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**Conserved IL-2Rγc Signaling Mediates Lymphopoiesis in Zebrafish**

Robert Sertori,∗† Clifford Liongue,∗† Faiza Basheer,∗† Kanako L. Lewis,‡
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The IL-2 receptor γ common (IL-2Rγc) chain represents the shared component of the receptors for the IL-2 family of cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, each of which regulates different aspects of immune development and function (1–4). Specifically, IL-2 stimulates the growth, differentiation, and activation of various T and NK cell populations (5); IL-4 is involved in B cell proliferation, Ig class switching, and Th2 cell development (6); and IL-7 contributes to the development, survival, and homeostatic proliferation of T cells, especially memory T cells (7). IL-9 exerts a wider range of effects, such as mediating the growth and functional activation of T cells and mast cells, and supporting the differentiation of hematopoietic stem cells (8), whereas IL-15 regulates the proliferation, differentiation, and survival of many cell types, including B cells, NK cells, mast cells, neutrophils, eosinophils, monocytes/macrophages, and dendritic cells (9, 10). Finally, IL-21 stimulates lymphoid cell proliferation and the differentiation of B cells to plasma cells, and it regulates apoptosis in B and NK cells (4).

Interleukin-2 receptor γ common (IL-2Rγc) chain is the shared component of the receptors for IL-2 family of cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, each of which regulates different aspects of immune development and function (1–4). Specifically, IL-2 stimulates the growth, differentiation, and activation of various T and NK cell populations (5); IL-4 is involved in B cell proliferation, Ig class switching, and Th2 cell development (6); and IL-7 contributes to the development, survival, and homeostatic proliferation of T cells, especially memory T cells (7). IL-9 exerts a wider range of effects, such as mediating the growth and functional activation of T cells and mast cells, and supporting the differentiation of hematopoietic stem cells (8), whereas IL-15 regulates the proliferation, differentiation, and survival of many cell types, including B cells, NK cells, mast cells, neutrophils, eosinophils, monocytes/macrophages, and dendritic cells (9, 10). Finally, IL-21 stimulates lymphoid cell proliferation and the differentiation of B cells to plasma cells, and it regulates apoptosis in B and NK cells (4).

The IL-2Rγc chain acts as the major signal transduction component of the IL-2 receptor family. Ligand binding stimulates the activation of the tyrosine kinase JAK3 associated with the intracellular region of IL-2Rγc, which activates a number of downstream intracellular pathways, including those involving STAT5, PI3K, and ERK, the latter lying downstream of MEK (11, 12). Inactivating mutations in human IL-2Rγc cause T B+ SCID, characterized by decreased numbers of T cells and a diminished immune response (13). Mice lacking IL-2rγc display a T B+ form of SCID (14), with reduced B and T cells (15), and have proved to be an invaluable model for a range of studies, especially relating to immunity and cancer (16–21).

The zebrafish is now established as an important alternate model for the study of vertebrate development and disease, with particular relevance to hematopoiesis and immunity (22, 23). Like mammals, zebrafish undergo distinct phases of hematopoiesis, with lymphoid progenitors seeding the thymus early in development following the establishment of definitive hematopoiesis (24). Zebrafish also shows broad conservation of cytokine receptors and downstream signaling pathway components (25, 26), particularly those known to facilitate the development of blood and immune cells (27–31).

We have previously identified two putative paralogs of the IL-2Rγc gene in zebrafish, il-2rγca and il-2rγcb (25), but their roles have not been elucidated. In this study, we show that il-2rγca, but not il-2rγcb, is involved in early zebrafish lym-
phosphorylation. The il-2rα gene is expressed in the developing thymus and adult lymphocytes, with ablation of il-2rα leading to a significant and specific reduction in T lymphopoiesis. This phenotype could be mimicked by targeting zebrafishJak3 by either morpholino-mediated knockdown or pharmacological inhibition, indicating a conserved role for IL-2Rα/JAK3 signaling across vertebrate lymphopoiesis, with contributions from the STAT5, PI3K, and MEK/ERK pathways also being identified.

Materials and Methods

Nomenclature conventions

Nomenclature rules for zebrafish, fugu, chicken, mouse, and humans and proteins differ. Gene and protein names are presented according to the respective nomenclature conventions (zebrafish and fugu: il-2rα, il-2rβ, and mouse: Il-2rα, Il-2rβ, and Il-2rγ).

