Optimized cell transplantation using adult rag2 mutant zebrafish

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Optimized cell transplantation using adult *rag2* mutant zebrafish

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Abstract

Cell transplantation into adult zebrafish has lagged behind mouse due to the lack of immune compromised models. Here, we have created homozygous *rag2*^{E450fs} mutant zebrafish that have reduced numbers of functional T and B cells but are viable and fecund. Mutant fish engraft zebrafish muscle, blood stem cells, and cancers. *rag2*^{E450fs} mutant zebrafish are the first immune compromised zebrafish model that permits robust, long-term engraftment of multiple tissues and cancer.

Cell transplantation of human and mouse cells into immune compromised mice has greatly enhanced our understanding of stem cell function, regeneration, and cancer. However, transplantation experiments in mice are expensive, routinely utilize small cohorts of animals, and engraftment is often difficult to visualize directly. By contrast, large-scale cell transplantation of fluorescent blood and cancer cells into syngeneic and irradiated zebrafish has now become routine [1–7]. However, these approaches require that donor cells are from the same strain of syngeneic zebrafish or that the recipient immune system is transiently ablated by whole body γ-irradiation two-days prior to transplantation. Irradiated recipients eventually recover their immune system by 20 days post-irradiation and kill engrafted cells [1,2,6], making long-term engraftment studies difficult. To date, immune compromised zebrafish have not been developed as a universal recipient for allograft cell transplantation.

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Author Contributions

Q.T., N.S.A., J.S.B., J.C.M., S.A.M., I.M.T., and D.M.L. designed and performed experiments. Q.T., J.C.M., and D.M.L. wrote the manuscript. F.E.M., R.L., I.M.T., M.S.I., and Y.H. contributed reagents and animals. Q.T., N.S.A., J.C.M., J.N.B., R.S.L., and D.M.L. performed data analysis and interpretation.

Competing Financial Interest

The authors declare no competing financial interest.

Capitalizing on recently developed gene inactivation methods using genome engineering [8], zinc finger nucleases were engineered to target the Plant Homeodomain (PHD) of the zebrafish *recombination activating gene 2 (rag2)* at similar residues commonly mutated in the Omenn syndrome [9,10] (Fig. 1a). Mutations in residues of the PHD domain disrupt the RAG2 protein interaction with trimethylated histone H3 to alter chromatin accessibility and to partially impair V(D)J recombination *in vivo* [11]. Omenn syndrome is an autosomal recessive severe combined immunodeficiency (SCID) that results in impaired T and B cell receptor rearrangement leading to reduced numbers of functionally mature lymphocytes in human patients. A mutant zebrafish line was generated in the AB-strain background and contained a frame-shift at amino acid E450 that resulted in premature termination (designated $rag2^{E450fs}$; Fig. 1b).

Heterozygous $rag2^{E450fs}$ mutant fish were incrossed and raised to adulthood. Animals were genotyped at 3 months of age, revealing expected Mendelian ratios (146:wild type, 265:heterozygous, and 129:$rag2^{E450fs}$ mutant). $rag2^{E450fs}$ mutant fish were similar in size to heterozygous and wild type sibling animals (Supplementary Fig. 1), survived fin clip, and remained healthy for >6 months when raised under standard laboratory conditions. Homozygous $rag2^{E450fs}$ mutant zebrafish could reproduce, albeit at reduced fecundity when compared with heterozygous sibling fish (Supplementary Table 1). Histological analysis of 90-day-old $rag2^{E450fs}$ mutant zebrafish revealed a striking reduction in thymic T cells and an altered thymic architecture including reduced numbers of epithelial cells with preponderance of adipocytes (n=5 of 6 mutant animals, Fig. 1c,d). This thymic involution is commonly observed in T cell deficient lines of mice [12] and was not detected in wild type siblings (n=6, p=0.008, Fisher's exact test). A similar reduction in thymocyte number was also noted in 5-day-old zebrafish (Supplementary Fig. 2). Analysis of whole kidney marrow revealed that adult homozygous $rag2^{E450fs}$ zebrafish contained all blood cell lineages (Fig. 1e,f); however, quantization revealed a striking 75% reduction in lymphocytes of $rag2^{E450fs}$ mutant zebrafish (Fig. 1g and Supplementary Table 2). Transcript expression for mature B and T cell markers was reduced in $rag2^{E450fs}$ mutant marrow, including *immunoglobulin m (igm), lymphocyte-specific protein tyrosine kinase (lck), t-cell receptor alpha* (*tcra*) and *t-cell receptor beta* (*tcrb*) (Fig. 1h). By contrast, *rag1* transcript levels were not reduced in marrow of mutant animals suggesting that early B cell precursors were not altered in $rag2^{E450fs}$ mutant zebrafish (Fig. 1h). Homozygous $rag2^{E450fs}$ mutants did not exhibit expression differences for *myeloperoxidase (mpx)* and *l-plastin (lcp1),* confirming that neutrophils, monocyte, macrophages, and other cell lineages were not altered in $rag2^{E450fs}$ mutant zebrafish (Fig. 1h and Supplementary Table 2).

