Forward Genetic Screens in Zebrafish Identify Pre-mRNA-Processing Pathways Regulating Early T Cell Development

Graphical Abstract

Highlights

- Forward genetic screens identify mutations affecting zebrafish T cell development
- Identification of il7r, jak3, and pole1 mutations validates specificity of screen design
- Mutations in pre-mRNA-processing factor genes reveal evolutionarily conserved pathway
- In zebrafish and mice, TNPO3 deficiency impairs T cell differentiation

Authors
Norimasa Iwanami, Katarzyna Sikora, Andreas S. Richter, ..., Connor P. O’Meara, Michael Schorpp, Thomas Boehm

Correspondence
boehm@immunbio.mpg.de

In Brief
Using forward genetic screens in zebrafish, Iwanami et al. identify evolutionarily conserved functions in T cell development of transcription factors (such as ikzf1), signaling components (such as il7r), DNA replication/repair genes (such as pole1), and certain pre-mRNA-processing factor genes (such as tnpo3).

Accession Numbers
GSE77480

Iwanami et al., 2016, Cell Reports 17, 2259–2270
November 22, 2016 © 2016 The Author(s).
http://dx.doi.org/10.1016/j.celrep.2016.11.003
Forward Genetic Screens in Zebrafish Identify Pre-mRNA-Processing Pathways Regulating Early T Cell Development

Norimasa Iwanami,1 Katarzyna Sikora,1,2 Andreas S. Richter,2,3 Maren Mönich,1,4 Lucia Guerri,1,6 Cristian Soza-Ried,1,6 Divine-Fondzenyuy Lawir,1 Fernando Mateos,1 Isabel Hess,1 Connor P. O’Meara,1 Michael Schorpp,1 and Thomas Boehm1,7,*

1Department of Developmental Immunology
2Bioinformatics Unit
Max Planck Institute of Immunobiology and Epigenetics, Stuebeweg 51, 79108 Freiburg, Germany
3Present address: Genedata AG, 4053 Basel, Switzerland
4Present address: Wilhelm Johannsen Centre for Functional Genome Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen, Denmark
5Present address: Laboratory of Neurogenetics, NIAAA, NIH, 5625 Fishers Lane, Rockville, MD 20852-1728, USA
6Present address: Fundacion Oncoloop, Antonio Varas 710, Santiago and Universidad Andrés Bello, Facultad de Medicina, República 330, Santiago, Chile
7Lead Contact
*Correspondence: boehm@immunbio.mpg.de
http://dx.doi.org/10.1016/j.celrep.2016.11.003

SUMMARY

Lymphocytes represent basic components of vertebrate adaptive immune systems, suggesting the utility of non-mammalian models to define the molecular basis of their development and differentiation. Our forward genetic screens in zebrafish for recessive mutations affecting early T cell development revealed several major genetic pathways. The identification of lineage-specific transcription factors and specific components of cytokine signaling and DNA replication and/or repair pathways known from studies of immunocompromised mammals provided an evolutionary cross-validation of the screen design. Unexpectedly, however, genes encoding proteins required for pre-mRNA processing were enriched in the collection of mutants identified here. In both zebrafish and mice, deficiency of the splice regulator TNPO3 impairs intrathymic T cell differentiation, illustrating the evolutionarily conserved and cell-type-specific functions of certain pre-mRNA-processing factors for T cell development.

INTRODUCTION

All vertebrates share the same basic principle of lymphocyte differentiation along distinct T-cell-like and B-cell-like lineages (Boehm, 2011; Hirano et al., 2013), suggesting that the genetic program regulating the developmental pathways of lymphocytes must have already existed in a common ancestor for all vertebrates. Taking advantage of the apparent evolutionary conservation of lymphocyte-based immunity, we conducted genetic screens in zebrafish aimed at identifying previously unknown regulators of T lymphocyte development. Zebrafish is particularly suited for such an analysis, as T cell development already begins in the embryo during the third day after fertilization (Lanegenau and Zon, 2005). During these early stages of embryonic development, maternal factors are expected to buffer, at least partially, the phenotypic consequences of zygotic defects in mutant fish unless particular cell types (T cells in the present case) exhibit a specific requirement for the unimpaired activity of a certain gene(s). We therefore expected that this unique biological feature of T cell development in zebrafish would allow us to identify cell-type-specific functions of genes that are also required for stages of development prior to the onset of lymphopoiesis. A similar approach would not be feasible in mammals, because their lymphocytes develop at a considerably later point in embryogenesis; as a consequence, in mice, for instance, embryonic lethality often masks subsequent lineage-specific functions of pleiotropically acting genes, for example Gata3 (Pandolfi et al., 1995).

In the mouse, specific networks of transcription factors have been shown to regulate the three major phases of T cell development. In the initial phase, T cell progenitors are generated and recruited to the thymus; subsequently, they are induced to adopt a T cell fate; finally, they become responsive to signals emanating from the T cell receptor (Yui and Rothenberg, 2014). Hence, assuming that these regulatory circuits emerged at an early stage in vertebrate evolution, a comprehensive genetic screen of T cell development in zebrafish would be predicted to identify at least some of the factors governing these three phases. In keeping with this expectation, we identified mutations in genes encoding lymphoid lineage-specific transcription factors, and components of cytokine signaling and DNA replication/repair pathways. Quite unexpectedly, however, pre-mRNA-processing factors were also found to play a specific role in T cell development. Using genetic interaction analysis and transcriptome profiling, we established a functional network of certain
components of the pre-mRNA splicing machinery and demonstrated that the role of this network for T cell development is evolutionarily conserved.

RESULTS

Outcome of Forward Genetic Screens in Zebrafish

Two genetic screens were conducted in zebrafish to identify recessive mutations affecting T lymphocyte development (Boehm et al., 2003; Schorpp et al., 2006). To this end, the expression of *rag1* was determined at 5 days post-fertilization (dpf) by RNA in situ hybridization. The product of the *rag1* gene is essential for T cell receptor assembly in developing T cells in the thymus, the first site of lymphopoiesis in zebrafish embryos. Only mutant fish with no overt developmental abnormalities apart from impaired intrathymic T cell development were considered for further characterization. Together with the Tübingen 2000 screen consortium, we screened F₂ clutches of 4,584 F₂ families, representing 4,253 mutagenized haploid genomes; so far, 42 lines carrying recessive mutations affecting *rag1* expression levels could be established. The Freiburg gynogenetic screen of 281 genomes led to the establishment of three lines, all of which were found to harbor recessive mutations. Owing to the considerable efforts associated with isolating mutated genes by positional cloning, we conducted an interim analysis after the identification of more than one-third of affected genes. The results of this analysis are reported here.

The pertinent features of the first 15 complementation groups, for which the affected genes were identified by linkage analysis and positional cloning (in two cases, aided by whole-genome sequencing), are summarized in Table 1. The fact that, among the first 17 of the 45 mutant lines analyzed here, two genes (*ikzf1* and *top3a*) were represented by two distinct alleles each suggests that our screen was near saturation. According to their known functions, it was possible to group the affected genes into three categories: regulators of hematopoiesis and lymphopoiesis; regulators of DNA replication, repair, and cell cycle; and regulators of pre-mRNA processing.

**Regulators of Pre-mRNA Processing**

The products of the five genes in the third functional group (*snapp3* [small nuclear RNA-activating protein complex protein 3], *lsm8* [like-Sm protein 8], *gemin5* [gem nuclear organelle associated protein 5], *tnpo3* [transportin 3], and *cstf3* [cleavage stimulation factor subunit 3]) are implicated in pre-mRNA processing. The mutations are predicted to affect different aspects of this multi-layered process, such as transcription of small nuclear RNAs (snRNAs) (*snapp3*) (Hernandez, 2001), formation of small nuclear RNA-containing ribonucleoprotein (snRNP) splicing complexes (*gemin5*; *lsm8*) (Friesen and Dreyfuss, 2000).
2000), nuclear import of splice regulators (tnpo3) (Kataoka et al., 1999), and polyadenylation (cstf3) (Xiang et al., 2014). As our screen was focused on T cell development, the prevalence of this group of ubiquitously expressed genes was unexpected, particularly because, to the best of our knowledge, there is no precedence for their hematopoietic/lymphopoietic roles from studies in mammals. Previous biochemical studies indicated that the number of spliceosome-associated factors is in the order of 170 (Wahl et al., 2009), whereas the gene ontology term “RNA processing” is associated with about 500 genes. Assuming a coding capacity of the zebrafish genome of about 26,000 genes (Kettleborough et al., 2013), and using a conservative estimate that a total of 2,000 genes encode the various components of pre-mRNA-processing pathways, the degree of enrichment of genes in this functional category in our screen (5/15) is significant (hypergeometric test, p = 0.0036).

The unexpected prevalence of mutations in genes encoding components of the pre-mRNA-processing machinery in our screen prompted us to examine whether the cell-type-specific functions of the gene products of this group are mirrored in functional similarities, i.e., whether they belong to the same genetic network and whether their functions are evolutionarily conserved.

**snRNP Function and T Cell Development**

Despite the fact that the identified mutant alleles of lsm8, gemin5, and snapc3 most likely encode non-functional variants, larval development and hematopoietic development initially proceeded normally apart from a pronounced defect in T cell development (Figures S1–S3). We monitored the presence of developing T cells in the thymus of 5 dpf larvae by evaluating the signal emanating from rag1-expressing thymocytes in both thymic lobes; on a per-cell basis, rag1 expression levels are the same in all genotypes analyzed, allowing us to use the rag1 hybridization signal as a proxy for the number of rag1-expressing thymocytes. The signal emanating from growth hormone (gh) gene-expressing cells in the hypophysis was found to be unchanged in mutant fish and thus subsequently used as an internal standard (Figure 1A). The extent of T cell development was then expressed as the ratio and referred to as the thymopoietic index. The double-probe RNA in situ analyses indicate severe reductions of rag1 signals in the thymic lobes of homozygous lsm8, gemin5, and snapc3 mutants; heterozygotes exhibit no detectable decrease in the thymopoietic indices (Figures 1B–1D).

In contrast, other tissues in the developing larvae were much less affected. For example, in lsm8 and gemin5 mutants, the development of lymphoid precursors—visualized in the ikaros:eGFP transgenic background (Hess and Boehm, 2012)—is initially not affected (Figures 1E and 2A). However, by 5 dpf, fewer lymphoid precursors are present in the thymus—as identified by ikaros expression (Figures 1E and 2B), in line with the reduced rag1 signal (Figures 1B, 1C, and 2C). By 8 dpf, T cell development in gemin5−/− fish has completely ceased (Figure 2D). This defect is lymphocyte intrinsic, because the stromal microenvironment of the thymus appears to be normal.
The mutant thymic anlage expresses foxn1 (Figure 2E), the gene encoding the master regulator of thymic epithelial cell differentiation (Nehls et al., 1996), and is colonized normally by transplanted wild-type lymphoid precursors (Figure 2F). In contrast to impaired T cell development, several other features indicate that gemin5−/− embryos and larvae initially develop normally. For instance, development of different types of neuronal tissues appears undisturbed, including the hypophysis (exhibiting normal numbers of growth-hormone expressing cells [Figure 2C]), the hindbrain (visualized by ikaros-expressing neurons [Figure 2G]), and the retina (exhibiting the characteristic multilayered organization [Figure 2H]). Moreover, the swim bladder develops normally and the body size is indistinguishable from that of wild-type siblings [Figure 2I]). Collectively, these observations suggest a surprisingly tissue-restricted consequence of gemin5 mutation during early stages of zebrafish development, well beyond the stages during which maternally supplied protein and/or mRNA could be expected to compensate for the loss of zygotic gemin5 function. Likewise, fish mutant for lsm8 (Figures 1 and S1) and snapc3 (Figure S3) also exhibit tissue-restricted phenotypes.

