Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia

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We have created a stable transgenic *rag2-EGFP-mMyc* zebrafish line that develops GFP-labeled T cell acute lymphoblastic leukemia (T-ALL), allowing visualization of the onset and spread of this disease. Here, we show that leukemias from this transgenic line are highly penetrant and render animals moribund by 80.7 ± 17.6 days of life (±1 SD, range = 50–158 days). These T cell leukemias are clonally aneuploid, can be transplanted into irradiated recipient fish, and express the zebrafish orthologues of the human T-ALL oncogenes *tal1/scl* and *lmo2*, thus providing an animal model for the most prevalent molecular subgroup of human T-ALL. Because T-ALL develops very rapidly in *rag2-EGFP-mMyc* transgenic fish (in which "mMyc" represents mouse c-Myc), this line can only be maintained by *in vitro* fertilization. Thus, we have created a conditional transgene in which the *EGFP-mMyc* oncogene is preceded by a loxed *dsRED2* gene and have generated stable *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic zebrafish lines, which have red fluorescent thymocytes and do not develop leukemia. Transgenic progeny from one of these lines can be induced to develop T-ALL by injecting *Cre* RNA into one-cell-stage embryos, demonstrating the utility of the Cre/lox system in the zebrafish and providing an essential step in preparing this model for chemical and genetic screens designed to identify modifiers of Myc-induced T-ALL.

lymphoma | *tal1/scl* | *lmo2*

T cell lineage acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy arising when immature T cells acquire mutations that cause differentiation arrest, rapid proliferation, and suppression of apoptosis within developing T lymphocytes (1). Our current understanding of the molecular basis of T cell malignancies has emerged largely from the analysis of recurrent chromosomal translocations, which typically juxtapose T cell oncogenes with strong promoter elements responsible for high expression levels of the T cell receptor (2, 3). These T cell oncogenes encode transcription factors, including (*i*) basic helix–loop–helix (bHLH) family members such as *TAL1/SCL*, *TAL2*, *LYL1*, and *BHLHB1*, (*ii*) LIM-only (LMO) domain genes such as *LMO1* and *LMO2*, and (*iii*) the orphan homeobox genes *HOX11/TLX1* and *HOX11L2/TLX3* (3–5). Up-regulation of T-ALL oncogene transcription factors can also occur in the absence of chromosomal translocations (6–8), presumably due to mutations that cause either monoallelic activation, through cis-acting mutations or deletions, or biallelic activation, from the disruption of upstream factors that normally suppress the expression of these genes in developing thymoctyes (8).

In human T-ALL, we have identified five distinct multistep molecular pathways based on the overexpression of (*i*) *TAL1/SCL* plus *LMO1* or *LMO2*, (*ii*) *LYL1* plus *LMO2*, (*iii*) *HOX11*, (*iv*) *HOX11L2*, and (*v*) *MLL-ENL* (1, 9), each of which is characterized by distinct molecular signatures (1, 10, 11). These subgroups are clinically relevant, with event-free survival differing among patient groups (1, 11, 12). For example, patients expressing both *TAL1/SCL* or *LYL1* and a LMO family member (*LMO1* or *LMO2*) have a worse prognosis than those expressing *HOX11* (1, 11, 12). Activation of these T cell oncogenes appears to be critical for thymocyte transformation, possibly by causing stage-specific arrest of T cell maturation (1). However, additional mutations are also found in leukemic cells from patients in each of the major subgroups, including those that affect pathways that control apoptosis, proliferation, and genomic instability. Recently, we have identified activating mutations in *NOTCH1* that result in increased NOTCH signaling and increased proliferation of developing thymocytes (13). In addition, four of five subgroups of human T-ALL express high levels of either *MYC* or *MYCN* (1), suggesting that MYC may be a central regulator of proliferation and/or genomic instability in this malignancy. Finally, most human T-ALLs biallelically delete the *CDKN2A* locus, which encodes both the p16(INK4A) and p14(ARF) tumor suppressors, thereby disrupting both the RB and p53 pathways and contributing to aberrant control of both cell cycle progression and programmed cell death (1, 14).