To directly assess the impact of the $rag2^{E450fs}$ mutation on mature T and B cells, whole kidney marrow cells were also assessed for *tcrb* and *igm* rearrangements [4,13]. Homozygous $rag2^{E450fs}$ mutant fish lacked *tcrb* rearranged T cells (n=7, Fig. 1i), while the B cell immune repertoire was reduced in 3 of 7 animals tested. Thus, the $rag2^{E450fs}$ mutation results in the production of a hypomorphic protein with reduced receptor recombination, leading to lack mature T cells and a variable reduction of functionally diverse B cells.

Immune compromised mice, including those deficient in *Rag2*, have been successfully used for adoptive transfer of mouse and human cells. Yet, immune compromised adult zebrafish

have not been developed that permit engraftment of a range of cells and tissues. Indeed, the first example of targeted gene knock out in zebrafish was the *rag1*-deficient fish [13]. *rag1* mutant fish lack all mature T and B cells, but have not been widely used for cell transplantation approaches likely due to reduced viability of adult fish and failure to thrive following fin clip and genotyping. To assess if hypomorphic, *rag2*-deficient zebrafish were amenable to cell transplantation, we first assessed if hematopoietic stem cell engraftment was enhanced in *rag2*^{E450fs} mutant lines. Specifically, *rag2*^{E450fs} mutant and wild type sibling adults were sub-lethally irradiated with 10 Gy irradiation to clear the hematopoietic stem cell niche. Animals were transplanted two days after irradiation treatment by intraperitoneal injection with either 5×10^4 or 3×10^5 whole kidney marrow cells from unrelated *ubiquitin-EGFP* donor zebrafish (*ubi-EGFP*, Fig. 2a)[14]. As expected, none of the wild type siblings engrafted blood cells (*n*=10), while all *rag2*^{E450fs} mutant fish exhibited robust, multi-lineage blood cell engraftment that persisted past 45 days post-transplantation (dpt, *n*=6) - a full 25 days after immune rejection is normally initiated in wild type, irradiated recipient fish (Fig. 2b,c, p=0.0002, Fisher's exact test; Supplementary Fig. 3, and Supplementary Table 3) [2,6]. Engrafted *rag2*^{E450fs} homozygous mutant fish also commonly exhibited GFP+ circulating cells by 30 dpt (Supplementary Movie 1). These data show that hypomorphic *rag2*^{E450fs} can robustly engraft hematopoietic cells from unrelated donors.

To assess the broad utility of the model for engraftment of regenerative tissues, fluorescently labeled muscle cells from adult *ubi-EGFP* transgenic fish were transplanted by intramuscular injection into non-irradiated recipient animals (5×10^4 cells/fish, 2μl/animal). Muscle cell viability was >85% following disassociation and single cell preparation (Supplementary Fig. 4). Remarkably, GFP+ muscle fibers were readily detected in all *rag2*^{E450fs} mutant animals by 30 dpt (*n*=3 of 3) but not wild type sibling fish (*n*= 4; p=0.03, Fisher's exact test; Fig. 2d,e and Supplementary Table 3). Fluorescent fibers persisted in *rag2*^{E450fs} mutant fish for up to 60 dpt. Histological analysis revealed that GFP+ muscle fibers were viable, contained muscle striations, and were indistinguishable from recipient muscle tissue (Supplementary Fig. 5). We next wanted to verify the specificity of our results using a muscle-specific, fluorescent transgenic line. Muscle cells were isolated from the dorsal musculature of *alpha-actin-RFP* transgenic fish [15] and injected into the dorsal musculature of recipient fish (2.5×10^5 cells/fish). Again, robust engraftment of fluorescent muscle was only observed in homozygous *rag2*^{E450fs} mutant fish by 30 dpt (*n*=5 of 5, Fig. 2f and Supplementary Table 3), but not heterozygous or wild type siblings (*n*=5 animals/genotype assessed, p=0.008, Fisher's exact test). These data show that homozygous *rag2*^{E450fs} mutant fish can engraft cells even in the absence of preconditioning with low-dose irradiation.