Analysis of il-2rα paralogs from zebrafish

Expressed sequence tags corresponding to the two putative Danio rerio (zebrafish) IL-2R-α paralogs, il-2rα and il-2rα, were identified with BLASTX (32), with additional sequences obtained from RT-PCR and 5′ RACE products. These sequences were assembled using Sequencer (Gene Codes Corporation) and corresponded to full-length reference sequences deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank/; accession number NP_001121743.1 il-2rα, NP_001116522.1 il-2rβ). Multiple alignment of the encoded protein sequences was performed using the CLUSTALX program (33), along with zebrachthy thymic stromal lymphopoietin receptor (TSLpr) (CAM88660.1), IL-13a1 (CAH09493.1) and IL-13a2 (NP_001107203.1), Homo sapiens IL-2Rγ (AA391451.1), thymic stromal lymphopoietin receptor (TSLPR) (NP_071431.2), IL-13Ra1 (EAW99893.1) and IL-13Ra2 (AAH07293.1), Mus musculus (mouse) IL-2Rγ (AAH14720.1), TSLPR (NP_051258.01.1), IL-13Ra1 (AAH59399.1) and IL-13Ra2 (EDL14709.1), Gallus gallus (chicken) IL-2Rγ (NP_098588.1), TSLPR (XP_416864.3), IL-13Ra1 (XP_420218.3) and IL-13Ra2 (NP_0014543.1), and Takifugu rubripes (fugu) il-2rα (NP_001128545.1) and IL-13ra2 (XP_00397136.1). A phylogenetic tree was derived from this alignment using the Neighbor-Joining algorithm and visualized with NjPlot (34). Genomic sequences corresponding to the zebrafish il-2rα and il-2rβ genes were identified using BLASTN, and the positions of exon/intron boundaries were determined by alignment with the corresponding zebrafish il-2rγ cDNA sequence, applying the ‘GT-AG’ rule (35).

Zebrafish husbandry and manipulation

Wild-type as well as Tg(lck:GFP-EGFP)1 (Lck:GFP) (36) and Tg(Cau:ighv-iglon:EGFP)1/2 (Ighv:GFP) (37) transgenic zebrafish were maintained using standard husbandry practices (38). Wild-type embryos at the 1–8-cell stage were injected with anti-sense morpholinos diluted in 1X Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM CaCl2, 50 mM HEPES, pH 9). Morpholinos were used: il-2rα (5′-CCTGTTT-CATTAATACATACCCGCCG, 3 mM), il-2rα (5′-CTTACGTGTGAA-CATCACAGTAC, 7 mM), il-2rβ (5′-GCACCGTAAACAATATA-TGATCG, 5 mM), jak3 (5′-TATAAAGGTTGTGATGCATC-AC, 2 mM), stat5a (5′-TGAACAAGCTGTAGTTAGCAGGTT, 1 mM), stat5b (5′-GTGACAGCAGGATCCATCCATC, 1 mM) and standard control morpholino (5′-CCTTACCTTACCTCACTTTAAT-7A, 1 mM as appropriate). In some experiments, embryos were co-injected with 100 pg/ml mRNA encoding eGFP or constitutively active version of zebrafish JAK3 A572V (this study) or Stat5a1 (H29R/N714F) (39) and standard control and il-2rα-2α morpholinos. Alternatively, I-cell embryos were injected with 100 pg/ml mRNA encoding transcription activator-like effector nucleases (TALENs) (40) targeting exon 3 of il-2rα, raised to adulthood intercrossed and progeny screened by RFLP with Ndel to identify mutants. Embryos were also treated with the JAK3 inhibitor tofacitinib (41) at 30 and 60 μM, MEK inhibitor PD098059 (42), 45 μM and PI3K inhibitor LY294002 (44) at 15 μM from 56 h postfertilization (hpf) and then fixed at the appropriate time points. Single-cell suspensions were prepared from the thymus of Lck:GFP and kidney of Ighv:GFP transgenic zebrafish, as described (45). Sytox Red Dead Cell Stain was used for live cell discrimination (Molecular Probes), and cell sorting was performed using a FACSaria II (BD Biosciences). National and institutional guidelines for the care and use of laboratory animals were followed in all studies.

