama, E., Zapata, A., Cortés, A., Perret, E., Machu, C., and Herbomel, P. (2008) Live imaging of emerging hematopoietic stem cells and early thymus colonization. Blood 111, 1147–1156 Medline

11. Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H. F., Handin, R. I., and Herbomel, P. (2006) Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. Immunity 25, 963–975 CrossRef Medline

12. Li, D., Xue, W., Li, M., Dong, M., Wang, J., Wang, X., Li, X., Chen, K., Zhang, W., Wu, S., Zhang, Y., Gao, L., Chen, Y., Chen, J., Zhou, B. O., et al. (2018) VCAM-1+ macrophages guide the homing of HSPCs to a vascular niche. Nature 564, 119–124 CrossRef Medline

13. Hess, I., and Boehm, T. (2012) Intravital imaging of thymopoiesis reveals dynamic lympho-epithelial interactions. Immunity 36, 298–309 CrossRef Medline

14. Georgopoulos, K., Moore, D. D., and Derfler, B. (1992) Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science 258, 808–812 CrossRef Medline

15. Molnár, A., and Georgopoulos, K. (1994) The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. Mol. Cell Biol. 14, 8292–8303 CrossRef Medline

16. Sun, L., Liu, A., and Georgopoulos, K. (1996) Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. EMBO J. 15, 5358–5369 CrossRef Medline

17. Klug, C. A., Morrison, S. J., Masek, M., Hahn, K., Smale, S. T., and Weissman, I. L. (1998) Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms, and Ikaros is localized to heterochromatin in immature lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 95, 657–662 CrossRef Medline

18. Payne, K. J., Huang, G., Sahakian, E., Zhu, J. Y., Barteneva, N. S., Barsky, L. W., Payne, M. A., and Crooks, G. M. (2003) Ikaros isoform x is selectively expressed in myeloid differentiation. J. Immunol. 170, 3091–3098 CrossRef Medline

19. Georgopoulos, K., Bigby, M., Wang, J. H., Molnár, A., Wu, P., Winandy, S., and Sharpe, A. (1994) The Ikaros gene is required for the development of all lymphoid lineages. Cell 79, 143–156 CrossRef Medline

20. Winandy, S., Wu, P., and Georgopoulos, K. (1995) A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. Cell 83, 289–299 CrossRef Medline

21. Wang, J. H., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A. H., Bigby, M., and Georgopoulos, K. (1996) Selective defects in the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity 5, 537–549 CrossRef Medline

22. Nichogiannopoulou, A., Trevisan, M., Neben, S., Friedrich, C., and Georgopoulos, K. (1999) Defects in hemopoietic stem cell activity in Ikaros mutant mice. J. Exp. Med. 190, 1201–1214 CrossRef Medline

23. Lopez, R. A., Schoetz, S., DeAngelis, K., O’Neill, D., and Bank, A. (2002) Multiple hematopoietic defects and delayed globin switching in Ikaros null mice. Proc. Natl. Acad. Sci. U.S.A. 99, 602–607 CrossRef Medline

24. Dumortier, A., Kirstetter, P., Kastner, P., and Chan, S. (2003) Ikaros regulates neutrophil differentiation. Blood 101, 2219–2226 CrossRef Medline

25. Papathanasiou, P., Perkins, A. C., Cobb, S. B., Ferrini, R., Sridharan, R., Hoyne, G. F., Nelms, K. A., Smale, S. T., and Goodnow, C. C. (2003) Widespread failure of hematopoietic differentiation caused by a recessive niche-filling allele of the Ikaros transcription factor. Immunity 19, 131–144 CrossRef Medline

26. Rao, K. N., Smuda, C., Gregory, G. D., Min, B., and Brown, M. A. (2013) Ikaros limits basophil development by suppressing C_EBP-α expression. Blood 122, 2572–2581 CrossRef Medline