Epistasis Analysis

In order to gain insight into the functional interrelationships between the three regulators of pre-mRNA processing identified here, epistasis analysis was performed. The SMN (survival of
motor neurons) complex (part of which is GEMIN5) contributes to the formation of U1, U2, U4, and U5 snRNPs, and to the formation of the spliceosomal U6 snRNP (containing the LSM2-8 protein ring) (Friesen and Dreyfuss, 2000). Hence, we tested whether genetic interaction was detectable between gemin5 and lsm8 mutations. Several features were notable. The phenotype of impaired intrathymic T cell development in gemin5−/− fish was considerably more severe when the fish originated from gemin5+/−; lsm8+/− double-heterozygous parents rather than from single heterozygous gemin5+/− (that is, gemin5−/−; lsm8+/−) parents; by contrast, the phenotype of lsm8−/− fish was not affected by parental heterozygosity of gemin5 (Figure 3A). The more severe phenotype of gemin5 mutants arising from lsm8 heterozygosity has a maternal origin (Figure 3B), suggesting that reduced levels of lsm8 mRNA and/or protein in the oocyte affect the function of gemin5 in the early stages of embryogenesis. By contrast, maternal contribution of gemin5 mRNA and/or protein appears to be less important than that of lsm8, at least with respect to T cell development, because the extent of T cell development in lsm8−/− fish was unaffected by parental gemin5 heterozygosity (Figure 3A). Impaired translation of maternal and zygotic lsm8 mRNA (using antisense oligonucleotides directed against the translational start site) phenocopies the genetic lsm8 defect and affects T cell development at later stages of development, whereas interference with splicing of zygotic lsm8 pre-mRNAs alone (using antisense oligonucleotides directed against the splice donor site of exon 3), has no effect on the thymopoietic index (Figures S4A and S4B). Collectively, these observations indicate that reduced levels of LSM8 in the early embryo have a long-lasting effect that becomes apparent at much later stages of development and specifically affects T cell differentiation. Of note, the effect of maternal heterozygosity of lsm8 was not observed in combination with a null mutation of the il7r gene, encoding a component of the il7 cytokine receptor (Figures S4C and S4D), which by itself also affects T cell development (Table 1) (Iwanami et al., 2011). Collectively, our observations support the notion that gemin5 and lsm8 function predominantly in separate pathways with redundant or complementary roles (Mani et al., 2008).

**Perturbed Splicing Patterns in snapc3 and lsm8 Mutants**

Next, we determined the effect of snapc3 and lsm8 mutations on the global splicing patterns in 4 dpf whole larvae using RNA sequencing (RNA-seq), as it is technically not feasible to isolate the few developing thymocytes for transcriptome analysis. The alterations of splicing patterns observed in both mutants are dominated by the occurrence of exon-skipping events (Figures 4A and 4B; Table S1). However, in lsm8 mutants, a significant number of genes (including tnpo3) were additionally affected by incomplete splicing events, resulting in the frequent occurrence of retained introns (Figure 4B; Table S2). Other types of splicing events, such as the aberrant usage of mutually exclusive exons and the use of alternative donor and acceptor sites, did not appreciably contribute to the global changes in either genotype (Figures 4A and 4B). Intriguingly, we observed a considerable degree of overlap between the genes that are affected by splicing aberrations in the two mutants (Figures 4C and 4D; Tables S1 and S2). Interestingly, in snapc3 mutant fish, skipping of the first coding exon of the gemin5 gene (ENSDARG00000053496; ENSDART00000142315; Table S1; inclusion levels, 0.86 ± 0.05 [mean ± SEM] for wild type; 0.46 ± 0.02 [mean ± SEM] for mutant; p = 0.002; t test, two-tailed) was detected, predicting the formation of an N-terminally truncated protein. Because GEMIN8 is, like GEMIN5, part of the thymopoietic index is very low; hence, it proved to be difficult to establish by analysis of compound mutants the presence and the direction (synthetic or alleviating) of genetic interactions between snapc3 and lsm8 mutations and snapc3 and gemin5 (data not shown).
SMN complex (Battle et al., 2006; Wahl et al., 2009), this observation supports the notion that \(\text{snapc}3\) and \(\text{gemin5}\) act in the same molecular pathway.

Changes in Gene Expression in \(\text{snapc}3\) and \(\text{Ism8}\) Mutants
To further address the functional consequences of the identified mutations, we also analyzed the transcriptomes of \(\text{snapc}3\) and \(\text{Ism8}\) mutants and their wild-type counterparts for changes in gene expression levels (≥2-fold; false discovery rate [FDR] < 5%) (Table S3). In \(\text{snapc}3\) mutants, 140 genes were significantly upregulated (Figure 4E), including 17 protein-coding genes with known functions in pre-mRNA splicing (Table S4). Most notable was the increase in expression levels for genes encoding components of the SM (\(\text{snrpa1}, \text{snrpd2}, \text{snrpd3}, \text{snrpe}, \text{snrpf}\)) and the LSM (\(\text{Lsm7}, \text{Lsm8}\)) heptameric complexes (Table S4). In \(\text{Ism8}\) mutants, the expression levels of 427 genes were increased (Figure 4E), including 35 protein-coding genes, the functions of which have been linked to pre-mRNA splicing (Table S4). As is the case in \(\text{snapc}3\) mutants, genes encoding components of...
Figure 5. Characterization of trnp03 Mutants, Related to Figure S5

(A) Reduction of ikaros-expressing cells in the thymus (encircled with dotted lines in middle and right panels) of homozygous trnp03 mutants (additionally transgenic for an ikaros:eGFP reporter). Note the normal numbers of ikaros-positive cells in general hematopoietic tissues at 1 dpf. Scale bars, 100 μm.

(B) Maturation block of thymocytes. Few ccr9b-positive thymocytes (insets in 4 dpf panels) in trnp03 mutants express rag1. Scale bars, 100 μm.

(C) Thymopoietic indices at 5 dpf. Each symbol represents one animal. The mean ± SEM is indicated. Scale bar, 100 μm.

(D) Analysis of ptprc isoforms by RT-PCR; size markers are indicated.

(E) Masking genetic interaction between gemin5 and trnp03 mutations in zebrafish. The thymopoietic indices (rag1/gh ratio; mean values ± SD) at 5 dpf are given for fish (genotypes denoted in bars) resulting from crosses of parents heterozygous for both gemin5 and trnp03; the observed thymopoietic index for (legend continued on next page)
the SM (snrpb, snrpd1, snrpd2, snrpd3, snrpe, snrpf) and LSM (lsm6, lsm7, lsm8) ring structures are upregulated (Table S4). Of the 58 genes upregulated in both snapc3 and lsm8 mutants, 16 (28%) encode splicing-related proteins (Figure 4F). This includes snapc4, encoding one component of the transcriptional activation complex of snRNA genes, and of genes (snrpd2, snrpd3, snrpe, snrpf, lsm7, lsm8) encoding 6 of the 14 components of the SM and LSM heptameric ring structures containing snRNAs (Table S4). In both lsm8 and snapc3 mutants, expression of the foxg1d gene is reduced (Table S3); the mouse homolog of this gene (Foxg1) is implicated in the regulation of thymic epithelial cell differentiation (Wei and Condie, 2011), possibly contributing to impaired T cell development.

We then tested the generality of the presumed transcriptional feedback regulation among components of snRNPs by examining transcriptional responses in gemin5 mutants, focusing on the expression levels of sm and lsm genes. In support of our hypothesis, the qPCR results indicate that snapc3, lsm8, and gemin5 mutations all result in strong upregulation of lsm7 and lsm8 mRNAs (Figure 4G), defining a core group of genes co-regulated by perturbation of snapc3, lsm8, and gemin5 functions. By contrast, expression levels of mRNAs of genes encoding the eight known GEMIN protein family members are largely unaffected in the three mutants (Table S5), supporting the notion of the specific nature of the transcriptional feedback regulation. Collectively, the present genetic interaction studies and transcriptome analyses suggest the presence of a network connecting snapc3, lsm8, and gemin5 genes encoding key components of snRNPs that appears to converge on the transcriptional regulation of lsm7 and lsm8 genes.

**tnpo3 as a Regulator of Early T Cell Development in Zebrafish**

TNPO3 has been shown to participate in the import of splice regulators to the nucleoplasm (Maertens et al., 2014). Hence, in contrast to the gene products of snapc3, lsm8, and gemin5, TNPO3 is predicted to indirectly affect pre-mRNA processing. In zebrafish tnpo3 mutant larvae, the number of lymphoid precursors in hematopoietic tissues is not affected at 1 dpf when definitive hematopoiesis has begun in zebrafish; at later stages of development, lymphoid progenitors are found in the thymus (albeit in somewhat reduced numbers), indicating that the homing process is largely unaffected by tnpo3 deficiency (Figures 5A and 5S). However, only few thymocytes in tnpo3 mutants express rag1 (Figures 5B and 5C), despite normal expression levels of a thymocyte marker, ccr9b (Figure 5B), suggesting that intrathymic defects contribute to impaired T cell development in tnpo3-deficient fish. At the time point of our analyses (5 dpf), only T cells are present in zebrafish larvae, because B cells develop considerably later in development (Danilova and Steiner, 2002). Hence, we reasoned that the splicing pattern of ptprc (encoding the zebrafish ortholog of the mammalian thymocyte maturation marker CD45) might provide further evidence for impaired intrathymic T cell differentiation in tnpo3-deficient larvae. Indeed, the mutant splicing pattern is dominated by lower molecular weight isoforms (Figure 5D) that are characteristically found in immature mouse thymocytes (Lefrancois and Goodman, 1987). At present, it is not possible to determine the cause-and-effect relationship of differential ptprc splicing in the absence of TNPO3 function. However, tnpo3-deficient zebrafish larvae exhibit aberrant splicing of the nck2b gene (Table S1), encoding the zebrafish homolog of the essential adaptor protein NCK required for proper TCR signaling in the mouse (Alarcon et al., 2003), suggesting that differential splicing of ptprc is an indirect effect of tnpo3 deficiency.

**Changes in Transcriptomes Common to snapc3, lsm8, and tnpo3 Mutants**

The presence of pervasive autoregulation affecting the processing of pre-mRNAs of splice regulator genes as a consequence of
mutations in snpac3, lsm8, and tnpo3 is suggested by our finding that among the 32 genes commonly affected by exon-skipping events (Figure 5G), two genes—srsf3a and ptbp3—encode known regulators of pre-mRNA processing; and of the four genes commonly affected by intron retention, two encode splice regulators (srsf1b, prpf39) (Table S2).

When we examined transcriptomes for changes common to all three mutants and potentially involved in T cell/thymus development, we noted that splicing of the dnmt4 (also known as dnmt3bb.1) gene, which is expressed in the hematopoietic lineage (Takayama et al., 2014), is similarly affected in all three mutants; the splice pattern suggests the presence of different compositions of the N-terminal non-catalytic domains of the predicted protein (Figure 5G; Table S6).

Many of the 34 downregulated genes (Figure 5I) are signature genes of the exocrine pancreas, identifying a second cell type exhibiting particular sensitivity to the malfunction of the pre-mRNA splicing regulators identified here. The lower expression levels of elastase, trypsin, amylase, and carboxypeptidase, etc. (ela3l, try, amy2a, cpa1) genes suggest that the downstream effects of snpac3, lsm8, and tnpo3 mutations converge on malfunction of the exocrine pancreas (Table S7). This conclusion is supported by the fact that in all three mutants, aberrant splicing of the NRSa2 transcription factor also occurs (Table S6), which is required for liver and exocrine pancreas development (Nissim et al., 2016). We also note that the gene encoding the brush-border membrane glycoprotein Trehalase (treh) is affected by intron retention in all three mutants, adding to impaired absorption of carbohydrates. Although more work is needed to assess the functional significance of these findings, we hypothesize that malfunction of the exocrine pancreas and the intestine at least partly underlies the phenotype of premature death in the mutant fish. These results also underscore the notion of cell-type-restricted functions of snpac3, lsm8, and tnpo3 genes.

mutations in snpac3, lsm8, and tnpo3 is suggested by our finding that among the 32 genes commonly affected by exon-skipping events (Figure 5G), two genes—srsf3a and ptbp3—encode known regulators of pre-mRNA processing; and of the four genes commonly affected by intron retention, two encode splice regulators (srsf1b, prpf39) (Table S2).

When we examined transcriptomes for changes common to all three mutants and potentially involved in T cell/thymus development, we noted that splicing of the dnmt4 (also known as dnmt3bb.1) gene, which is expressed in the hematopoietic lineage (Takayama et al., 2014), is similarly affected in all three mutants; the splice pattern suggests the presence of different compositions of the N-terminal non-catalytic domains of the predicted protein (Figure 5G; Table S6).

Many of the 34 downregulated genes (Figure 5I) are signature genes of the exocrine pancreas, identifying a second cell type exhibiting particular sensitivity to the malfunction of the pre-mRNA splicing regulators identified here. The lower expression levels of elastase, trypsin, amylase, and carboxypeptidase, etc. (ela3l, try, amy2a, cpa1) genes suggest that the downstream effects of snpac3, lsm8, and tnpo3 mutations converge on malfunction of the exocrine pancreas (Table S7). This conclusion is supported by the fact that in all three mutants, aberrant splicing of the NRSa2 transcription factor also occurs (Table S6), which is required for liver and exocrine pancreas development (Nissim et al., 2016). We also note that the gene encoding the brush-border membrane glycoprotein Trehalase (treh) is affected by intron retention in all three mutants, adding to impaired absorption of carbohydrates. Although more work is needed to assess the functional significance of these findings, we hypothesize that malfunction of the exocrine pancreas and the intestine at least partly underlies the phenotype of premature death in the mutant fish. These results also underscore the notion of cell-type-restricted functions of snpac3, lsm8, and tnpo3 genes.

the mutant phenotype to impaired TCR signaling. This finding is reminiscent of the features of impaired TCR signaling identified in the tnpo3-deficient zebrafish larvae. The loss of single-positive cells in mutant thymi does not appear to be due to increased apoptosis, because the fractions of TUNEL-positive and Annexin V-positive thymocytes remained unchanged (data not shown); these findings call for further mechanistic studies addressing migration, survival, etc., of mutant T cells in the periphery. As expected for the lymphopenic condition in Tnpo3-deficient mice (Figure 6D), increased proliferation levels of peripheral T cells are observed (Figure 6E), compatible with the notion that TNPO3 deficiency per se does not impair cell proliferation.
Collectively, the present data suggest an evolutionarily conserved role of TNPO3 in T cell differentiation.