RT-PCR, quantitative RT-PCR, and 5′ RACE

Total RNA was extracted from 30 zebrafish embryos using Trizol reagent (Life Technologies) following the manufacturer’s recommendations and then resuspended in nuclease-free water. This RNA was subjected to semiquantitative RT-PCR with the following primers: il-2rα 5′-CAGCGTACGAGCACTTACAG and 5′-CTTACTATACCTAC- GACTGG (time course/sequencing), 5′-GAAGATCTGTTATAGGACC- TG and 5′-TGTCGGTACCTGGCGTGAAC (sequencing), 5′- TATGGTAAGAATATGGAGGA and 5′-AAGCCTACCTATCATCATCTGC- GGTTAGCT and 5′-GTTGGTTTCTCACCATACTG (sequencing), il-2rγ, 5′-TGAAGAAGCAGAACAGAGAGAC and 5′-GAAGACCC- GGCAAGATGGAGAGAGGAG (sequencing), jak3 5′-AACCTGAGAGACACCTTCAGA and 5′-GTTGAGACCCCTTCCCTTC (morphology titration), 5′-CAAGCTATTGTCACAGAGGCTCT and 5′-GAGCAGGTTTACCGAGAGAGG (gsp1), 5′-GGTGTGACTTGTGCAGACATC (gsp2), 5′-AACCCTTGCGTGGTG (new gsp1), il-2rβ, 5′-AAGACGCGTTCGTTG (gsp1), 5′-GATGAGGTTTACCGAGAGAGG (gsp2) and il-2rβ 5′-GAAAGTGTGTCGTCGTC (gsp3).

Total RNA was also extracted from zebrafish embryos, larvae, and pooled adult zebrafish tissues with RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol for animal tissues. This was subjected to semiquantitative RT-PCR with previously published primers for TCR-β chains (Vb1, S17,5, Vb12) and Ig heavy chains (igVH1, igVH4) (46, 47). Total RNA was also subjected to quantitative RT-PCR (QRT-PCR) with the following primers: il-2rα 5′-GTCATGCTGTGTATGCTGTG and 5′-GTCTTCACTATCCTGTGTCGTTG, il-2rγ 5′-AGAAAGACCCAGCAAGAG- GT and 5′-ATCTTCTCATTCATCCAGAC, jak3 5′-AACAGAGC- GACAGCAAGAGAG and 5′-GTGTCGACCACTTCCCTCC, pik3cy 5′-AGGGGCTGAGGTTGACCTT and 5′-CTTCACTCCTATCATC- CATCAGGACTGCT and map2k1 and map2k2 (48). Data were normalized to β-actin, and fold change was calculated using the ΔΔCt method.

Total RNA was extracted from sorted cells using Trizol reagent and DirectZol RNA MiniPrep spin columns (ZymoResearch). cDNA was prepared using QuantiTect Reverse Transcription Kit (Qiagen) and subjected to QRT-PCR with il-2rα, il-2rβ, lck, igm, and efa1 primers (37). Each primer set flanked split sites such that amplification of contaminating genomic DNA would produce considerably larger fragments in each case. Data were normalized to efa1, and fold change was calculated using the ΔΔCt method. Each primer set flanked split sites such that amplification of contaminating genomic DNA would produce considerably larger fragments in each case.

Genomic DNA analysis

Genomic DNA was obtained from pooled F1 embryos with QuickExtract following the manufacturer’s instructions. The following kit was used with specific primers for il-2rα and analyzed by RFLP with Ndel and by Sanger sequencing at the Australian Genome Research Facility.

Whole-mount in situ hybridization (WISH) and histochemistry

Embryos were dechorionated and fixed for 1-2 d in 4% (w/v) paraformaldehyde at 4°C prior to whole-mount in situ hybridization (WISH) with DIG-labeled anti-sense probes, as described (49, 50). Freshly anesthetized em-

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Transient expression and analysis in human 293T cells
Human 293T cells were grown to 50–80% confluency before transfection with pBKCMV or pBKCMV expressing zebrafish Jak3 and/or Stat5.1 using lipofectamine reagent (Life Technologies). After incubation at 37°C in 10% (v/v) CO₂ for 2 d, a total cell lysate was prepared and subjected to Western blot analysis with anti-phospho Stat5.1 (Millipore 05-495) and anti-GAPDH (Millipore CB1001).