We next wanted to assess the utility of our model to engraft a diversity of transgenic zebrafish cancers including T cell acute lymphoblastic leukemia (TALL), embryonal rhabdomyosarcoma (ERMS), and melanoma. Specifically, primary *Myc*-induced T-ALL was generated in a mixed Tubingen/AB-strain background [16,17] and transplanted into the peritoneal cavity of recipient animals (Fig. 3a,e,i). Homozygous *rag2*^{E450fs} mutant fish robustly engrafted T-ALL by 30 dpt even in the absence of prior immune suppression by γ-irradiation (*n*=2 of 2, 1×10^5 cells/fish, Fig. 3i). By contrast, heterozygous and wild type

siblings failed to engraft T-ALL (n≥7 per genotype, p<0.03, Fisher's exact test, Fig. 3e). Similar results were observed using three serially-passaged T-ALL from syngeneic CG1-strain zebrafish (1×10^5 cells/fish). In total, 39 of 39 *rag2*^{E450fs} mutants engrafted T-ALL arising from CG1-strain fish while 0 of 22 wild type siblings engrafted leukemia (p<0.0001, Fisher's exact test, Fig. 3b,f,j and Supplementary Table 4), suggesting that engraftment is not restricted to zebrafish strain or major histocompatibility complex (MHC) matching between fish. Homozygous *rag2*^{E450fs} mutant zebrafish also successfully engrafted fluorescently labeled *kRAS*^{G12D}-induced ERMS generated in both the CG1 and AB-strain background (*n*=4 independent tumors analyzed, 1×10^4–1×10^6 cells/fish). In total, 24 of 27 homozygous *rag2*^{E450fs} mutant zebrafish engrafted ERMS while 0 of 7 wild type siblings engrafted disease (p<0.0001, Fisher's exact test, Fig. 3c,g,k and Supplementary Table 4)[18]. Finally, melanomas that harbor a *p53* mutation and over-express both *mitfa* and *BRAF*^{V600E} could successfully engraft into *rag2*^{E450fs} mutant zebrafish but not wild type siblings (*n*=4 tumors analyzed, 5×10^5–1×10^6 cells/fish) [19,20]. In total, 25 of 25 homozygous *rag2*^{E450fs} mutant fish engrafted melanoma while 0 of 16 wild type siblings engrafted disease (p<0.0001, Fisher's exact test, Fig. 3d,h,l and Supplementary Table 4). No instances of tissue rejection or tumor regression were observed in engrafted *rag2*^{E450fs} mutant fish (*n*=104), showing that homozygous *rag2*^{E450fs} mutant zebrafish can robustly engraft cells from a diversity of genetic backgrounds and even in the absence of preconditioning of recipient animals with γ-irradiation.

Our experiments highlight the use of hypomorphic *rag2*^{E450fs} mutant fish as the first universal recipient for allograft cell transplantation into adult fish, ushering in a new era of large-scale cell transplantation studies to directly visualize and assess stem cell self-renewal within normal tissues and clonal heterogeneity, therapeutic responses, and growth in cancer.