DISCUSSION

Our unbiased genetic screens identified an extended genetic network linking genes of several distinct functional categories (hematopoietic differentiation; DNA replication/repair; pre-mRNA processing) required for early stages of intrathymic T cell development in zebrafish. The present study has established additional animal models for mammalian (particularly human) immunodeficiency syndromes and presents previously unknown candidate genes whose mutations might underlie failing immune function. Indeed, we note that some of the genes that we have identified are implicated in human immunodeficiency disorders, such as il7r (Puel et al., 1998), jak3 (Russell et al., 1995), and pole1 (Pachlornik Schmid et al., 2012) (Table 1).

The value of forward genetic screens to identify candidate genes is underscored by our finding of an enrichment of genes encoding factors involved in pre-mRNA processing. This outcome was unexpected, because mutations in this group of genes have so far been associated mostly with neuronal disease, such as spinal muscular atrophy or retinitis pigmentosa (Singh and Cooper, 2012). Mutations in splicingosoma factors manifest themselves as hypomorphic alleles (Singh and Cooper, 2012), indicating that the resulting phenotypes most likely arise from subtle perturbations in the multi-component protein complexes regulating pre-mRNA splicing. For example, insufficient levels of the SMN complex are associated with impaired maturation of snRNPs and aberrant splicing reactions in neuronal tissues (Challen et al., 2008, 2013), whereas complete loss of SMN function is lethal (Hsieh-Li et al., 2000). However, because defects in early development are buffered by maternal factors, our results also illustrate how the unique biological features of the zebrafish model allow the identification of tissue-restricted functions of pleiotropic genes also in the case of null alleles, which in the mammalian system can only be studied by conditional mutagenesis.

Although the results of the present epistasis analysis are consistent with those in previously published molecular studies on snRNP assembly and function (Wahl et al., 2009), an unexpected finding in the in vivo studies described here is the presence of a feedback loop connecting impaired snapc3, lsm8, and geminin5 function with the transcriptional regulation of the lsm7 and lsm8 genes. In contrast to many other genes in zebrafish, lsm genes do not possess paralogs. Hence, the transcriptional response in our mutants is unlikely to be part of a direct compensatory mechanism, but rather appears to be a general consequence of snRNP biogenesis perturbation, because expression levels of lsm7 and lsm8 are also upregulated in zebrafish larvae mutant for the p110 splicing regulator (Trede et al., 2007), which is required for recycling of the U4/U6 snRNP from singular U4 and U6 snRNPs (Bell et al., 2002). Another notable feature of transcriptional changes emerging from our studies is the presence of pervasive autoregulatory loops affecting the splicing of pre-mRNAs-encoding splice regulators, as illustrated here by, for instance, the srsf5a gene, a homolog of the mammalian Srsf3 gene previously shown to be regulated by variable inclusion of so-called poison-cassette exons (Lear et al., 2007). On a more general level, our findings provide evidence for the emerging notion of tissue-restricted roles played by ubiquitous components of basic cellular pathways. Future work may thus reveal an “RNA splicing code” of context- and cell-type-dependent functions of some general-purpose factors involved in pre-mRNA processing; our mutants provide an entry point into the identification of the precise mechanism(s) by which pre-mRNA splicing affects T cell development.

With respect to the mechanism(s) underlying failing T cell development in zebrafish larvae, our work indicates that, despite mechanistic differences, certain commonalities exist among the aberrations caused by mutations in the snapc3/lsm8/gemin5/Tnpo3 network. One particularly notable observation is the apparent association with DNA methylation, because the splicing of several genes encoding putative de novo DNA methylases is affected by perturbations in this network; most importantly, aberrant processing of the dnmt3bb.1 gene is a common feature of snapc3, lsm8, and Tnpo3 mutants. We consider this mechanistic link to be particularly intriguing, because our screen has independently uncovered a missense mutation in the gene encoding the maintenance DNA methylase dnmt1 (Table 1). Given that the status of DNA methylation affects the differentiation of hematopoietic stem cells (Challen et al., 2014) and modulates immune functions including T cell differentiation (Jin et al., 2008), it will be interesting to examine the possible presence of common changes in gene-specific DNA methylation patterns to identify those genes whose methylation signatures are crucial to normal T cell development.

Finally, an important aspect of our present work addresses the evolutionarily conserved function of the identified network of splice regulators with respect to T cell differentiation. We chose Tnpo3 to verify that the observations made in the zebrafish model also hold for the mammalian system. Our conditional mouse Tnpo3 knockout model exhibits a phenotype of impaired T cell differentiation, resembling the phenotype observed in Tnpo3-deficient zebrafish larvae. The comparative analysis of mutant zebrafish tnpo3 and mouse Tnpo3 genes highlights the advantages of the zebrafish model to identify cell-type-restricted functions of pleiotropic regulators, because Tnpo3-deficient mice exhibit early embryonic lethality. Future work will have to be aimed at addressing the degree of functional conservation for the other members of the identified genetic networks and the precise molecular mechanism(s) underlying the exquisite sensitivity of T cells to perturbed pre-mRNA splicing.

EXPERIMENTAL PROCEDURES

Animals

The zebrafish (Danio rerio) strains Ekkwill (EKK), Tüpfel long fin (TL), wild-type-in-Kalkutta (WIK), AB, Assam (ASS), and Tübingen (TÜ) are maintained in the animal facility of the Max Planck Institute of Immunobiology and Epigenetics. The ikaros:eGFP transgenic line was described previously (Hess and Boehm, 2012). The floxed Tnpo3 allele of the mouse was maintained on the C57BL/6J background. All animal experiments were approved by the institute’s review committee and conducted under licenses from the local government (Regierungspräsidium Freiburg).
ENU Mutagenesis of Zebrafish and Mutant Recovery
A detailed description of the screen design, coverage, complementation analysis, and mutant identification by positional cloning and whole genome sequencing can be found in Supplemental Experimental Procedures.

**Trnp3 Conditional Knockout Mouse**
The ESC line EPD0318_3_G02 (genetic background: C57BL/6N Agouti(A/a); allele name: Trnp3(+/+ΔCOPFWIN)) was obtained from the KOMP Repository and used to derive chimeric mice using standard procedures.

**Morphants and Phenotypic Rescue**
Morphants were generated as described (Schropp et al., 2009). For rescue experiments, mRNAs were injected into fertilized eggs and analysis carried out by gh and rag1 RNA in situ hybridization at various time points after injection.

**Cell Transplantation and Live Microscopy**
EGFP+ cells were sorted from single-cell suspensions of kidney marrow cells isolated from adult ikaros:eGFP transgenic zebrafish and injected into the sinus venosus of embryos from heterozygous intercrosses at 2 dpf. GFP+ cells in the thymic region were counted using Imager.Z1 (Zeiss) at 3 days post-injection. The procedures for live imaging using the ikaros:eGFP transgenic background were described previously (Hess and Boehm, 2012).

**Flow Cytometry**
For analysis of Trnp3 mutant mice, analytical flow cytometry was carried out as described in Calderón and Boehm (2012).

**RNA In Situ Hybridization**
Procedures for RNA in situ hybridization and probes were described previously (Schropp et al., 2009). The determination of rag1/gh ratios was carried out as described in Supplemental Experimental Procedures.

**RNA Extraction, cDNA Synthesis, and qPCR**
Total RNA was extracted using TRI Reagent (Sigma) following the manufacturer’s instructions. After treatment with Cloned DNase (Takara), RNA extraction using TRI Reagent was repeated. Superscript II Reverse Transcriptase (Invitrogen) and random hexamer primers were used for cDNA synthesis from total RNA. qPCR was carried out as described (Rode and Boehm, 2012); primers are listed in Table S8.

**RNA Sequencing and Computational Analysis of RNA-Seq Data**
Total RNA was extracted from whole zebrafish larvae at 4 dpf and subjected to transcriptome analysis. The libraries were sequenced in paired-end 75-bp mode on 0.8 lanes per sample on an Illumina HiSeq 2500 instrument. The high-throughput RNA sequencing analysis pipeline, version 0.4.2, written by Fabian Kilpert (https://github.com/kilpert/rna-seq-qc) was applied as described in Supplemental Experimental Procedures. RNA-seq data are deposited in NCBI’s GEO (Edgar et al., 2002) and are accessible through GEO: GSE77480.

**Statistics**
For single comparisons of independent groups, Student’s t test or the Mann-Whitney test was performed depending on the sample size and distribution. Analyses were performed using Prism software. The statistical models applied to RNA-seq data are described in Supplemental Experimental Procedures.

**ACCESSION NUMBERS**
The accession number for the RNA-seq data reported in this paper is GEO: GSE77480.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.003.

**AUTHOR CONTRIBUTIONS**
N.I., M.M., L.G., C.S.-R., D.-F.L., F.M., and M.S. identified and characterized zebrafish mutants by positional cloning. N.I., M.M., L.G., C.S.-R., D.-F.L., I.H., C.P.O., and M.S. determined the phenotypes of zebrafish mutants. N.I. established and characterized the Trnp3-mutant mice. K.S. and A.S.R. carried out the bioinformatic analysis of RNA-seq data. N.I., M.S., and T.B. conceived and designed the study. N.I. and T.B. wrote the paper. N.I., K.S., A.S.R., M.M., L.G., C.S.-R., D.-F.L., F.M., I.H., C.P.O., M.S., and T.B. contributed to the writing of the manuscript.

**ACKNOWLEDGMENTS**
This project has received funding from the Max Planck Society and the European Research Council under the European Union’s Seventh Framework Programme (FP7/2007–2013) ERC Grant Agreement 323126. The mutant screen was conducted in collaboration with the Tubingen 2000 Screen Consortium and the Freiburg Screening Group; their members are listed in Supplemental Experimental Procedures. We thank W. Wiest for initial identification of the WW18/10 mutant, B. Kanzler for help in the establishment of the mouse Tnpo3 mutant, D. Bönisch for excellent animal care, and U. Bönisch, E. Betancourt, and S. Diehl for help with next-generation sequencing.

Received: April 24, 2016
Revised: September 5, 2016
Accepted: October 25, 2016
Published: November 22, 2016

**REFERENCES**
Alarcón, B., Gil, D., Delgado, P., and Schamel, W.W. (2003). Initiation of TCR signaling: regulation within CD3 dimers. Immunol. Rev. 191, 38–46.
Battle, D.J., Lau, C.-K., Wan, L., Deng, H., Lotti, F., and Dreyfuss, G. (2006). The Gemin5 protein of the SMN complex identifies snRNAs. Mol. Cell 23, 273–279.
Bell, M., Schreiner, S., Damianov, A., Reddy, R., and Bindereif, A. (2002). p110, a novel human U6 snRNP protein and U4/U6 snRNP recycling factor. EMBO J. 21, 2724–2735.
Boehm, T. (2011). Design principles of adaptive immune systems. Nat. Rev. Immunol. 11, 307–317.
Boehm, T., Bleul, C.C., and Schropp, M. (2003). Genetic dissection of thymus development in mouse and zebrafish. Immunol. Rev. 195, 15–27.
Calderón, L., and Boehm, T. (2012). Synergistic, context-dependent, and hierarchical functions of epithelial components in thymic microenvironments. Cell 149, 159–172.
Challen, G.A., Sun, D., Mayle, A., Jeong, M., Luo, M., Rodriguez, B., Mallaney, C., Celik, H., Yang, L., Xia, Z., et al. (2014). Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. Cell Stem Cell 15, 350–364.
Danilova, N., and Steiner, L.A. (2002). B cells develop in the zebrafish pancreas. Proc. Natl. Acad. Sci. USA 99, 13711–13716.
den Dunnen, J.T., and Antonarakis, S.E. (2000). Mutation nomenclature extension and suggestions to describe complex mutations: a discussion. Hum. Mutat. 15, 7–12.
Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30, 207–210.
Friesen, W.J., and Dreyfuss, G. (2000). Specific sequences of the Sm and Sm-like (Lsm) proteins mediate their interaction with the spinal muscular atrophy disease gene product (SMN). J. Biol. Chem. 275, 26370–26375.
Hess, I. (2012). Intravital imaging of thymopoiesis reveals dynamic lympho-epithelial interactions. Immunity 36, 298–309.
Hirano, M., Guo, P., McCurley, N., Schorpp, M., Das, S., Boehm, T., and Cooper, M.D. (2013). Evolutionary implications of a third lymphocyte lineage in lampreys. Nature 507, 435–438.