Results
Two IL-2Rγc paralogs in zebrafish
We have previously identified two putative zebrafish paralogs of the mammalian IL-2Rγc gene, called il-2rγc.a and il-2rγc.b (25). The full-length sequences of these genes were assembled from expressed sequence tags and predicted mRNA sequences present in gene databases, supplemented by sequencing of RT-PCR and 5'RACE products, and the encoded zebrafish protein sequences deduced. A phylogenetic tree was generated using the human, mouse, chicken, fugu, and zebrafish IL-2Rγc sequences along with those of the closely related thymic stromal lymphopoietin receptor (TSLPR), with the more divergent IL-13Rα sequences used as an out-group (Fig. 1A). This analysis grouped the zebrafish Il-2rγc.a and Il-2rγc.b sequences in a clade with the other IL-2Rγc chains supported by strong bootstrapping values, which was distinct from the related TSLPR clade. Of the two zebrafish sequences, Il-2rγc.b was the more divergent.

Alignment of the human, mouse, chicken, fugu, and zebrafish IL-2Rγc chains confirmed the conservation of key domains in both zebrafish sequences including an extracellular cytokine homology domain, a transmembrane domain and an intracellular domain (data not shown), including a Box 1 motif (Fig. 1B). Importantly, Il-2rγc.a showed conservation of three of the four intracellular tyrosines present in mammalian counterparts, with one additional nonconserved tyrosine, whereas Il-2rγc.b possessed only one conserved tyrosine and showed considerable divergence in its extended cytoplasmic region.

Expression of zebrafish IL-2Rγc paralogs
RT-PCR and QRT-PCR were used to characterize the expression of il-2rγc.a and il-2rγc.b during zebrafish embryogenesis. Expression of il-2rγc.a was first apparent at 16 hpf and then increased particularly from 4 days postfertilization (dpf) during the establishment of lymphopoiesis (Fig. 1C) (24). Conversely, il-2rγc.b showed biphasic expression with strong expression at 8–24 hpf, which waned before increasing from 5 dpf to maximal expression at 6 dpf (Fig. 1D). WISH was performed using probes for il-2rγc.a and il-2rγc.b. Distinct expression within the thymus was only evident for il-2rγc.a (Fig. 1F), which was not observed for il-2rγc.b (Fig. 1H) or sense controls (Fig. 1E, 1G). In adult zebrafish, il-2rγc.a was broadly expressed, but with highest expression in the thymus, kidney, and spleen (Fig. 1I), which represent key lymphoid organs (45, 52), whereas il-2rγc.b expression was highest in the spleen (Fig. 1J). Further analysis in transgenic zebrafish lines revealed that expression of both il-2rγc.a and il-2rγc.b was higher in the lckb⁺ T cell population within the thymus (Fig. 1K) and the igm⁺ B cell population within the kidney (Fig. 1L).

Knockdown of il-2rγc.a but not il-2rγc.b affects lymphopoiesis
To investigate the functions of zebrafish il-2rγc.a and il-2rγc.b, a morpholino-mediated knockdown strategy was used (53), with morpholinos designed to interfere with splicing of the premRNA in the region encoding the cytokine homology domain (Supplemental Fig. 1A–C). The level of gene knockdown was determined by RT-PCR, which confirmed robust knockdown in embryos injected with 3 mM il-2rγc.aSS1, 7 mM il-2rγc.aSS2, and 5 mM il-2rγc.bSS morpholino, compared with those injected with standard control morpholin at the equivalent concentration (Supplemental Fig. 1D, 1E). Sequence analysis of alternate transcripts seen with il-2rγc.aSS1 and il-2rγc.aSS2 confirmed that these would encode severely truncated proteins in each case (Supplemental Fig. 1F).

Morpholino-injected embryos showed no overt phenotypes; however, analysis by WISH with an early lymphocytic marker, ikaros (54), revealed significantly decreased expression from 3 dpf in the thymus of embryos injected with il-2rγc.aSS1 (Fig. 2B, 2E) and il-2rγc.aSS2 (Fig. 2C, 2F) compared with those injected with the standard control morpholino (Fig. 2A), which continued at 5 dpf (Fig. 2I, 2K, and data not shown). In contrast, ikaros expression in the thymus was not significantly altered in il-2rγc.bSS-injected embryos at any time point tested (Fig. 2D, 2G, 2J, 2L).