Online Methods

Animal use and creation of hypomorphic *rag2*^{E450fs} mutant zebrafish

Zebrafish studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, under protocol #2011N000127.

rag2^{E450fs} mutant zebrafish were created using the previously described Zinc Finger Nuclease (ZFN) pair that targeted the PHD domain of the zebrafish *rag2* gene[8]. Specifically, RNA was prepared for each ZFN arm and microinjected into AB strain zebrafish (500 ng/μl). F0 injected animals were raised to adulthood and single male by female matings performed. Resultant progeny were arrayed into 96-well plates and genomic DNA extracted (*n*=12 individual embryos per cross). Polymerase Chain Reaction (PCR) was performed using forward primer (5′-ACTGCTCTAGTTGCAATTCCT) and reverse primer (5′-AGCTGGGGTCATCTTCAGT) to produce a 585 base pair (bp) PCR amplicon. The PCR cycle parameters were 1) denaturation: 94°C for 30 sec., 2) annealing: 54°C for 30 sec., and 3) elongation: 68°C for 45 sec., repeated for 35 cycles. PCR samples were purified and sent for Sanger sequencing. From this analysis, one line was identified with a *rag2* frame shift mutation that starts at amino acid E450 and results in a premature stop codon mutation (designated *rag2*^{E450fs} in this manuscript with an official ZFIN.org allele designation of

rag2^{fb101}). F1 progeny were subsequently raised to adulthood and heterozygous *rag2*^{E450fs} fish identified by genotyping (see below).

Genotyping of *rag2*^{E450fs} mutant zebrafish

The *rag2*^{E450fs} mutant line is best maintained through heterozygous incrossing (Supplementary Table 1) and produces progeny at the expected Mendelian ratios. The *rag2*^{E450fs} allele introduces a *de novo XcmI* site, allowing for restriction enzyme mediated identification of the mutant allele (diagramed with representative results in Supplementary Fig. 6). Specifically, adult 2–4 month-old fish were fin clipped and genomic DNA prepared using the modified HotSHOT method[21]. Individual genomic DNA was diluted ten-fold and 2 μl used in a standard 25 μl volume PCR using Taq DNA Polymerase (New England Biolabs, Cat# M0273L) with forward primer (5′-ACTGCTCTAGTTGCAATTCCT) and reverse primer (5′-AGCTGGGGTCATCTTCAGT) to produce a 585 base pair (bp) PCR amplicon. The PCR cycle parameters are 1) denaturation: 94°C for 30 sec., 2) annealing: 54°C for 30 sec., and 3) elongation: 68°C for 45 sec., repeated for 35 cycles. For enzymatic digestion, 15 μl of non-purified PCR reaction was combined with 0.3 μl *XcmI* + 2 μl 10X NEB buffer 2.1 + 2.7 μl water. The 20 μl reaction was incubated at 37°C for ≥4 hrs. Enzymatic digestion was visualized by electorphoresis on a 2% Tris-Acetate-EDTA (TAE) agarose gel containing ethidium bromide. Wild type fish produced a single band at 585 bp. Heterozygous *rag2*^{E450fs} fish produced three bands at 585, 372 and 212 bp due to digestion of the mutant allele, with the two higher molecular weight bands being most distinctive. Homozygous fish produced two bands at 372 and 212 bp. A representative image of genotyping analysis is shown in Supplementary Fig. 6.

Characterization of the *rag2*^{E450fs} mutant line

RNA *in situ* hybridization of larval fish (Supplementary Fig. 2) and histological analysis of thymus of adult fish (Fig. 1c,d) were completed as previously described[22]. Two-tailed Student's *t*-test analysis was performed to assess larval thymus size differences (Supplementary Fig. 1c). For hematopoietic cell quantification (Fig. 1g), cytospins of whole kidney marrow from both *rag2*^{E450fs} mutant fish and wild type siblings were reviewed by clinical hematopathologists (J.S.B.) and (R.S.L.). At least five 400x magnification fields were analyzed per slide and > 200 cells counted per animal. Sample identification was blinded during counts and genotype only revealed after cell counting was completed. Two-tailed Student's *t*-test analysis was performed to assess changes in relative numbers of each blood cell lineage between wild type and *rag2*^{E450fs} mutant fish. Quantitative real-time PCR (Fig. 1h) and nested PCR for *tcra* and *igm* rearrangements (Fig. 1i) were completed using primers shown in Supplementary Tables 5 and 6, essentially as previously described[4,23]. Two-tailed Student's *t*-test analysis was performed to assess expression differences in hematopoietic marker genes using quantitative PCR (Fig. 1h). A threshold of $p \leq 0.05$ was considered significant for two-tailed Student's *t*-test.