Hsieh-Li, H.M., Chang, J.-G., Jang, Y.-J., Wu, M.-H., Wang, N.M., Tsai, C.H., and Li, H. (2000). A mouse model for spinal muscular atrophy. Nat. Genet. 24, 66–70.

Iwanami, N., Mateos, F., Hess, I., Riffel, N., Soza-Ried, C., Schorpp, M., and Boehm, T. (2011). Genetic evidence for an evolutionarily conserved role of IL-7 signaling in T cell development of zebrafish. J. Immunol. 186, 7060–7066.

Jin, B., Tao, Q., Peng, J., Soo, H.M., Wu, W., Ying, J., Fields, C.R., Delmas, A.L., Liu, X., Qiu, J., and Robertson, K.D. (2008). DNA methyltransferase 3B (DNMT3B) mutations in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating development, neurogenesis and immune function. Hum. Mol. Genet. 17, 690–709.

Katoaka, N., Bachorik, J.L., and Dreyfuss, G. (1999). Transportin-SR, a nuclear import receptor for SR proteins. J. Cell Biol. 145, 1145–1152.

Kettleborough, R.N.W., Busch-Nentwich, E.M., Harvey, S.A., Dooley, C.M., de Bruijn, E., van Eeden, F., Sealy, I., White, R.J., Herd, C., Nijman, I.J., et al. (2014). Structural basis for nuclear import of splicing factors by human Transportin 3. Proc. Natl. Acad. Sci. USA 111, 2728–2733.

Langenu, D.M., and Zon, L.I. (2005). The zebrafish: a new model of T-cell and thymic development. Nat. Rev. Immunol. 5, 307–317.

Lareau, L.F., Inada, M., Green, R.E., Wengrod, J.C., and Brenner, S.E. (2007). Unproductive splicing of SR genes associated with highly conserved and ultra-conserved DNA elements. Nature 446, 926–929.

Lefrancois, L., and Goodman, T. (1987). Developmental sequence of T200 antigen modifications in murine T cells. J. Immunol. 139, 886–889.

Mani, R., St Onge, R.P., Hartman, J.L., 4th, Giaever, G., and Roth, F.P. (2008). Defining genetic interaction. Proc. Natl. Acad. Sci. USA 105, 3461–3466.

Mönch, M., Hess, I., Wiest, W., Bachrati, C., Hickson, I.D., Schorpp, M., and Boehm, T. (2010). Developing T lymphocytes are uniquely sensitive to a lack of IL7R expression in T cell development of zebrafish. J. Immunol. 184, 2259–2270, November 22, 2016.

Orban, P.C., Chui, D., and Martí, J.D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. Proc. Natl. Acad. Sci. USA 89, 6861–6865.

Pachlopnik Schmid, J., Lemoine, R., Nehme, N., Cornier-Daire, V., Revy, P., Debeurme, F., Debré, M., Nitschke, P., Bole-Feysoit, C., Legeai-Mallet, L., et al. (2012). Polymerase ε1 mutation in a human syndrome with facial dysmorphism, immunodeficiency, livedo, and short stature (“FILS syndrome”). J. Exp. Med. 209, 2323–2330.

Pandolfi, P.P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D., and Lindenbaum, M.H. (1995). Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat. Genet. 11, 40–44.

Puel, A., Ziegler, S.F., Buckley, R.H., and Leonard, W.J. (1998). defective IL-7R expression in T–B NK+ severe combined immunodeficiency. Nat. Genet. 20, 394–397.

Rode, I., and Boehm, T. (2012). Regenerative capacity of adult cortical thymic epithelial cells. Proc. Natl. Acad. Sci. USA 109, 3463–3468.

Russell, S.M., Tayebi, N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Migone, T.-S., Noguchi, M., Markert, M.L., Buckley, R.H., et al. (1995). Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. Science 270, 797–800.

Schorpp, M., Blaiack, 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.

Singh, R.K., and Cooper, T.A. (2012). Pre-mRNA splicing in disease and therapeutics. Trends Mol. Med. 18, 472–482.

Soza-Ried, C., Hess, I., Netuschil, N., Schorpp, M., and Boehm, T. (2010). Essential role of c-myc in definitive hematopoiesis is evolutionarily conserved. Proc. Natl. Acad. Sci. USA 107, 17304–17308.

Takayama, K., Shimoda, N., Takanaga, S., Hozumi, S., and Kikuchi, Y. (2014). Expression patterns of dnmt3a, dnmt3ab, and dnmt4 during development and fin regeneration in zebrafish. Gene Expr. Patterns 14, 105–110.

Trede, N.S., Medenbach, J., Damianov, A., Hung, L.H., Weber, G.J., Paw, B.H., Zhou, Y., Hersey, C., Zapata, A., Keefe, M., et al. (2007). Network of cor-regulated spliceosome components revealed by zebrafish mutant in recycling factor p110. Proc. Natl. Acad. Sci. USA 104, 6608–6613.

Wahl, M.C., Will, C.L., and Lührmann, R. (2009). The spliceosome: design principles of a dynamic RNP machine. Cell 136, 701–718.

Wei, Q., and Condie, B.G. (2011). A focused in situ hybridization screen identifies candidate transcriptional regulators of thymic epithelial cell development and function. PLoS One 6, e26795.

Xiang, K., Tong, L., and Manley, J.L. (2014). Delineating the structural blueprint of the pre-mRNA 3′-end processing machinery. Mol. Cell. Biol. 34, 1894–1910.

Yui, M.A., and Rothenberg, E.V. (2014). Developmental gene networks: a triathlon on the course to T cell identity. Nat. Rev. Immunol. 14, 529–545.

Zhang, Z., Lotti, F., Dittrmar, K., Yousif, I., Wan, L., Kasim, M., and Dreyfuss, G. (2008). SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell 133, 585–600.

Zhang, Z., Pinto, A.M., Wan, L., Wang, W., Berg, M.G., Oliva, I., Singh, L.N., Dengler, C., Wei, Z., and Dreyfuss, G. (2013). Dysregulation of synaptogenesis genes antecedes motor neuron pathology in spinal muscular atrophy. Proc. Natl. Acad. Sci. USA 110, 19348–19353.
Supplemental Information

Forward Genetic Screens in Zebrafish

Identify Pre-mRNA-Processing Pathways

Regulating Early T Cell Development

Norimasa Iwanami, Katarzyna Sikora, Andreas S. Richter, Maren Mönnich, Lucia Guerri, Cristian Soza-Ried, Divine-Fondzenyuy Lawir, Fernando Mateos, Isabell Hess, Connor P. O’Meara, Michael Schorpp, and Thomas Boehm
Figure S1

(A) Representative sequence traces indicating the G>T transition at nucleotide position 8611002 (Zv9) on chromosome 4; conceptual translation of the nucleotide sequence is indicated in three-letter code (stop codon is marked by *).

(B) Deduced protein structure of wild-type and predicted mutant (E72X) proteins.

(C) Phenotypic rescue of lsm8 mutation by injection of wild-type zebrafish (Dr) lsm8 mRNA as determined by the extent of thymopoietic activity (as measured by the ratio of hybridization signals obtained for rag1 and growth hormone [gh]); error bars represent S.E.M.; the significance level of the difference is indicated (t-test; two-tailed). The number (n) of embryos analyzed is indicated.

(D) Functionally competent thymic rudiment in lsm8 mutants. At 5 dpf, expression of the thymopoietic marker foxn1 in mutant embryos is indistinguishable from that in wild-type embryos (circles; left panels). To examine the receptive capacity of thymic rudiments, purified ikaros-positive hematopoietic kidney marrow cells from adult wild-type ikaros:eGFP transgenic fish were injected into the sinus venosus of wild-type and heterozygous embryos (collectively designated as +/± genotypes) and lsm8 mutant embryos at 2 dpf. 72 hours later, the numbers of green cells in the rudiment were counted; a total of 40 lsm8+/± and 10 lsm8−/− embryos were successfully injected; transplantation failed in 47 (54%) and 28 (74%) embryos, respectively. Scale bar, 10 μm.

(E) Characterization of early hematopoiesis. Whole mount RNA in situ hybridization was carried out with the indicated probes at various time points. runx1, 36 hpf. cmyb, 36 hpf; gata1, 24 hpf; t-plastin, 24 hpf. Shown are overviews (left panels) and magnifications (right panels). Scale bars, 50 μm.
Figure S2. Characterization of *gemin5* mutants. Related to Figure 1

(A) Representative sequence traces indicating the T>G transition at nucleotide position 35,739,415 (Zv9) on chromosome 21; conceptual translations are indicated in three-letter code (stop codon is marked by *).

(B) Deduced protein structure of wild-type and predicted mutant (Y437X) proteins. The truncation occurring in WD repeat 8 is indicated.

(C) Rescue of *gemin5* mutation by injection of wild-type mouse *Gemin5* mRNA (encoding protein ENSMUSP00000131842), but not the equivalent mutant (Y454X) mouse mRNA. The ratio of thymopoietic activity (as measured by the ratio of hybridization signals obtained for *rag1* and growth hormone ([gh]) of injected relative to un-injected embryos of the indicated genotypes is shown; error bars represent S.E.M.; significance level of differences is indicated (t-test; two-tailed). The number (n) of embryos analysed is indicated.
Figure S3. Characterization of *snapc3* mutants. Related to Figure 1

(A) Representative sequence traces indicating the G>T transition at nucleotide position 8611002 (Zv9) on chromosome 1; conceptual translation in three-letter code (stop codon is marked by *).

(B) Deduced protein structure of wild-type and predicted mutant (C297X) proteins.

(C) Diagnostic whole-mount RNA *in situ* hybridization pattern in *snapc3* mutants using *rag1* and *gh* at 5 days post fertilization (dpf). Scale bars, 10 µm.

(D) Quantification of thymopoietic activity in *snapc3* mutants expressed as a ratio of *rag1* to *gh* signals derived from analyses illustrated in (C).

(E) Phenocopy of the *snapc3* mutation by antisense morpholino oligonucleotides; splice acceptor (SA) morpholino, transcriptional start site (ATG) morpholino. Scale bars, 10 µm.

(F) The *rag1/gh* ratios as a measure of thymopoietic activity exemplified in (E) are quantified at 4 dpf (right panel); ***, P<0.001 (t-test; two-tailed).

(G) In contrast to similar numbers of *ikaros*-expressing neurons in the hindbrain, the number of *ikaros*-expressing thymocytes is greatly reduced in *snapc3* mutants at 5 dpf. Scale bars, 10 µm.

(H) Characterization of early hematopoiesis. Whole mount RNA *in situ* hybridization was carried out with the indicated probes at various time points. *runx1*, 36 hpf; *cmyb*, 36 hpf; *gata1*, 24 hpf; *l-plastin*, 24 hpf. Shown are overviews (left panels) and magnifications (right panels). Scale bars, 50 µm.
Figure S4

(A) Maternal *lsm8* transcripts are required for normal T cell development. Differential effect of morpholino oligonucleotides directed against the translation start site (ATG morpholino) and splice donor site (SD morpholino) (the number [n] of embryos analysed is indicated).

(B) Despite partially impaired splicing of zygotic *lsm8* transcripts at 1.5 dpf (right panel), T cell development at 5 dpf is not impaired; splicing of *actb2* transcripts (size of cDNA 261 bp) is not impaired in *lsm8* morphants. The white dot indicates primer dimers.

(C) Lack of genetic interaction between *lsm8* and *il7r* mutations. Thymopoietic activity as measured by the ratio of *rag1* and *gh* RNA in situ hybridization signals in embryos at 5 dpf for the indicated genotypes; the genotypes of the parental generation are indicated above the bars. The *rag1/gh* values for double-mutant fish are not significantly different from the expected values under a multiplicative model of genetic interaction (Mani et al., 2008) (t-test, two-tailed), suggesting that the parental *lsm8* genotype does not affect the contribution of *il7r* mutation to the resulting phenotype.

(D) Lack of detectable genetic interaction between *lsm8* and *il7ra* mutations. The observed *rag1/gh* values are compared against expected values calculated from data in (C) under the multiplicative model of genetic interaction (Mani et al., 2008).
Characterization of *tnpo3* zebrafish mutants. Related to Figure 5.

(A) Representative sequence traces indicating the C>T transversion at nucleotide position 12,656,091 (Zv9); conceptual translation of the nucleotide sequence is indicated in three-letter code (stop codon is marked by *).

(B) Schematic of the deduced wild-type and mutant TNPO3 protein structures. The 15th HEAT repeat that interacts with RS domains is indicated.