The zebrafish has recently emerged as an important vertebrate model of Myc-induced T-ALL (15); however, studies have not been performed to assess how closely zebrafish T-ALL mimics the human disease. In addition, the promise of the zebrafish T-ALL model lies in its utility for chemical (16–18) and genetic modifier screens (19–21), marking the emergence of the zebrafish as a unique vertebrate model with which to identify enhancers that accelerate disease or suppressors that curb tumor growth. Here, we show that transgenic *rag2-EGFP-mMyc* zebrafish (in which "mMyc" represents mouse c-Myc) develop T-ALLs that faithfully model the most common and most treatment-resistant subtype of human T-ALL, in which *SCL* and *LMO1/2* are coexpressed. However, these *rag2-EGFP-mMyc* transgenic fish are often severely diseased by the time they reach reproductive maturity, making this line difficult to breed and maintain. Thus, conditional transgenic approaches are needed to establish zebrafish leukemia models that are amenable to forward genetic and small molecule suppressor screens. Our current results indicate that the molecular mechanisms underlying zebrafish T-ALL are remarkably similar to those found in the human disease and establish Cre/lox strategies in transgenic zebrafish that provide a general means to develop conditional models of cancer for genetic analysis in this model organism.

Materials and Methods

Isolation of *lmo1* and *p16*. RNA was obtained from 1- to 5-day-old embryos and made into cDNA, and degenerate PCR primers were used to amplify a fragment of the *lmo1* and *p16* gene. RACE PCR was used to isolate the full-length *lmo1* (GenBank accession no.

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Abbreviations: T-ALL, T cell acute lymphoblastic leukemia; mMyc, mouse c-Myc; LMO, LIM-only; TCR, T cell receptor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF398514).

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AF398514). By contrast, the *p16* sequence fragment was used to search the zebrafish genome (www.sanger.ac.uk/Projects/D_rerio) and identify a putative full-length ORF for the *p16* locus (R. Stewart and A.T.L., unpublished data).

Penetrance of Disease in *rag2-EGFP-mMyc* Stable Transgenic Zebrafish. Stable transgenic *rag2-EGFP-mMyc* fish have been generated previously and develop GFP-labeled T-ALL (15). To determine the penetrance of disease in stable transgenic *rag2-EGFP-mMyc* fish, sperm was harvested from 10- to 20-week-old leukemic male fish and used for *in vitro* fertilization of AB WT eggs. The resulting progeny were scored for leukemia onset at 30–60 days of life as determined by infiltration of GFP-labeled leukemic cells into sites adjacent to the thymus. At 3 months of age, nonleukemic sibling fish were analyzed for the presence of the *mMyc* transgene as determined by PCR of genomic DNA isolated from the tail fin as described in ref. 15.

Collection of Leukemias and Determination of DNA Content. Leukemic *rag2-EGFP-mMyc* fish were killed, and the heads were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The remaining portion of fish was diced over 5 ml of ice-cold 0.9 × PBS plus 5% FBS. The suspension was filtered over a 40-μm filter, washed, and subsequently (*i*) transplanted into irradiated recipient fish (1×10^6 cells per fish, 2–3 days after receiving 23 Gy of total body irradiation from a ^{137}Cs source), (*ii*) analyzed for DNA content as determined by DNA flow cytometry, (*iii*) frozen in 10 million-cell aliquots, and/or (*iv*) analyzed by FACS to determine the percentage of GFP-labeled leukemic cells contained within each sample. DNA flow cytometric analysis was completed essentially as described in ref. 15. Specifically, tumor cells and WT nucleated red blood cells were stained with propidium iodide in hypotonic sodium-citrate buffer and analyzed for cellular DNA content by flow cytometry alone and as a mixture with normal control cells.

RT-PCR Analysis. RNA was isolated (with TRIzol, GIBCO/BRL) from leukemia cells and control FACS-sorted, GFP-positive thymocytes from *rag2-GFP* and *lck-GFP* transgenic fish (15, 22, 23). RNA was treated with DNaseI before reverse transcription, and RT-PCR was performed. (PCR primers and thermocycling conditions are described in detail in the *Supporting Text* and Table 1, which are published as supporting information on the PNAS web site.)

To confirm that expression of the *scl* and *lmo2* transcripts was confined to the leukemic lymphoblasts, RNA *in situ* hybridization was completed on paraffin-embedded sections from transgenic *rag2-EGFP-mMyc* fish essentially as described in refs. 15 and 22. PCR primers used to generate probes for *in situ* hybridization analysis are described in the *Supporting Text*.

To determine whether *scl* and *lmo2* expression resulted from transcription of one or both alleles, PCR was used to amplify the 3′ untranslated regions of *scl* and *lmo2* (Table 1). PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and sequenced. cDNA was obtained from zebrafish T-ALL samples having polymorphic alleles for either *scl* or *lmo2* and subjected to PCR. PCR fragments were purified and sequenced.