Generation of transgenic zebrafish cancers

T-ALL and ERMS were created by co-injection of either *rag2-Myc* or *rag2-kRAS*^{G12D} along with fluorescent reporters under the same promoter into one-cell stage animals respectively,

which have been previously described[16].
Tg(mitfa:GFP);mitfa$^{-/-}$*;p53*$^{-/-}$*;Tg(mitfa:BRAF(V600E))* melanoma bearing fish were generated as previously described[19,20].

Whole kidney marrow transplantation

Donor *ubi-EGFP* fish were euthanized by tricaine overdose (Tricaine-S, Western Chemical Inc.,). Kidney tissue was dissected, placed into a 1.5 ml Eppendorf tube containing 100 μl of suspension solution (0.9X PBS + 5% FBS), and manually pipetted ≥20 times using a 1 ml pipette tip. The kidney tissue suspension was filtered through a 40 μm strainer (Fisher Scientific, Cat# 352340). Total number of viable cells was calculated by Trypan blue (Life Technologies, Cat# 15250061) staining and hemocytometer counts. Cells were centrifuged at 1,000 g for 10 minutes and then resuspended in desired volumes for cell transplantation (Supplementary Table 3). Recipient fish were preconditioned with 10 Gy γ-irradiation (Cs137 irradiation), 2 days prior to transplantation. Recipient fish were transplanted with the indicated number of viable kidney marrow cells (Supplementary Table 3) injected into the peritoneal cavity using a 26s gauge Hamilton 80366 syringe (Sigma-Aldrich, 20779).

Whole kidney cell engraftment was assessed using whole body epi-fluorescence imaging (Olympus stereo microscope model MVX10, Olympus DP72 microscope digital camera, DP2-BSW software version 2.2), direct visualization of circulating cells within the tail vasculature beginning at 20 days post-transplantation (dpt), and/or FACs. FACs of kidney marrow was completed in the presence of propidium iodide (PI) to exclude dead cells. The GFP+ cell fraction was isolated from the marrow of engrafted *rag2*^{E450fs} fish and analyzed following cytospin preparation.

Muscle cell transplantation

Dorsal musculature of donor *ubi-EGFP* or *α-actin-RFP* fish was excised from tricaine overdosed animals. Specifically, the dorsal musculature of the tail posterior to the anus was harvested for muscle transplantation. The muscle tissue was combined from 10–12 animals and mechanically disassociated by maceration by repeated dicing using a razor blade for 5 minutes on a 10 cm petri dish containing 500 μl of suspension solution (0.9X PBS + 5% FBS). Samples were then supplemented with 5 ml of 0.9X PBS + 5% FBS and a 5 ml serological pipette was used to suspend the homogenized tissue by repeated pipetting ≥30 times. Cells were then filtered through a 40 μm cell strainer and washed with 5 mls of 0.9X PBS + 5% FBS. Total number of viable cells was calculated by Trypan blue staining and hemocytometer counts. Cells were then centrifuged at 1,000 g for 10 minutes and resuspended in desired volumes (Supplementary Table 3) prior to transplantations. No irradiation preconditioning was used prior to transplantation of muscle cells into *rag2*^{E450fs} mutant fish. Muscle cell transplantations were completed by injecting 2 μl of donor cell preparation into the dorsal musculature on the left side of the recipient fish, using a 26s gauge Hamilton 80366 syringe.

Engraftment was assessed by visualization of fluorescently labeled muscle fibers using epi-fluorescence imaging at 10, 20, 30, 45, and 60 dpt. Engraftment of *ubi-EGFP* muscle fibers were confirmed by immunohistochemistry on section slides using an anti-EGFP antibody

(JL-8, Living Colors, Cat# 632381). Viability of the muscle fibers was confirmed by the absence of cleaved caspase-3 expression (Cell Signaling, Cat# 9664) and TUNEL staining (customized Millipore S7100 kit, Specialized Histopathology & TMI Core at Dana-Farber Cancer Institute).