(C) Rescue of *tnpo3* mutation by injection of wild-type mouse *Tnpo3* mRNA (encoding protein ENSMUSP0000012679), but not mutant (R203X) mouse mRNA. The ratio of thymopoietic activity (as measured by the ratio of hybridization signals obtained for *rag1* and growth hormone [gh]) for injected relative to uninjected embryos of the indicated genotypes is shown; error bars represent S.E. M.; the significance level of difference is indicated (t-test, two-tailed). The number (n) of embryos analysed is indicated.

(D) Absence of eye and craniofacial abnormalities in *tnpo3* mutant embryos, as determined by RNA *in situ* hybridization to *dlx2* and staining of cartilage with alcian blue. Panels are representative of at least 3 animals. Scale bars, 100 µm.

(E) Functionally competent thymic rudiment in *tnpo3* mutants. To examine the receptive capacity of thymic rudiments, purified whole kidney marrow cells from adult wild-type *ikaros:eGFP* transgenic fish were injected into the sinus venosus of *tnpo3* wild-type (+/+) and heterozygous (+/-) embryos and mutant (-/-) embryos at 2 dpf. After an additional 72 hours, the numbers of green cells in the rudiment were counted (mean±S.E.M.). A total of 80 *tnpo3*+/+ and 27 *tnpo3*−− embryos were injected; transplantation failed in 52 (65%) and 18 (67%) of these embryos, respectively; the difference between the groups is not significant (t-test; two-tailed).

(F) Reduced numbers of alcian blue-positive goblet cells in the intestine (arrows) of *tnpo3* mutants. Panels are representative of at least 3 animals. Scale bar, 100 µm.

(G) Characterization of early hematopoiesis. Whole mount RNA *in situ* hybridization was carried out with the indicated probes at various time points. *runx1*, 36 hpf. *cmyb*, 36 hpf; *gata1*, 24 hpf; *l-plastin*, 24 hpf. Shown are overviews (left panels) and magnifications (right panels). Scale bars, 50 µm.
Figure S6. Characterization of conditional mouse Tnpo3 mutants. Related to Figure 6.

(A) Differential exon usage between mRNA transcript isoforms in heterozygous (Tnpo3<sup>fl/+</sup>; Lck:Cre) and mutant (Tnpo3<sup>fl/fl</sup>; Lck:Cre) DP and CD4<sup>+</sup> thymocytes as determined by RT-PCR. The absence of mRNAs containing exon 7 (coordinates on chromosome 6: nt 29,578,463 to 29,578,601) in the mutant cells indicates complete deletion of this exon in the mutant mRNA of CD4<sup>+</sup> thymocytes. Note that the control cells are heterozygous for the floxed allele of Tnpo3 gene, yielding an expected inclusion level of 0.5 for the floxed exon 7. The RT-PCR results indicate that the aberrant transcript is not subject to nonsense-mediated decay, although the lack of exon 7 sequences (nt. 1274 to nt. 1413 in Genbank accession number NM_177296) gives rise to a frame-shift and a stop codon immediately in exon 8.

(B) Deduced structures of wild-type and mutant TNPO3 proteins for zebrafish (D.r.) and mouse (M.m.). The predicted mouse mutant protein consists of 291 amino acids. Residue 291 in the mouse TNPO3 protein is equivalent to residue 290 in the zebrafish protein.

(C) Altered ratio of γδ T cells and αβ T cells in the spleen of mutant Tnpo3<sup>fl/fl</sup>; Lck:Cre mice (n=3); note, however, that the absolute number of gd T cells remains unchanged compared to the control genotype (Tnpo3<sup>fl/+</sup>; Lck:Cre mice (n=4) (P=0.1; Welch-test, two-tailed) (left panels). Flow cytometric profile representative of 3 or 4 experiments.
LIST OF SUPPLEMENTAL TABLES

Table S1. Exon skipping events in snapc3, lsm8, and tnpo3 zebrafish mutants.
Table S2. Intron retention events in snapc3, lsm8, and tnpo3 zebrafish mutants.
Table S3. Differential gene expression in snapc3, lsm8, and tnpo3 zebrafish mutants.
Table S4. Genes encoding pre-mRNA splice regulators upregulated in snapc3, and lsm8 mutants.
Table S5. Expression levels of lsm and gemin genes in snapc3, lsm8, and gemin5 zebrafish mutants.
Table S6. Genes affected by exon skipping events in snapc3, lsm8, and tnpo3 zebrafish mutants.
Table S7. Genes downregulated in snapc3, lsm8, and tnpo3 zebrafish mutants.
Table S8. Primers for real-time RT-PCR of zebrafish genes.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals. The zebrafish (D. rerio) strains Ekkwill (EKK), Tüpfel long fin (TL), wildtype-in-Kalkutta (WIK), AB, Assam (ASS) and Tübingen (TÜ) are maintained in the animal facility of the Max Planck Institute of Immunobiology and Epigenetics. The ikaros:eGFP transgenic line was described previously (Hess and Boehm, 2012). The mouse floxed Tnpo3 allele was maintained on the C57BL/6J background. All animal experiments were approved by the institute’s review committee and conducted under licenses from and approved by the local government (Regierungspräsidium Freiburg). All animal experiments were approved by the institute’s review committee and conducted under licenses from the local government.

ENU mutagenesis of zebrafish and mutant recovery. The Tübingen 2000 screen was initiated by the Department of Genetics (Max Planck Institute of Developmental Biology, Tübingen, Germany) and conducted together with Artemis Pharmaceuticals GmbH, an Exelixis company (Cologne, Germany), collectively referred to as the Tübingen 2000 Screen Consortium. Contributors to the Tübingen 2000 Screen Consortium are from the Max Planck Institute of Developmental Biology: F. van Bebber, E. Busch-Nentwich, R. Dahm, H. G. Frohnhöfer, H. Geiger, D. Gilmour, S. Holley, J. Hooge, D. Jülich, H. Knaut, F. Maderspacher, H.-M. Maischein, C. Neumann, T. Nicolson, C. Nüsslein-Volhard, H. H. Roehl, U. Schönberger, C. Seiler, C. Söllner, M. Sonawane, A. Wehner, C. Weiler; from Exelixis Germany: P. Erker, H. Habeck, U. Hagner, C. Nennen, E. Kaps, A. Kirchner, T. Koblietzek, U. Langheinrich, C. Loeschke, C. Metzger, R. Nordin, J. Odenthal, M. Pezzuti, K. Schlombs, J. deSatana-Stamm, T. Trowe, G. Vacun, B. Walderich, A. Walker, and C. Weiler. The dedicated screen for thymus abnormalities was conducted by the Freiburg Screening Group at the Max Planck Institute of Immunobiology and Epigenetics (Freiburg, Germany). Members of the Freiburg Screening Group were: M. Bialecki, T. Boehm, D. Diekhoff, T. Franz, M. Held, M. Leicht, E. Nold, T. Nolting, C. Riegger, M. Schorpp, and W. Wiest. Briefly, adult zebrafish males of the Tü line were incubated in buffered E3 medium containing ENU as previously reported (Haffter et al., 1996). After mutagenesis, males were repeatedly mated to normal females to generate over 10,000 F1 fish. Such F1 fish were crossed with each other to generate F2 families that underwent brother-sister crosses to generate homozygous larvae in the resulting F3 clutches. Twenty to 30 larvae of each F3 clutch were screened via rag1 RNA in situ hybridization. Based on the results of a small-scale pilot screen (Schorpp et al., 2000), we used the extent and pattern of rag1 staining in larvae at 120 h post fertilization (hpf) as a suitable marker to detect alterations in thymus and/or T cell development. At this point in larval development, rag1 staining is confined to the thymus rudiment (Schorpp et al., 2000; Willett et al., 1997). rag1 staining was expected to be reduced or absent in the case of perturbed lymphoid development and also in the case of aberrant development of the thymic stromal microenvironment and secondary impairment of thymopoiesis. A clutch was considered positive when 20–30% of the larvae exhibited a similar alteration in the rag1 expression pattern or intensity. To confirm and recover mutations, F2 pairs producing putative mutants among their F3 offspring were crossed out, and the resulting new F3 families underwent the same inbreeding and screening procedure. The gynogenetic screen was carried out by the Freiburg Screening Group at the Max Planck Institute of Immunobiology and Epigenetics (Freiburg, Germany) as follows: Adult zebrafish males of the AB line were incubated in buffered E3 medium containing ENU as previously reported (Haffter et al., 1996). After mutagenesis, males were repeatedly mated with normal females to generate F1 fish. The eggs of 1413 females were subjected to early pressure-induced gynogenesis (Johnson et al., 1995) and the embryos subjected to rag1 RNA in situ hybridization.

Screen coverage. (a) Tübingen 2000 screen. In total, F3 clutches of 4,584 F2 families, representing 4,253 mutagenized haploid genomes, were screened. This represents 1.5 times more mutagenized haploid genomes than in the first Tübingen large-scale screen (Haffter et al., 1996). A total of 141 mutants with reduced rag1 signals but without severe craniofacial defects were detected in the primary analysis. Ultimately, 42 mutant lines could be established, corresponding to approximately 1% of the number of genomes screened. (b) 281 genomes were analysed in the Freiburg gynogenetic screen; in 25 instances, clutches were observed, in which about 50% of the embryos exhibited abnormal rag1 signals without severe craniofacial defects. Ultimately, 3 mutant lines could be established, corresponding to approximately 1% of the number of genomes screened; this number is identical to the screening efficiency in the aforementioned Tübingen 2000 F3 screen. One gene (top3a) was identified in both the F3 and gynogenetic screens (Table 1); two alleles of ikzf1/ikaros were identified in the F3 screen (Table 1).
Initial mutant characterization. Prior to detailed phenotypic analysis, mutant carrier fish were out-crossed with wild-type fish for several generations to eliminate potentially confounding background mutations. Using molecular probes, all mutants were subsequently analyzed by RNA in situ hybridization for potential abnormalities of hematopoietic cells, development of pharyngeal endoderm and ectoderm, and structures derived from neural crest at various time points during the first 5 days of embryonic development. Differentiation of hematopoietic cells in the intermediate cell mass, a site of embryonic blood formation, was assessed by hybridization with probes specific for tall/scl (a gene that specifies hematopoietic and vascular progenitor cells) (Gering et al., 1998), gata1a (a gene required for red blood cell development) (Lyons et al., 2002), kpl1/-plastin, (a marker of the myeloid lineage) (Bennett et al., 2001), and ikzf1/ikaros (a putative marker of lymphoid progenitors in zebrafish) (Willett et al., 2001). The arrival and early differentiation of T cell progenitors in the thymic rudiment was assessed by hybridization with probes specific for ikzf1/ikaros, ccr9b (the zebrafish homolog of the mammalian chemokine receptor 9, a marker of early T cells in the mouse) (Benz and Bleul, 2005; Benz et al., 2004), rag1 (a marker of immature lymphoid cells rearranging their antigen receptor loci) (Greenhalgh and Steiner, 1995; Schorpp et al., 2000; Willett et al., 1997), and TCR (tcrb and tcrd) as markers of ab and gd T cells, respectively (Schorpp et al., 2006). Development of the pharyngeal arches was analyzed using gcm2, the zebrafish homolog of the mouse Gcm2 gene as a marker for zebrafish pharyngeal ectoderm (Hogan et al., 2004), and foxn1 (Schorpp et al., 2002), the zebrafish homolog of the mouse Foxn1 gene that is required for differentiation of thymic epithelial cells (Nehls et al., 1996) and expressed in endodermal derivatives. Neural crest development was assessed by dlx2 expression (Akimenko et al., 1994) and cartilage formation by alcin blue staining.

Linkage analysis. The genomic localization of zebrafish mutations was determined using the Tübingen marker set for genome scans (version 4) on F2-Tübingen x WIK crosses of the mutant carriers. Primer sequences are available from zfin.org. For fine-scale mapping, new markers were generated. For each of the five mutants discussed in detail here, the most informative markers are listed in the section summarizing their pertinent features. Sequence coordinates for genome assembly Zv9 are available at http://www.ensembl.org/Danio_rerio/Info/Index?db=core.

Complementation analysis. After assignment of mutants to particular linkage groups, complementation analysis was carried out for those mutations that mapped to the same chromosomal region to determine whether two mutations causing similar phenotypes reside in the same or in two different genes. To this end, a heterozygous fish carrying one mutation was crossed with a heterozygous fish carrying the other mutation. In general, allelic mutations fail to complement each other in trans-heterozygous embryos, which exhibit the mutant phenotype like homozygotes of either allele. If the mutations are in different genes, the double heterozygous offspring are expected to exhibit a wild-type phenotype, unless epistatic interactions modify the phenotype.