To confirm these effects, embryos were analyzed with a range of other lymphocyte markers. Significantly decreased expression of rag1, a marker of more mature lymphoid cells (55), was also observed 4–6 dpf in embryos injected with il-2rγc.aSS1 (Fig. 2N, 2Q, 2U, 2W, and data not shown) and il-2rγc.aSS2 (Fig. 2O, 2R, and data not shown), but not those injected with il-2rγc.bSS (Fig. 2P, 2S, 2V, 2X). This effect on lymphopoiesis was confirmed with the decreased expression of other lymphocyte-specific markers, tcrα and lck (36, 56), at 5 dpf in embryos injected with il-2rγc.aSS1 (Fig. 2Z, 2D') or il-2rγc.aSS2 (Fig. 2A', 2E') relative to controls (Fig. 2Y, 2C'), which was not observed with il-2rγc.bSS (Fig. 2B', 2F').

To analyze the effects of il-2rγc.a ablation on mature T and B cells, TALEN-mediated genome targeting was used because the effects of morpholinos are only transient (57, 58). Specific TALENs directed to exon 3 (Supplemental Fig. 2A) were injected into one-cell embryos, which were raised to adulthood and intercrossed. One pair produced ~25% progeny that recapitulated the loss of rag1 at 5 dpf (Supplemental Fig. 2E–H compared with Supplemental Fig. 2B–D), which were shown to have both il-2rγc.a alleles mutated by RFLP (Supplemental Fig. 2I) and sequence analysis (Supplemental Fig. 2J). Siblings from the cross were raised to 28 dpf and analyzed for the expression of rearranged TCR-β and Ig genes, as markers of mature T and B cells, respectively (46) (Fig. 2G'), and also genotyped (Fig. 2H'). This demonstrated the presence of mature T and B cells in wild-type larvae, as expected. In contrast, larvae carrying two il-2rγc.a mutant alleles lacked mature T cells, although mature B cells were present, albeit less consistently than in wild-type larvae.

It was important to determine whether the role of il-2rγc.a was confined to lymphopoiesis or whether it also exerted an effect on hematopoiesis more generally. Therefore, embryos were stained with O-dianisidine to visualize hemoglobin as a measure of erythrocytes (59) and subjected to WISH with lysozyme (lys) to mark leukocytes (60). No significant differences in either the extent of O-dianisidine staining (Supplemental Fig. 3A, 3B and data not shown) or the number of lyz⁺ cells (Supplemental Fig. 3C, 3D) were observed in embryos injected with either il-2rγc.aSS1 or il-2rγc.aSS2 compared with standard control morpholin. Similarly, expression of c-myb, a marker of hematopoietic stem–progenitor cells (61), at 3 dpf, was equivalent between il-2rγc.a morphants and controls (Supplemental Fig. 3E, 3F, and data not shown). Collectively, this result indicates a lymphocyte-specific function for IL-2rγc.a.

IL-2Rγc signaling components are conserved
Having identified a conserved functional role for the Il-2rγc.a paralog in zebrafish lymphopoiesis, it was of interest to determine
whether the downstream signaling pathways were also conserved. IL-2Rγc signals via JAK3, with zebrafish previously shown to possess a single, highly conserved jak3 gene (26). This signaling component, which was also strongly expressed in the developing thymus (Supplemental Fig. 4), was targeted by two approaches. In the first approach, embryos were treated with the JAK3-specific inhibitor tofacitinib (41) compared with a DMSO control from 56 hpf, prior to the onset of lymphopoiesis (62) (Fig. 3A–3D). Alternatively, embryos were injected with a morpholino targeting a splice site within jak3 (Fig. 3E–3H). Both approaches substantially reduced expression of rag1 (Fig. 3A–3G), along with other lymphocyte markers (data not shown).