Cancer cell transplantation

Donor fish with T-ALL, ERMS and melanoma from different transgenic backgrounds were euthanized by tricaine overdose. Animals were imaged using epi-fluorescence microscopy and tumor cells isolated as previously described[24]. Specifically, tumors were excised from diseased animals and placed in 500 μl of 0.9X PBS + 5% FBS on a 10 cm petri dish. Single-cell suspensions were obtained by maceration of tissue with a razor blade followed by manual pipetting to disassociate cell clumps. Cells were filtered through a 40 μm filter, centrifuged at 1,000g for 10 minutes, and resuspended at the correct volume (Supplementary Table 4) in a total volume of 5 μl per recipient fish for cell transplantation. Cell viability ranged from 58% to 66% assessed by PI staining and FACS analysis of ERMS tumor cells. 5 μl of tumor cells were transplanted into the peritoneal cavity of each recipient fish using a 26s Hamilton 80366 syringe. No irradiation preconditioning was utilized for tumor cell transplantations outlined in this work.

Tumor engraftment was assessed at 10, 20, 30, and 45 dpt by epi-fluorescence microscopy. Recipient fish were sacrificed when moribund or at 45 days post-transplantation for animals that failed to engraft disease (wild type). A subset of transplanted animals were photographed, and either 1) fixed in 4% paraformaldehyde for sectioning or 2) peripheral blood samples were collected for cytospins and histological examination.

Statistics

When possible, cell transplantation experiments were completed using ≥3 animals per arm to facilitate analysis using the Fisher's exact test. No animals were excluded from our analysis shown in Supplementary Table 3 and 4, with exception to *rag2*^{E450fs} mutant fish that engrafted *ubi-EGFP*+ marrow and were scored for circulating GFP+ cells in the tail at ≥20 dpt. We did not include these animals in Supplementary Table 3 because they were sectioned and marrow analysis by FACS was not possible.

Cytospin analysis shown in Fig. 1e–g and Supplementary Table 2 was reviewed by clinical hematopathologists (J.S.B.) and (R.S.L.). Genotype was only revealed after cell counting was completed.

Two-tailed Student's t-test analysis was performed to assess 1) changes in relative numbers of each blood cell lineage between wild type and *rag2*^{E450fs} mutant fish (Fig. 1g), 2) expression differences in hematopoietic marker genes using quantitative PCR (Fig. 1h), and 3) thymus size differences (Supplementary Fig. 2c). Fisher's exact tests were performed to assess 1) differences in adult thymus phenotypes between wild type and *rag2*^{E450fs} mutant fish (Fig. 1c,d), 2) engraftment rates of whole kidney marrow and muscle transplantations between genotypes (Fig. 2 and Supplementary Table 3) and 3) tumor cell transplantations

(Fig. 3 and Supplementary Table 4). A threshold of $p \leq 0.05$ was considered significant for all statistical methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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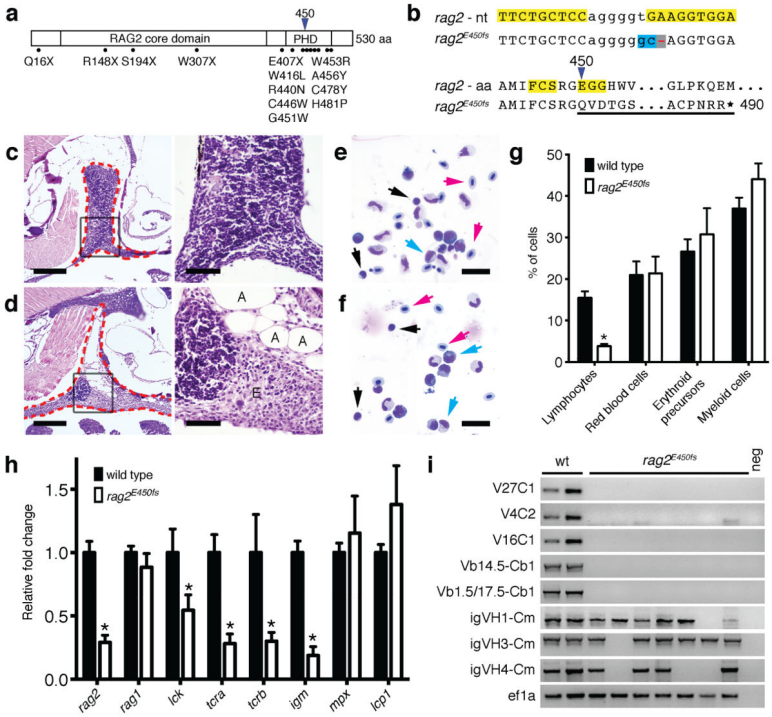