Gene identification. For all but one of the mutations identified here, linkage analysis identified critical intervals often containing less than a dozen candidate genes. Their coding exons (including flanking regions) were then sequenced after PCR amplification from genomic DNA of phenotypically wild-type (that is, a mixture of wild-types and heterozygous fish) and mutant embryos, which were identified by prior RNA in situ hybridization with the rag1 probe. Primer sequences used for these analyses are available upon request. In one case (KW059), meiotic recombination rates were found to be greatly reduced, resulting in an unusually large critical interval. Therefore, whole genome sequence analysis was used to identify the critical mutation in this line. Whole genome sequencing was also applied in the characterization of the II032 mutant to explore the applicability of novel bioinformatic procedures for the detection of mutated genes (manuscript in preparation). According to their known functions, it was possible to group the affected genes into three functional categories (Table 1). The first group of five mutants comprises key regulators of hematopoiesis and lymphopoiesis. cmnb encodes a transcription factor that has emerged as one of the key regulators of vertebrate hematopoiesis (Greig et al., 2008); in mice, it is dispensable for primitive stages of blood cell development but essential for definitive hematopoiesis (Mucenski et al., 1991) and self-renewal of mouse hematopoietic stem cells (Lieu and Reddy, 2009). This function is evolutionarily conserved (Soza-Ried et al., 2010). ikzf1/ikaros encodes a transcription factor thought to prime the lymphoid transcriptional programme in hematopoietic stem cells (HSCs) and to concomitantly repress the transcriptional programs characteristic of stem cell and non-hematopoietic lineages downstream of the HSC (Yoshida et al., 2010). The phenotype of the zebrafish ikzf1 alleles identified here suggests that ikaros plays similar roles in both fish and mammalian lymphopoiesis (Schorpp et al., 2006). The identification of several genes encoding components of the IL7 signalling pathway (i.e.,
il7r, jak1, and jak3) is not unexpected, given that IL-7 signalling is critical for T cell proliferation and survival (Ceredig and Rolink, 2012). The fact that mutations in the mammalian homologues of some of these genes, such as il7r and jak3, are also associated with hematopoietic and/or lymphopoietic abnormalities (Casanova et al., 2012) indicates their evolutionarily conserved functions. Collectively, the identification of many regulators of vertebrate hematopoiesis and lymphopoiesis validated the screening protocol employed in the present study, which focused on aberrations of early T cell development. The second group comprises five genes, the products of which are known to play a role in DNA replication and repair, as well as in the regulation of the cell cycle. The product of the top3a gene is a component of the RTR complex, which has critical functions in faithful DNA replication, recombination, and chromosome segregation (Oie et al., 1975). polel encodes the catalytic subunit and is a member of the B family of DNA polymerases (Pursell and Kunkel, 2008); reduced expression of the human POLE1 protein is associated with facial dysmorphism, immunodeficiency, livedo, and short stature ("FILS syndrome") (Pachlopnik Schmid et al., 2012). Minichromosome maintenance protein 10 (Mcm10) is required during both initiation and elongation phases of DNA replication, although its precise role is still controversial; in the present context, it is important to note that it has been shown to genetically interact with polel (Thu and Bielinsky, 2013), suggesting functional links between the gene products of this category of mutants. In mice, lymphoid differentiation fails when hematopoietic stem cells express reduced amounts of Dmnt1 (Bröske et al., 2009), providing an intriguing example of cell type-specific effects resulting from altered function of a general regulator and providing a functional link to the genes identified in the first group of mutants (i.e., cmymb, etc.). Finally, in mice, conditional ablation of Zbb17 in specific lineages of mice indicates its tissue-specific role in lymphopoiesis, regulating IL-7 signalling both in B cells (Kosan et al., 2010; Phan et al., 2005) and T cells (Saba et al., 2011a; Saba et al., 2011b). Interestingly, despite ubiquitous expression of the identified general regulators of DNA replication and repair, as well as the cell cycle, it appears that T cell development is particularly sensitive to the phenotypic effects of them being mutated. The products of the five genes in the third group (snapc3, lsm8, gemin5, tupo3, and cstf3) are all implicated in pre-mRNA processing. The first four representatives of this group of genes are the subject of this report. 

(a) lsm8 mutant. The WW18/10 mutant line (allele designation fr100) was identified in the Freiburg gynogenetic screen (Schorpp et al., 2000) as a result of severely reduced numbers of rag1-expressing cells in the thymus at 5 dpf (Figure 1B; Figure S1); however, immature lymphoid progenitors colonize the mutant thymic rudiment as revealed in the ikaros:eGFP (Hess and Boehm, 2012) transgenic background, albeit in reduced numbers (Figure 1A). The mutation is autosomal recessive and maps to chromosome 4; the marker closest to the mutation exhibits a genetic distance of <0.05 cM (18-10_114). The following markers were used (sequence coordinates refer to the zebrafish genome assembly [Zv9.70]): 18-10_125 (ATCTTGGAAAGAGCCTACAG [nt 8,600,970 to 8,600,989] and CAAGAGTTTCCTACAGCCC [nt 8,600,813 to 8,600,832]); 18-10_114 (AGCCACAGTGATGCTGCT [nt 8,642,638 to 8,642,657] and GACGGACTGTGGGTCTTCT [nt 8,625,139 to 8,625,158]); 18-10_127 (TCCAGCTGTGGGCTTACA [nt 8,655,054 to 8,655,073] and TGCTTTGACGCGATTCCAC [nt 8,655,542 to 8,655,561]). The number of recombination events between genetic markers and the mutation are as follows: 18-10_125 (1 recombination event in 2,060 meioses); 18-10_114 (0/2,060); 18-10_127 (2/2,060). Among the genes mapped to the critical interval, a non-sense mutation (G>T transition at nucleotide 4:8611002; Zv9.70) (Figure S1) was identified in lsm8 (ENSDARG00000091656); it converts residue 72 (ENSDARP00000104545) to a stop codon (E72X). Genotyping of fish was subsequently performed by PCR on genomic DNA using primers TCGAGCAGGTGGTCTG GGA and GCTGCTTATAGATGCCTGCAG (amplicon size 322 bp); sequencing w

(b) Genotyping of fish was subsequently performed by PCR on genomic DNA using primers TCGAGCAGGTGGTCTG GGA and GCTGCTTATAGATGCCTGCAG (amplicon size 322 bp); sequencing was carried out using primer TCGAGCAGGTGGTCTG GGA. The phenotype of WW18/10 mutants was rescued by injection of mRNA encoding wild-type zebrafish lsm8 (Figure S1). This rescue indicates that the mutation in lsm8 causes the observed phenotype.

Maternal lsm8 transcripts partially compensate the lack of zygotic lsm8 mRNA during T cell development, as indicated by the differential effect on rag1 expression in morphants analysed at 5 dpf injected with morpholino oligonucleotides directed against the translation start site (GGACATCTTTTCTGCTGCTG; final concentration in injection buffer, 50nm) and splice donor site of exon 3 (GGAGTTTAAACTACACATTAC; final concentration in injection buffer, 400nm) (Figure S4). The effect of the splice morpholino was determined by RT-PCR on RNA extracted at 1.5 dpf using the following primers: forward TCGAGCAGGTGGTCT GGA (located in exon 3), reverse CTGTCCATCAATGCACACACAC (located in exon 4) (Figure S4). The thymic epithelium, a derivative of pharyngeal endoderm expressing the transcription factor gene foxn1, appears to be functional, as indicated by its undisturbed colonization by wild-type cells after their transplantation into lsm8 mutant embryos (Figure S1). Collectively, these data indicate that the lsm8 mutation results in a cell type-specific defect, most pronounced in developing haematopoietic cells.
Other observations support the notion that, at least initially, the aberrations in lsm8 mutants are tissue-specific; the early steps of haematopoiesis in mutant larvae are indistinguishable from those of their wild-type siblings as revealed by the pattern of fluorescent cells in the ikaros:eGFP transgenic background (Figure 1); although only few ikaros-expressing cells accumulate in the thymus, the formation of ikaros-expressing neural structures appears to be normal (Figure 1).

(b) *gemin5* mutant. The KL069 mutant line (allele designation t26393) was identified in the Tübingen 2000 screen as a result of the lack of *rag1*-expressing cells in the thymus at 5 dpf (Figure 2); however, immature lymphoid progenitors colonize the mutant thymid rudiment as revealed in the ikaros:eGFP (Hess and Boehm, 2012) transgenic background, albeit in reduced numbers (Figure 2). The mutation is autosomal recessive and maps to chromosome 21; the markers closest to the mutation exhibit genetic distances of <0.15 cM (KL069_433, KL069_436). The following markers were used (sequence coordinates refer to the zebrafish genome assembly [Zv9.70]): KL069_437 (GCACGTTTACACAGCTTAGC and CAGGAGAAGCTTGTTTAGC [nt 35,633,322 to 35,633,341 and nt 35,633,837 to 35,633,856]); KL069_433 (TAAGTGAGTGGCCATGCAAC and GCATTGCCACGCTGTGAC [nt 35,730,346 to 35,730,365 and 35,729,805 to 35,729,824]); KL069_436 (GCTCACGCGCACAAGTGTCGTG [nt 35,757,383 to 35,757,402] and AGTTTGCACCCCTGTTCC); KL069_442 (GTGTAACCTGTGAAACACAGC and AGAGCCAACGCTGACTGAG [nt 35,833,521 to 35,833,541 and 35,833,796 to 35,833,815]). The number of recombination events between genetic markers and the mutation are as follows: KL069_437 (2 recombination events in 674 meioses); KL069_433 (0/674); KL069_436 (0/674); KL069_442 (1/674). Among the genes mapped to the critical interval, *gemin5* (ENSDARG00000079257) encodes a protein of 1440 amino acid residues (ENSDARP00000113067); a non-sense mutation (T>G transition at nucleotide 21,357,301; Zv9.70) converts residue 437 to a stop codon (Y437X) (2 recombination events in 674 meioses); KL069_433 (0/674); KL069_436 (0/674); KL069_442 (6/948). Using information obtained from whole genome sequencing, the coding regions of the mapped interval were manually examined and compared to the sequences of three wild-type strains. *snapc3* (ENSDARG00000071237) was the only gene found to carry a deleterious mutation. *snapc3* encodes a protein of 391 amino acid residues (ENSDARP00000110598); a non-sense mutation (T>A transition at nucleotide 1:27,053,807; Zv9.70) in exon 7 converts residue 297 to a stop codon (C297X) (Figure S3). The mutant mRNA is stable (data not shown). Genotyping of fish was subsequently carried out by PCR on genomic DNA using primers CTATGGTTTTCAGATCATGTGC and CAAACAGTCATCCTGATGCC (amplicon size 495 bp); sequencing was carried out using primer CAAACAGTCATCCTGATGCC. The phenotype of KW059 mutants was phenocopied by morpholino oligonucleotides targeting the splice acceptor of
exon 3 (TGC-GTAACGACGAAGCAGCATAA; final concentration in injection buffer, 200mM) and the translational start site (TCTTGGTAGATCTCCGACCTAA; final concentration in injection buffer, 200mM); the differential effects of blocking the splicing of zygotic mRNAs and translation suggests that maternal snapc3 mRNA can at least partially compensate the lack of zygotic snapc3 mRNA during T cell development (Figure S3). The aberrations in snapc3 mutant embryos and larvae appear to be tissue-specific. The expression patterns of marker genes for neural crest (dlx2; assayed at 3 dpf), ectoderm (gcm2; assayed at 3 dpf) and endoderm (foxn1; assayed at 5 dpf) are normal, as is the structure of craniofacial cartilage as revealed by alcian blue staining (4 dpf). Embryonic haematopoiesis appears to proceed normally, as determined by RNA in situ hybridization with gata1, lcp1/II-plastin, and ikaros-ikaros-specific probes at 1 dpf. The thymus does not contain ikaros-expressing cells despite the presence of ikaros-positive neural structures (Figure S3).

(d) *tnpo3* mutant. The HA343 mutant line (allele designation t22074) was identified in the Tübingen 2000 screen as a result of significantly reduced numbers of ragl-expressing cells in the thymus at 5 dpf. The mutation is autosomal recessive and maps to chromosome 4, close to marker 3751_2 (genetic distance from mutant locus ~ 0.3cM; 3 recombination events in 948 meioses); two other markers were found to be closely linked: z9247 (Genbank accession number G40755; 8 recombination events in 948 meioses); 3751_2 (CCAAGCGTTGTTGCAATGG and T CCTATCGAATCTCCCTATTG [nt 12,634,333 to 12,634,452 and 12,634,178 to 12,634,197]). Among the genes mapped to the critical interval, *tnpo3* (ENSDARG00000045680) encodes a protein of 923 amino acid residues (ENSDARP00000067160); a non-sense mutation (C>T transversion at nucleotide 4:12,656,091; Zv9.70) converts residue 203 to a stop codon (R203X) (Figure S5). Genotyping of fish was carried out by PCR on genomic DNA using primers AGGACTCCCTGGAAGACCTG and TGTGATCAGGTGACGTGTG (amplicon size 364 bp); sequencing was carried out using primer AGGACTCCCTGGAAGACCTG. When HA343 mutants were injected with mRNA encoding wild-type mouse *Tnpo3*, higher thymopoietic indices were observed; no rescue was observed when the corresponding mutant form was injected (Figure S5). This inter-specific rescue indicates substantial conservation of protein function between fishes and mammals and that the mutation in *tnpo3* causes the observed phenotype. The aberrations in *tnpo3* mutants appear to be tissue-specific. The early steps of haematopoiesis in mutant larvae are indistinguishable from those in wild-type siblings as revealed by the pattern of fluorescent cells in the *ikaros:eGFP* transgenic background (Figure S5) and expression patterns of early hematopoietic markers (Figure S5); the expression patterns of a marker gene for neural crest (dlx2) was normal (Figure S5). Finally, although *ikaros*-expressing cells fail to accumulate in the thymus, the formation of *ikaros*-expressing neural structures appears to be normal (Figure S5). The thymic epithelium, a derivative of pharyngeal endoderm expressing the transcription factor gene *foxn1*, appears to be functional, as indicated by its undisturbed colonization by wild-type cells after their transplantation into *tnpo3* mutant embryos (Figure S6).