FIGURE 1. Characterization of zebrafish il-2rγc genes and their targeting with morpholinos. (A) Phylogenetic analysis of IL-2Rγc proteins. A phylogenetic tree was constructed from a ClustalX multiple alignment of zebrafish (Dr) IL-2rγc.a, IL-2rγc.b, IL-13rα1, IL-13rα2, and Ts1pr along with the human (Hs), mouse (Mm), chicken (Gg), and fugu (Tr) IL-2Rγc, IL-13Rα1, IL-13Rα2, and TSLPR using the Neighbor-Joining algorithm with 1000 replicates. The IL-2Rγc clade is highlighted with a red box and bootstrap values >90% are bolded. (B) Conserved elements within IL-2Rγc intracellular domains. The intracellular regions of human, mouse, chicken, fugu, and zebrafish IL-2Rγc sequences were aligned using ClustalX. The sequences around specific motifs are shown with similar residues colored the same, and conservation across the six sequences indicated (identical *, highly similar:., similar .). (C and D) Expression of il-2rγc genes during zebrafish embryogenesis. Embryos were harvested during embryogenesis at the times indicated and analyzed by RT-PCR (left) and QRT-PCR (right) with primers specific for il-2rγc.a (C) or il-2rγc.b (D) along with β-actin controls with the sizes of amplified products indicated. Representative RT-negative controls are shown (7 dpf) to confirm the absence of genomic contamination in RT-PCR, with QRT-PCR displayed as a percentage of maximal expression. (E–H) WISH expression of il-2rγc.a and il-2rγc.b. Embryos at 5 dpf were subjected to WISH with il-2rγc.a and il-2rγc.b sense (S) and antisense (AS) RNA probes. Staining in the thymus with il-2rγc.a is indicated with an arrow. (I and J) Adult expression of il-2rγc.a and il-2rγc.b. Adult zebrafish tissues were analyzed with QRT-PCR with primers specific for il2rγc.a (I) or il2rγc.b (J), with levels displayed as a percentage of maximal expression. (K and L) Expression of il-2rγc.a and il-2rγc.b in adult lymphoid cells. GFP + lymphocytes were sorted from the thymus of Lck:eGFP (K) and kidney of IgM1:eGFP (L) transgenic fish. Shown are the normalized relative transcript levels of il-2rγc.a (black bars), il-2rγc.b (white bars), and lck or ighm (checked bars), relative to unsorted cells, as determined with QRT-PCR (mean ± SEM of three technical replicates). *p < 0.05, **p < 0.001.
Zebrafish possess two STAT5 paralogs, *stat5.1* and *stat5.2* (65), which were ablated separately with morpholinos (Fig. 3I–3L). Only knockdown of Stat5.1 resulted in a significant, albeit small, decrease in *rag1* expression compared with the controls. Finally, zebrafish also possess both PI3K and ERK (66, 67), which were investigated with LY294002, a PI3K inhibitor (68), and PD98059, an inhibitor of MEK, which lies upstream of ERK (69). The extent of *rag1* expression was significantly decreased for both inhibitors compared with a DMSO control (Fig. 3M–3P). Notably the JAK3 inhibitor reduced the expression of *jak3* (1.5 ± 0.1-fold; *p* = 0.029), as would be expected because this is a marker of developing lymphocytes (Supplemental Fig. 4). The PI3K inhibitor also reduced expression of *pi3kcg* (3.0 ± 0.3-fold; *p < 0.0001), which is
known to be highly expressed in lymphocytes (70). In contrast, the MEK inhibitor had no effect on expression of \textit{map2k1} (1.00 ± 0.04-fold; \( p = 0.7662 \)) and increased the expression of \textit{map2k2} (1.2-fold ± 0.02; \( p = 0.002 \)).

Finally, coinjection of mRNA encoding a constitutively active Jak3 and to a lesser extent Stat5.1 rescued \textit{rag1} expression in embryos injected with \textit{il-2r\_gc.a}\textsuperscript{sS1} morpholino (Fig. 3Q–3U), and zebrafish Jak3 could phosphorylate Stat5.1 in vitro (Fig. 3V).
Discussion