27. Zhang, J., Jackson, A. F., Naito, T., Dose, M., Seavit, J., Liu, F., Effer, E. J., Kashiwagi, M., Yoshida, T., Gounari, F., Petrie, H. T., and Georgopoulos, K. (2011) Harnessing of the nucleosome-remodeling-deacetylase complex
controls lymphocyte development and prevents leukemogenesis. *Nat. Immunol.* **13**, 86–94 CrossRef Medline
28. Morgan, B., Sun, L., Aviathl, N., Andrikopoulos, K., Ikeda, T., Gonzales, E., Wu, P., Neben, S., and Georgopoulos, K. (1997) Aiolos, a lymphoid-restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J.* **16**, 2004–2013 CrossRef Medline
29. Hahn, K., Cobb, B. S., McCarty, A. S., Brown, K. E., Klug, C. A., Lee, R., Akashi, K., Weissman, I. L., Fisher, A. G., and Smale, S. T. (1998) Helios, a T cell-restricted Ikaros family member that quantitatively associates with Ikaros at centromeric heterochromatin. *Genes Dev.* **12**, 782–796 CrossRef Medline
30. Schorpp, M., Bialecki, M., Diekhoff, D., Walderich, B., Odenthal, J., Maischein, H. M., Zapata, A. G., and Boehm, T. (2006) Conserved functions of Ikaros in vertebrate lymphocyte development: genetic evidence for distinct larval and adult phases of T cell development and two lineages of B cells in zebrafish. *J. Immunol.* **177**, 2463–2476 CrossRef Medline
31. Iwanami, N., Sikora, K., Richter, A. S., Mönnich, M., Guerr, L., Soza-Ried, C., Lawir, D. F., Mateos, F., Hess, I., O’Meara, C. P., Schorpp, M., and Boehm, T. (2016) Forward genetic screens in zebrafish identify pre-mRNA-processing pathways regulating early T cell development. *Cell Rep.* **17**, 2259–2270 CrossRef Medline
32. He, Q., Zhang, C., Wang, L., Zhang, P., Ma, D., Lv, J., and Liu, F. (2015) Inflammatory signaling regulates hematopoietic stem and progenitor cell emergence in vertebrates. *Blood* **125**, 1098–1106 CrossRef Medline
33. North, T. E., Guo, S., Walkley, C. R., Lengerke, C., Kopani, K. R., Lord, A. M., Weber, G. J., Bowman, T. V., Jang, I. H., Grosser, T., Fitzgerald, G. A., Daley, G. Q., Okrin, S. H., and Zon, L. I. (2007) Prostaglandin E2 regulates vertebrate haematopoetic stem cell homeostasis. *Nature* **447**, 1007–1011 CrossRef Medline
34. Page, D. M., Wittamer, V., Bertrand, J. Y., Lewis, K. L., Pratt, D. N., Delgado, N., Schale, S. E., McGue, C., Jacobsen, B. H., Doty, A., Pao, Y., Yang, H., Chi, N. C., Magor, B. G., and Traver, D. (2013) An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood* **122**, e1–e11 CrossRef Medline
35. Renshaw, S. A., Loynes, C. A., Trushell, D. M., Elworthy, S., Ingham, P. W., Ald, G. A., Daley, G. Q., Orkin, S. H., and Zon, L. I. (2007) Prostaglandin E2 regulates hematopoietic stem and progenitor cell self-renewal of myeloproliferative neoplasm-like syndrome via Mertk signaling in zebrafish. *J. Immunol.* **177**, 991–1000 CrossRef Medline
36. Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S., and Lukyanov, K. A. (2006) Engineering of a monomeric green-to-red photoactivatable fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12651–12656 CrossRef Medline
37. Brass, A. L., Yan, B., Shi, Y. Q., Zhang, W. Q., and Wen, Z. L. (2012) Live imaging of multilineage engraftment in zebrafish embryos. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 10858–10863 CrossRef Medline
38. Zhang, W., Huang, Y., Zhang, Y., Li, Y., Sun, J., Zhang, Y., Wen, Z., Li, H., Huang, H., Ruan, H., Liao, L., and Li, X. (2013) Genome editing with RNA-guided Cas9 nuclease in zebrafish. *Science* **341**, 167–171 CrossRef Medline
39. Lui, C., Saito, F., Liu, Z., Lei, Y., Uehara, S., Love, P., Lipp, M., Kondo, S., Manley, N., and Takahama, Y. (2006) Coordination between CCGr7- and CCR9-mediated chemokine signals in prevascular fetal thymus coloniza-