Loss of *tnpo3* does not generally impair proliferation, survival and or differentiation of mutant cells; for instance, rapidly proliferating cells in the central nervous system (here exemplified by *ikaros*-expressing neurons in the hindbrain, growth hormone-expressing neurons in the hypophysis, the eye (Figure S5) and cranifacial structures (Figure S5). The defects associated with *tnpo3* deficiency are not restricted to developing T cells; for instance, in *tnpo3*-deficient zebrafish larvae, the number of goblet cells in the intestine is significantly reduced (Figure S5), further emphasizing the cell-type specific requirements of *tnpo3* function in some but not all cell types, and possibly related to the aberrations in the gene expression patterns associated with the digestive system.

(e) Other genes: The detailed characterization of cstf3, pole1, mcm10, dmt1, and zhb17 zebrafish mutants will be described elsewhere.

Whole zebrafish genome sequencing. To generate a generic library for the KW059 line, a total of 100 mutant 5 dpf embryos (as judged by reduced ragl signals after RNA in situ hybridization) from 10 different mating pairs were pooled. After purification of genomic DNA, 5µg were sheared to ~200 bp fragment size using a Covaris S2 sonicator. Libraries were constructed with the NEBNext Ultra DNA library Prep Kit for Illumina (New England Biolabs), according to the manufacturer's recommendations. Quality controls included assessment of size distribution using an Agilent Bioanalyzer, determination of DNA concentration using Qubit and a KAPA Library Quant Illumina kit (Pepqlab). The fragments were sequenced in paired-end 50 bp mode on 2 lanes of an Illumina HiSeq 2500 instrument. The most frequent depth coverage was ~12X, as determined by the "create a histogram of genome coverage" tool from BEDtools (Quinlan and Hall, 2010).

For genomic libraries of wild-type strains, 25 embryos each of TÜ, WIK and ASS fish were used. After purification of genomic DNA, 3µg were sheared to ~300 bp fragment size using a Covaris S220 sonicator. Sonication was followed by a clean-up step by adding the same volume of Agencourt
AMPure XP magnetic beads (Beckman Coulter). Libraries were constructed with the NEXTflex PCR-free DNA Sequencing Kit (Illumina compatible) (Bio Scientific), according to the manufacturer’s recommendations followed by quality control assessment as described above. Sequencing was carried out in the paired-end 100 bp run mode on an Illumina HiSeq 2500 instrument using 2 lanes/sample. The most frequent depth coverage was ~50X for the TU strain, and ~55X each for the WIK and ASS strains. The genomic library for the I032 mutant line was generated with 50 mutant embryos, pooled from 4 different mating pairs at 5dpf. The library was prepared in the same way as for the WT strains; sequencing of the I032 mutant genome was carried out using 1 lane of the HiSeq 2500. Sequencing reads were mapped to the Zv9.70 reference genome using the Bowtie 2 program (Langmead and Salzberg, 2012). Mapping duplicates were removed using Picard Mark Duplicates feature (http://picard.sourceforge.net) in Galaxy (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010). BAM files from KW059 and the three wild-type strains were visualized in parallel on IGV (Integrative Genomic Viewer) (Robinson et al., 2011; Thorvaldsdóttir et al., 2013) using a threshold of 1 for the minimum mapping quality and Zv9.70 as reference genome. For the KW059 mutant, the coding regions within the critical interval were assessed manually. In the genomic sequence of the I02 mutant, a non-sense mutation was identified in the ikzf1/ikaros gene (allele designation t25880) using a customary bioinformatic pipeline (manuscript in preparation) and verified by complementation analysis with the IT325 mutant.

Morphants. Morphants were generated by injection of anti-sense morpholino oligonucleotides (Gene Tools, Philomath, OR) encompassing the sequences of either initiation codons to block translation of both maternal and zygotic mRNAs, or splice donor and/or acceptor sites to block processing of zygotic mRNAs (leaving processed maternal mRNAs intact). Stock solutions were diluted as needed and the final concentration in the injection buffer indicated; approximately 1-2 nl of solution were injected into fertilized eggs as described (Schorpp et al., 2006).

Preparation of mRNAs for phenotypic rescue. For rescue experiments, mRNAs were injected into fertilized eggs and analysis carried out by gh and rag1 RNA in situ hybridization at various time points after injection.

(a) lsm8. A cDNA fragment containing the complete zebrafish lsm8 coding region was amplified using primers CCGGATCCGTATTATCGCTTCCGCTC and CCGGAATTCCGCTGCTGTTATAGATGCCTGCAG, digested with BamHI and EcoRI (restriction sites underlined in primer sequences), and subcloned into BamHI and EcoRI sites of the pCS2+ expression vector (Invitrogen). After linearization by NotI digestion, the plasmid was transcribed from the SP6 promoter using the mMESSAGE mMACHINE kit (Ambion). RNA was dissolved at 0.1mg/ml and 1-2 nl injected into 1-cell embryos from lsm8−/− intercrosses.

(b) gemin5. A full-length mouse Gemin5 cDNA (Clone ID C330013N08 [Imagenes, Berlin, Germany]) in the pFLCI vector was used as the source of wild-type Gemin5. To create the mouse equivalent of the zebrafish gemin5 non-sense mutation, nucleotide 1362 (T) (equivalent to nucleotide 1448 in Genbank accession number NM_001166669) was substituted for G using primer-directed mutagenesis to create the Y454X mutation. After linearization by PciI digestion, the wild-type and mutant Gemin5 plasmids were transcribed from the T7 promoter using the mMESSAGE mMACHINE kit (Ambion). RNAs were dissolved at 0.3mg/ml and 1-2 nl injected into 1-cell embryos from gemin5−/− intercrosses.

(c) tnpo3. A full-length mouse Tnpo3 cDNA (clone ID: 6313452, Source Biosciences) in the pCMV-SPORT6.1 vector was used as a wild-type form of Tnpo3. To create the mouse equivalent of the zebrafish non-sense mutation, nucleotide 607 (C) (equivalent to nucleotide 729 in Genbank accession number NM_177296) was substituted for T using primer-directed mutagenesis to create the R203X mutation. After linearization by NotI digestion, the wild-type and mutant Tnpo3-containing plasmids were transcribed from the SP6 promoter using the mMESSAGE mMACHINE kit (Ambion). RNAs were dissolved at 0.2mg/ml and 1-2 nl injected into 1-cell embryos from tnpo3−/− intercrosses.

Cell transplantation. EGFP+ cells were sorted from single-cell suspensions of kidney marrow cells isolated from adult ikaros:egFP transgenic zebrafish using FACS Aria (Becton & Dickinson) flow cytometer. 5-10 nl of 20,000 cells/ml of sorted cells in 0.9x Dulbecco’s PBS containing 1% rhodamine B-isothiocyanate Dextran (Sigma) were injected into the sinus venosus of embryos from heterozygous intercrosses at 2 dpf. GFP+ cells in the thymic region were counted using Imager.Z1 (Zeiss) at 3 days post injection.
RNA in situ hybridization. Procedures for RNA in situ hybridization and probes were described previously (Schorpp et al., 2006). The runx1 probe consisted of nucleotides 116 to 1796 in Genbank accession number NM_131603.

Thymopoietic index. Determination of rag1/gh ratios was carried out as follows: After RNA in situ hybridization with rag1 and gh probes, ventral images of 4-5 dpf zebrafish larvae were taken on an MZFL.III (Leica) microscope using a digital camera DFC300FX (Leica), essentially generating a two-dimensional projection of the three-dimensional structure. The areas of rag1 and gh signals were measured using ImageJ (NIH), and the ratio of the average of the rag1-positive area vs gh-positive area was calculated as a measure of thymopoietic activity. After photographic documentation of the RNA in situ hybridization signal, larvae were processed for genomic DNA extraction for subsequent genotyping, where required.

Live microscopy of fish. The procedures for live imaging using the ikaros:eGFP transgenic background were described previously (Hess and Boehm, 2012).

Tnpo3 conditional knock-out mice. The ES cell line EPD0318_3_G02 (genetic background: C57BL/6N Agouti(A/a); allele name: Tnpo3\(^{\text{fl/\text{flox}}}[\text{KOMP}^{\text{Wtsi}}]\) was obtained from the KOMP Repository and used to derive chimaeric mice using standard procedures. Chimaeras were crossed with mice constitutively expressing FLP recombinase (B6:SJL-Tg[ACTFLPe]9205Dym/J [Jackson Laboratory stock number 003800]) (Rodriguez et al., 2000) to remove the lacZ/neo cassette which is flanked by FRT sites. As a result, the ES-derived allele of Tnpo3 possesses an exon 7 flanked by loxp sites. Constitutive deletion of exon 7 was achieved by crossing heterozygous mice with mice with a universal Cre-deleter strain (B6.C-Tg(CMV-cre)1Cgn/J [Jackson Laboratory stock number 006054]) (Schwenk et al., 1995); Tnpo3 deficiency is embryonic lethal (data not shown). T cell-specific deletion was achieved by crosses with mice transgenic for an Lck:Cre construct (Orban et al., 1992). Wild-type and floxed alleles of Tnpo3 were amplified from genomic DNA using primers GGAATTCAGTGCTCTGTACC and TCCAGCTGGATCCATGC (amplicon sizes: wild-type allele, 227 bp; floxed allele, 342 bp); the deleted allele was amplified using primers GGAATTCAGTGCTCTGTACC and CAATTCCTGAAGCCACCTGT (amplicon sizes: deleted allele, 266 bp; wild-type allele, 947 bp; floxed allele, 1,072 bp). Partial Tnpo3 cDNAs were amplified using primers CGAAGCTGCTTCAGACTGTG (located in exon 6) and ATGTTCTCCTAGTCGGTACC (located in exon 8); amplicon size of the wild-type form, 331 bp; of the mutant form, 192 bp. The phenotype of Tnpo3\(^{\text{fl/\text{flox}}}\); Lck:Cre and Tnpo3\(^{\text{fl/\text{flox}}}\); Lck:Cre mice is indistinguishable (data not shown).

Flow cytometry. For analysis of Tnpo3 mutant mice, analytical flow cytometry was carried out as described in(Calderón and Boehm, 2012) using the following antibodies: FITC-conjugated anti-CD4 (clone GK1.5; BioLegend); PE-conjugated anti-CD8 (clone Ly-2; eBioscience); PerCP-Cy5.5-conjugated anti-CD8epison (clone 145-2C11; Biolegend); APC-conjugated anti-CD19 (clone MB19-1; eBioscience); FITC-conjugated anti-TCRgammadelta (clone eBioGL3; eBioscience); PE-conjugated anti-TCRb (clone H57-597; eBioscience). PE-conjugated ati-CD5 (clone 53-7.3, eBiosciences), FITC-conjugated Annexin V (eBiosciences). For preparative flow cytometry, the following antibodies were used: APC-Cy7-conjugated anti-CD4 (clone GK1.5; Biolegend); FITC-, or APC-conjugated anti-CD8 (clone 53.6.7; eBioscience); PerCP-Cy5.5-conjugated anti-TCRb (clone H57-597; Biolegend); PE-Cy7-conjugated anti-CD24 (clone M1/69; eBioscience).

TUNEL staining. To detect apoptotic cells in situ, tissue sections of thymus, spleen and lymph nodes were examined using the TACS 2 TdT-DAB in situ apoptosis detection kit (Trevisgen) according to the manufacturer’s instructions. For thymus and lymph node sections, Mn\(^{2+}\) cations, for spleen sections, Co\(^{2+}\) cations were used in the reaction.