The IL-2R family has been studied extensively in mammals, where it has an important role in immune cell development (7), with mutations of the shared IL-2Rcytc signaling chain leading to a T−B− SCID in humans (14) and a T−B−CD in mice (15). A range of bioinformatic approaches confirmed the presence of two IL-2Rcytc paralogs in zebrafish, il-2rca, and il-2rcb. The Il-2rca protein showed higher sequence conservation, particularly within the intracellular region. Peak expression of il-2rca also occurred coincidentally with embryonic lymphopoiesis, when it was evident in the developing thymus, whereas il-2rcb was not expressed at this site. The il-2rca gene was broadly expressed in adult tissue, but showed highest expression in the spleen, thymus, and kidney with its expression demonstrated in the lymphoid cell populations of these thymus and kidney, whereas il-2rcb showed peak expression in the spleen. Knockdown of Il-2rca— but not Il-2rcb—substantially inhibited embryonic T lymphopoiesis, but did not affect other aspects of hematopoiesis. Mature T cells were also absent in il-2rca ablative larvae, although mature B cells were present, recapitulating human SCID. This finding suggests that Il-2rca has a conserved role in lymphopoiesis, with the function of Il-2rcb currently unknown, consistent with the divergent roles observed for many teleost genes (71–73).

Mammalian IL-2Rcytc signals specifically through JAK3K (74–76), which then activates a number of downstream pathways. Zebrafish Il-2rca possessed a conserved JAK3K docking site and was coexpressed with Jak3 in the developing thymus. In addition, ablation of zebrafish Jak3 recapitulated the effects of il-2rca knockdown, consistent with Jak3K loss also causing SCID in both humans and mice (77–79), whereas a constitutively active Jak3K could rescue the effects of il-2rca knockdown. Lying downstream of IL-2Rcytc/ Jak3K are a number of signaling molecules, including STAT5, PI3K, and ERK, each of which have been shown to contribute to lymphopoiesis. Thus, Stata5a/Stab5b mice showed reduced B lymphocytes because of decreased proliferative responses to IL-2 (80–83), Erk1/2 mice exhibited defective thymic T cell maturation (84, 85), whereas PI3K knockout mice displayed a range of immune defects, including impaired proliferation of T cells in p85a or p110δ knockout and a reduction in thymocyte numbers in p110δ knockouts (86). Ablation or inhibition of these molecules in zebrafish also produced a reduction in lymphocytes suggesting multiple pathways contribute to lymphocyte development. Of the two zebrafish STAT5 proteins, Stata5.1 appeared to play the key role in this regard, whereas a constitutively active Stata5.1 was able to rescue il-2rca morphants partially, with zebrafish Jak3 shown to phosphorylate Stat5.1.

Having determined the importance of IL-2rca/Jak3 signaling in zebrafish lymphopoiesis, it remains of interest to determine which receptor complexes are involved. In zebrafish, ligand-specific receptor subunits have been identified for all IL-2R family components, except IL-2Rα and IL-9Rα (25). Motifs important for downstream pathways are also conserved on several receptor subunits, including the conserved STAT5 docking sites (YXXV/L/M) on IL-2Rγc, IL-4Rα, and IL-7Rα (87–89), and a docking site for SHC, which contributes to activation of the PI3K and ERK pathways, on IL-2Rγc (90) (data not shown). Ligands of the IL-2R family identified in zebrafish include IL-2, IL-15, IL-15-like, and IL-21 (29, 90), with potential IL-2 (91–93) and IL-7 ligands (94) characterized in other teleosts, but not IL-9 (95). Therefore, the phenotypes observed following knockdown of il-2rca and jak3 could potentially be due to defective signaling via several cytokines. Zebrafish IL-2 has been shown to stimulate B cell proliferation and Ab production in adult fish (29), but its role in T cell development has not been determined.

However, zebrafish carrying an il-7ra mutation showed decreased rag1 expression at 5 dpf (28), indicating that IL-7–mediated signaling represents an important contributor for early T cell lymphopoiesis. This article also showed contributions from both zebrafish Jak1 and Jak3 in mediating these effects, consistent with the data presented in this study.

Finally, disruption of zebrafish IL-2rca signaling resulted in ablation of lymphopoiesis with similar characteristics to human SCID (74, 96). This result provides important proof-of-principle evidence that the generation of a zebrafish SCID model by specific gene targeting is feasible. Although a number of husbandry challenges need to be overcome, such a SCID zebrafish would represent an invaluable new resource to investigate aspects of immunity and provide a platform for transplantation and cancer studies.

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Disclosures

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