Figure 1. ***rag2*^{E450fs} mutant zebrafish lack mature T cells and have a reduced B cell repertoire**

(**a**) Human RAG2 protein with known SCID mutations denoted. Arrow denotes ZFN target region. (**b**) Nucleotide (top) and protein sequence (bottom) for the *rag2*^{E450fs} mutation. Yellow denotes ZFN target sites, blue nucleotide additions, gray nucleotide deletions, and arrow amino acid change at E450. Underlining shows amino acid sequence following frame shift with termination amino acid numbered. (**c,d**) Thymus sections of 90-day-old wild type (**c**) and *rag2*^{E450fs} mutant zebrafish (**d**). Red dashed lines denote thymus (left). Right panels are amplified views of boxed regions with adipocytes (A) and vacant thymic epithelium (E) shown. (**e,f**) Whole kidney marrow cytospins of wild type (**e**) and *rag2*^{E450fs} zebrafish (**f**) with lymphocytes (black arrows), erythrocytes (magenta arrows) and granulocytes (cyan arrows) denoted. Scale bars are 200 μm (**c,d** – left); 50 μm (**c,d** – right); 20 μm (**e,f**). (**g**) Cell counts from cytospins performed on whole kidney marrow. Error bars represent ± 1 s.d., $p < 0.05$ by Student's *t*-test. (**h**) Gene expression analysis of whole kidney marrow cells. Error bars represent s.e.m, $p < 0.05$ by Student's *t*-test. (**i**) PCR analysis for *tcrb* and *igm* rearrangement of whole kidney marrow cells from wild type (wt) and *rag2*^{E450fs} zebrafish. negative control (neg).

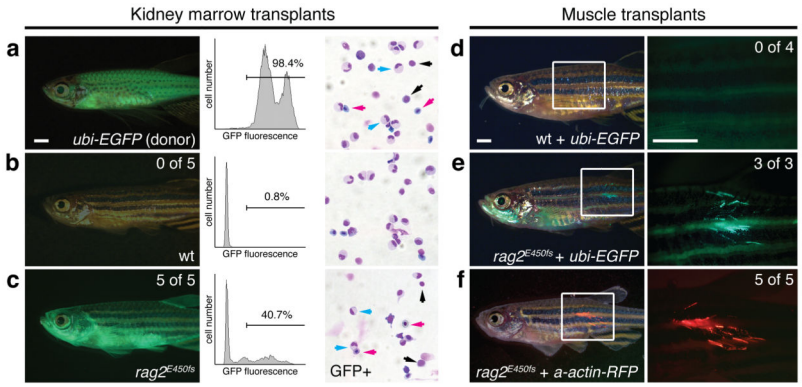

Figure 2. *rag2*^{E450fs} mutant fish engraft hematopoietic and muscle stem cells

(**a**) *ubi-EGFP* transgenic donor fish. (**b–c**) Wild type (wt, **b**) or homozygous *rag2*^{E450fs} recipient fish (**c**) transplanted with GFP-labeled marrow and imaged at 45 days post-transplantation. Fluorescent image of whole fish (left) with engraftment rates noted. FACS (middle) and cytospin analysis (right) of whole kidney marrow from donor (**a**) and recipient fish (**b–c**). FACS sorted GFP+ cells are shown for cytospin analysis in **c**. Lymphocytes (black arrows), red blood cells (magenta arrows), granulocytes (cyan arrows). (**d–e**) Fluorescent image of wild type (wt) and *rag2*^{E450fs} mutant fish engrafted with muscle cells from *ubi-EGFP* transgenic fish imaged at 30 days post-transplantation. (**f**) *rag2*^{E450fs} fish engrafted with muscle cells from *alpha-actin-RFP* transgenic fish at 30 days post-transplantation. Engraftment rates for **d–f** are shown on magnified image panels to the right. Scale bars are 2 mm.