EdU staining. To measure cell proliferation, mice were injected intraperitoneally with 200\(\mu\)l of 10mM EdU. Cells were harvested 16 hours later and processed using the Click-iT Edu Flow Cytometry Assay Kit (Invitrogen) according to the manufacturer’s recommendation.
**RNA isolation and sequencing.** Total RNA was extracted from purified mouse CD4⁺ CD8⁻ TCRβ₁ CD24⁺ high (hereafter referred to as DP) and CD4⁺ CD8⁻ TCRβ₁ high CD24⁻ low (hereafter referred to as CD4⁺ SP) cells using RNeasy Mini kit (Qiagen), including a DNaseI treatment step.

**RNA extraction and cDNA synthesis.** Total RNA extracted using TRI Reagent (Sigma) following the manufacturer's instructions. After treatment with Cloned DNaseI (Takara), RNA extraction using TRI Reagent was repeated. Superscript II Reverse Transcriptase (Invitrogen) and random hexamer primers were used for cDNA synthesis from total RNA.

**RT-PCR.** Quantitative PCR was carried out as described (Rode and Boehm, 2012) using SYBR Premix Ex Taq (Takara) and 7500 fast real-time PCR system (Applied Biosystems); primers are listed in Table S8; primer sequences for zebrafish rag1 and actb1 genes were taken from Lam et al. (Lam et al., 2004). RT-PCR for ptprc splice forms was carried out using a forward primer (TTTGGTCCCTATGCTTGGTCC) located in exon 1 and a reverse primer (CAGTTGTCAACGTGGATAGTG) located in exon 4.

**RNA sequencing.** Total RNA was extracted from whole zebrafish larvae at 4 dpf and subjected to transcriptome analysis. RNA quality was determined using the 2100 Bioanalyzer instrument (Agilent) and RNA concentration was determined using Qbit 2.0 Fluorometer (Life Technologies). To examine gene expression levels and splicing patterns, 350 ng of total RNAs (using three biological replicates per condition) were used for library preparation using the TruSeq Stranded Total RNA kit with Ribo-Zero (Human/Mouse/Rat; Illumina). The libraries were sequenced in paired-end 75 bp mode on 0.8 lanes per sample on an Illumina HiSeq 2500 instrument.

**Computational analysis of RNA-seq data.** The high throughput RNA sequencing analysis pipeline v0.4.2 written by Fabian Kilpert (https://github.com/kilpert/rna-seq-qc) was applied as outlined below:  
(a) Alignment of RNA-seq reads. The raw RNA-seq reads were mapped against the zebrafish genome assembly Zv9 with the spliced read aligner TopHat2 version 2.0.13 (Kim et al., 2013). Reads were mapped to the transcriptome provided by the zebrafish gene annotation of Ensembl v78 (Harrow et al., 2012). The mean length of the sequenced fragments and the standard deviation of the distribution as well as the type of the strand-specific library were determined on a subsample of 1 million reads with RSeQC version 2.4 (Wang et al., 2012) and provided to TopHat2. All other TopHat2 parameters were set to default. 
(b) Analysis of differential gene expression. The numbers of sequenced fragments per annotated gene (Ensembl release 78 gene models) were quantified with featureCounts version 1.4.5-p1 (Liao et al., 2014). Fragments were only counted if both ends aligned on the same chromosome with a minimum mapping quality of 10 and in agreement with the strand orientation of the annotated gene. Differentially expressed genes were called using the Bioconductor package limma version 3.26.3 (Ritchie et al., 2015). Although the wild-type fish replicates originated from distinct genetic backgrounds (n=3), only one of which was matching the mutant fish replicates (n=3), the low number of samples in the combination of the genotype and background factors (minimum n=1) resulted in reduction of degrees of freedom and statistical power after fitting a combined model. Therefore, a univariate model was fitted with limma, chosen for its stringency to extract differentially expressed genes highly consistent between replicates, and thus not affected by their individual genetic backgrounds. The differentially expressed gene list was filtered for FDR<5% and absolute log 2-fold change ≥ 1. Functional enrichment analysis of genes with significant alternative splicing events was performed with the DAVID Functional Annotation Clustering tool (database version 6.7) (Huang et al., 2009a, b) using the mouse orthologs of zebrafish genes extracted from Ensembl v78 annotation. 
(c) Analysis of differential alternative splicing events. The BAM files with TopHat2 aligned reads were filtered to include only those reads that overlap with annotated transcripts in the correct strand orientation. The filtered BAM files for different genotypes were then used as input for the differential alternative splicing (AS) analysis with rMATS version 3.0.9 (Shen et al., 2012). Of all reads, rMATS only considers unique, properly paired reads that are mapped without insertions and deletions. The mean fragment size of the paired-end samples and its standard deviation were determined as described above and provided to rMATS. All other parameters were set to default. The reported AS events are of the following types: exon skipping, retained intron, mutually exclusive exons, alternative 5’ donor or 3’ acceptor splice sites. We selected for each type all AS events with at least 5% difference in the mean...
exon inclusion levels (mutant vs. wild-type) that were identified by reads spanning splice junctions at 1% FDR. Functional enrichment analysis of genes with significant AS events was performed with the DAVID Functional Annotation Clustering tool as described above (Huang et al., 2009a, b). For each class of AS events in rMATS output (e.g. exon skipping), DAVID term enrichment was performed by comparing gene set representation in the list of genes with significant AS events (foreground) and to the list of genes with all tested AS events of that class (background). RNA-seq data are deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO accession number GSE77480.
SUPPLEMENTAL REFERENCES

Akimenko, M.-A., Ekker, M., Wegner, J., Lin, W., and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. J. Neurosci. 14, 3475-3486.

Bennett, C.M., Kanki, J.P., Rhodes, J., Liu, T.X., Paw, B.H., Kieran, M.W., Langenau, D.M., Delahaye-Brown, A., Zon, L.I., Fleming, M.D., et al. (2001). Myelopoiesis in the zebrafish, Danio rerio. Blood 98, 643-651.

Benz, C., and Bleul, C.C. (2005). A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. J. Exp. Med. 202, 21-31.

Benz, C., Heinzel, K., and Bleul, C.C. (2004). Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. Eur. J. Immunol. 34, 3652-3663.

Blankenberg, D., Von Kuster, G., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., Nekrutenko, A., and Taylor, J. (2010). Galaxy: a web-based genome analysis tool for experimentalists. Curr. Protoc. Mol. Biol. 19, 10.11-19.10.21.

Bröske, A.M., Vockentanz, L., Kharazi, S., Huska, M.R., Mancini, E., Scheller, M., Kuhl, C., Enns, A., Prinz, M., Jaenisch, R., et al. (2009). DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. Nat. Genet. 41, 1207-1215.

Casanova, J.-L., Holland, S.M., and Notarangelo, L.D. (2012). Inborn errors of human JAKs and STATs. Immunity 36, 515-528.

Ceredig, R., and Rolink, A.G. (2012). The key role of IL-7 in lymphopoiesis. Semin. Immunol. 24, 159-164.

Gering, M., Rodaway, A.R.F., Göttgens, B., Patient, R.K., and Green, A.R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. EMBO J. 17, 4029-4045.

Giardine, B., Riemer, C., Hardison, R.C., Burhans, R., Elnitski, L., Shah, P., Zhang, Y., Blankenberg, D., Albert, I., Taylor, J., et al. (2005). Galaxy: a platform for interactive large-scale genome analysis. Genome Res. 15, 1451-1455.

Goecks, J., Nekrutenko, A., Taylor, J., and The Galaxy Team (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol. 11, R86.

Greenhalgh, P., and Steiner, L.A. (1995). Recombination activating gene 1 (Rag1) in zebrafish and shark. Immunogenetics 41, 54-55.

Greig, K.T., Carotta, S., and Nutt, S.L. (2008). Critical roles for c-Myb in hematopoietic progenitor cells. Semin. Immunol. 20, 247-256.

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J.M., Jiang, Y.-J., Heisenberg, C.-P., et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development 123, 1-36.

Harrow, J., Frankish, A., Gonzalez, J.M., Tapanari, E., Diekhans, M., Kokocinski, F., Aken, B.L., Barrell, D., Zadissa, A., Searle, S., et al. (2012). GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 22, 1760-1774.

Hogan, B.M., Hunter, M.P., Oates, A.C., Crowhurst, M.O., Hall, N.E., Heath, J.K., Prince, V.E., and Lieschke, G.J. (2004). Zebrafish gcm2 is required for gill filament budding from pharyngeal ectoderm. Dev. Biol. 276, 508-522.
Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1-13.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44-57.

Johnson, S.L., Africa, D., Horne, S., and Postlethwait, J.H. (1995). Half-tetrad analysis in zebrafish: mapping the ros mutation and the centromere of linkage group I. Genetics 139, 1727-1735.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Kosan, C., Saba, I., Godmann, M., Herold, S., Herkert, B., Eilers, M., and Möröy, T. (2010). Transcription factor Miz-1 is required to regulate interleukin-7 receptor signaling at early commitment stages of B cell differentiation. Immunity 33, 917-928.

Lam, S.H., Chua, H.L., Gong, Z., Lam, T.J., and Sin, Y.M. (2004). Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Dev. Comp. Immunol. 28, 9-28.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357-359.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923-930.

Lieu, Y.K., and Reddy, E.P. (2009). Conditional c-myb knockout in adult hematopoietic stem cells leads to loss of self-renewal due to impaired proliferation and accelerated differentiation. Proc. Natl. Acad. Sci. USA 106, 21689-21694.

Lyons, S.E., Lawson, N.D., Lei, L., Bennett, P.E., Weinstein, B.M., and Liu, P.P. (2002). A nonsense mutation in zebrafish gata1 causes the bloodless phenotype in vlad tepes. Proc. Natl. Acad. Sci. USA 99, 5454-5459.

Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J., Jr., and Potter, S.S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell 65, 677-689.

Oie, S., Lowenthal, D.T., Briggs, W.A., and Levy, G. (1975). Effect of hemodialysis on kinetics of acetaminophen elimination by anephric patients. Clin. Pharmacol. Ther. 18, 680-686.

Phan, R.T., Saito, M., Basso, K., Niu, H., and Dalla-Favera, R. (2005). BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. Nat. Immunol. 6, 1054-1060.

Pursell, Z.F., and Kunkel, T.A. (2008). DNA polymerase ε: A polymerase of unusual size (and complexity). Prog. Nucleic Acid Res. Mol. Biol. 82, 101-145.

Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26, 841-842.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 43, e47.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttmman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. 29, 24-26.
Rodríguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-\textit{loxP}. Nat. Genet. 25, 139-140.

Saba, I., Kosan, C., Vassen, L., Klein-Hitpass, L., and Möröy, T. (2011a). Miz-1 is required to coordinate the expression of TCR\(\beta\) and p53 effector genes at the pre-TCR "\(\beta\)-selection" checkpoint. J. Immunol. 187, 2982-2992.

Saba, I., Kosan, C., Vassen, L., and Möröy, T. (2011b). IL-7R-dependent survival and differentiation of early T-lineage progenitors is regulated by the BTB/POZ domain transcription factor Miz-1. Blood 117, 3370-3381.

Schorpp, M., Leicht, M., Nold, E., Hammerschmidt, M., Haas-Assenbaum, A., Wiest, W., and Boehm, T. (2002). A zebrafish orthologue (\textit{whnb}) of the mouse \textit{nude} gene is expressed in the epithelial compartment of the embryonic thymic rudiment. Mech. Dev. 118, 179-185.

Schorpp, M., Wiest, W., Egger, C., Hammerschmidt, M., Schlake, T., and Boehm, T. (2000). Genetic dissection of thymus development. Curr. Top. Microbiol. Immunol. 251, 119-124.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A \textit{cre}-transgenic mouse strain for the ubiquitous deletion of \textit{loxP}-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23, 5080-5081.

Shen, S., Park, J.W., Huang, J., Dittmar, K.A., Lu, Z.X., Zhou, Q., Carstens, R.P., and Xing, Y. (2012). MATS: a Bayesian framework for flexible detection of differential alternative splicing from RNA-Seq data. Nucleic Acids Res. 40, e61.

Thorvaldsdóttir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. 14, 178-192.

Thu, Y.M., and Bielinsky, A.-K. (2013). Enigmatic roles of Mcm10 in DNA replication. Trends Biochem. Sci. 38, 184-194.

Wang, L., Wang, S., and Li, W. (2012). RSeQC: quality control of RNA-seq experiments. Bioinformatics 28, 2184-2185.

Willett, C.E., Kawasaki, H., Amemiya, C.T., Lin, S., and Steiner, L.A. (2001). Ikaros expression as a marker for lymphoid progenitors during zebrafish development. Dev. Dyn. 222, 694-698.

Willett, C.E., Zapata, A.G., Hopkins, N., and Steiner, L.A. (1997). Expression of zebrafish \textit{rag} genes during early development identifies the thymus. Dev. Biol. 182, 331-341.

Yoshida, T., Ng, S.Y.-M., and Georgopoulos, K. (2010). Awakening lineage potential by Ikaros-mediated transcriptional priming. Curr. Opin. Immunol. 22, 154-160.