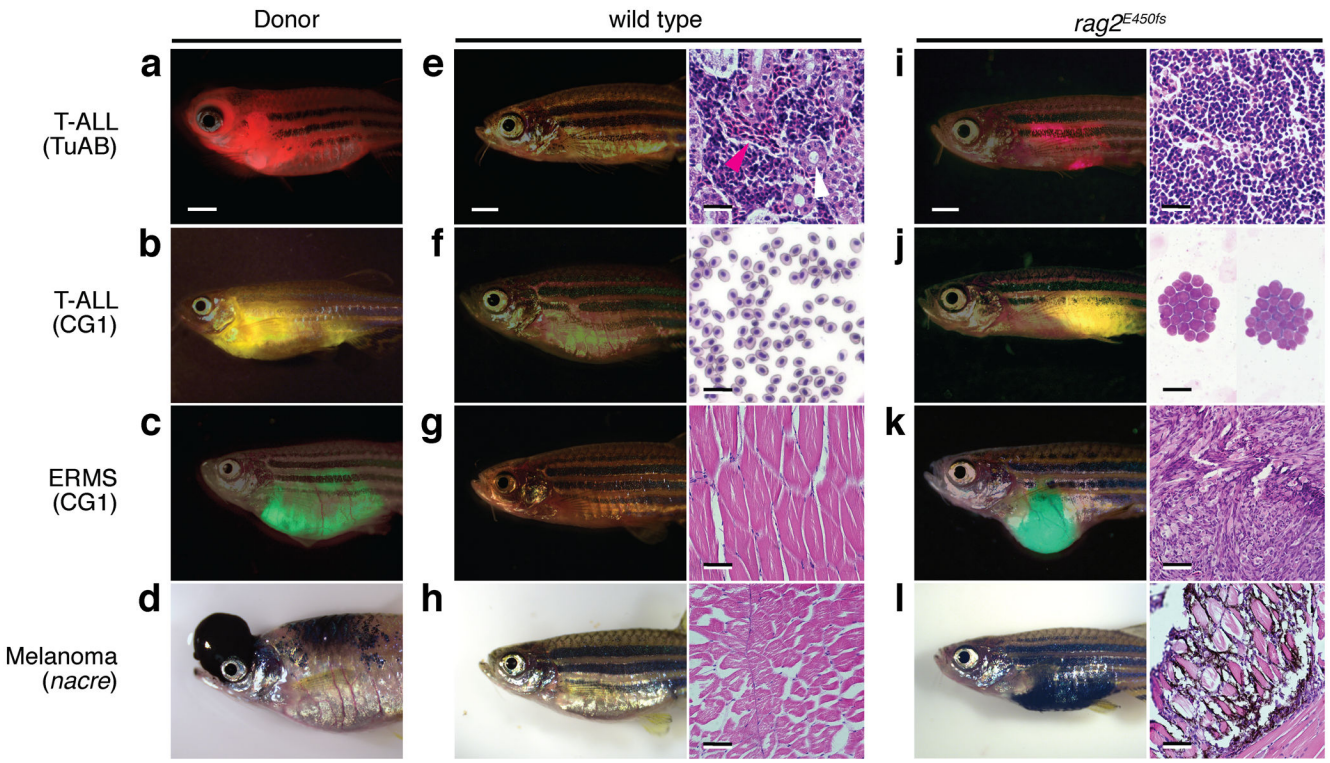

Figure 3. Engraftment of zebrafish tumors into *rag2*^{E450fs} mutant fish

(**a–d**) Donor tumors used in cell transplantation studies. dsRED-labeled *Myc*-induced T-ALL arising in TuAB strain fish (**a**), zsYellow-labeled T-ALL from CG1-strain fish (**b**), GFP-labeled embryonal rhabdomyosarcoma (ERMS) from CG1-strain fish (**c**), and *mitfa* and *BRAF*^{V600E} induced melanoma arising in *p53*-deficient *nacre* strain fish (**d**). Merged bright-field and fluorescent images of wild type (**e–h**) or *rag2*^{E450fs} mutant fish (**i–l**) at 30 days post-transplantation. Images of whole kidney marrow sections (**e,i**), peripheral blood cytospins (**f,j**), and skeletal muscle (**g,k,h,i**). Red blood cells denoted by magenta arrows and renal tubules white arrows (**e**). Scale bars in fish images are 2 mm and histopathology images are 25 μm (**e,i**), 20 μm (**f,j**), and 50 μm (**g,k,h,l**).