Southern Blot Analysis to Determine Clonality and to Assess Loss of the *p16* Locus. Southern blot analysis was used to determine whether leukemia cells have *T cell receptor (TCR)-α* or *IgM* receptor rearrangements and whether the *p16* genomic locus is lost. Southern blot analysis was performed as described in refs. 15 and 24.

Sequencing of the *p53* Locus. Genomic DNA was isolated from zebrafish leukemic samples and subjected to PCR amplification of exons 4–9 of the zebrafish *p53* gene. Fragments were purified and sequenced as described in ref. 25 and Table 1.

Cre/lox Strategies. To verify that Cre/lox-mediated strategies work in the zebrafish, an expression vector was created that contains loxP sites flanking the *dsRED2* transgene and the polyadenylation site contained within the dsRED2-N1 vector (Clontech). The loxed *dsRED2* transgene was cloned into the EGFP-N1 expression vector upstream of the *EGFP* ORF (Fig. 3*A*). One-cell-stage embryos were injected with either the *CMV-loxP-dsRED2-loxP-EGFP* plasmid (50 ng/μl) alone or in combination with *Cre* RNA (25 ng/μl). *Cre* RNA was made by *in vitro* transcription by using the *pCS2+Cre* vector and SP6 RNA polymerase. Transiently injected embryos were analyzed 26 h postfertilization for GFP and dsRED2 expression as determined by fluorescent microscopy.

Developing Conditional Transgenic Zebrafish. The loxed *dsRED2* coding sequence was cloned into the *rag2-EGFP-mMyc* plasmid upstream of the *EGFP-mMyc* transgene by using *Bam*HI restriction enzyme sites (Fig. 4*A*). The resulting *rag2-loxP-dsRED2-loxP-EGFP-mMyc* plasmid was linearized with *Xho*I, phenol/chloroform-extracted, and ethanol-precipitated. Linearized DNA was injected into one-cell-stage AB strain embryos [100 ng/μl DNA in 0.5× TE buffer (10 mM Tris/1 mM EDTA, pH ≈ 7.0) containing 100 mM KCl. Primary injected adult fish were screened for the ability to produce offspring that contained the transgene as determined by detection of dsRED2 fluorescence within developing thymocytes at 6 days postfertilization. Two stable transgenic *rag2-loxP-dsRED2-loxP-EGFP-mMyc* zebrafish lines were identified (lines G7 and G16, AB strain). F1 and F2 *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic fish were injected with 25 ng/μl *Cre* RNA and analyzed for leukemia onset.

Analysis of Leukemias from *Cre*-Injected *rag2-loxP-dsRED2-loxP-EGFP-mMyc* Fish. Leukemic cells from *Cre*-injected stable transgenic *rag2-loxP-dsRED2-loxP-EGFP-mMyc* fish were harvested and (*i*) transplanted into irradiated adult fish, (*ii*) analyzed by cytospin and Giemsa/May–Grunwald staining to confirm lymphoblast morphology, (*iii*) subjected to FACS analysis to assess levels of GFP and dsRED2 expression within lymphoblasts, and/or (*iv*) extracted for genomic DNA and analyzed for CRE recombination as detected by PCR (forward primer specific to the *rag2* promoter region, ATGCTAATTTGAAGCACTAGCA; reverse primer specific to the EGFP coding sequence, GTGCAGATGAACTTCAGGGT).

Results

Complete Penetrance of T-ALL in a *rag2-EGFP-mMyc* Stable Transgenic Line. We have previously described a *rag2-EGFP-mMyc* stable transgenic zebrafish line in which the onset and progression of T cell malignancy can be monitored by fluorescence microscopy (15). The disease is first detected as an expansion of GFP-labeled T cells in the thymus, and, subsequently, malignant cells infiltrate regions adjacent to the thymus in a phase comparable to human T cell lymphoblastic lymphoma. Then, transformed T cells rapidly spread throughout the skeletal musculature, visceral organs, and kidney marrow, leading to widely disseminated T-ALL (Fig. 1 and Fig. 4 of ref. 15). In a series of 106 stable transgenic *rag2-EGFP-mMyc* fish, all animals developed T-ALL and succumbed to death by 80.7 ± 17.6 days of life (±1 SD, range = 50–158 days). Sixty-four nonleukemic siblings were raised until 3 months of age, and none harbored the *mMyc* transgene in somatic DNA. Taken together, these results indicate that Myc-induced leukemias are fully penetrant in our *rag2-EGFP-mMyc* transgenic line.

Myc-Induced Leukemias Are of T Cell Origin and Oligoclonal. Because *rag2* is expressed in both immature T and B cells (22), we wanted to assess whether leukemias arising in our stable transgenic zebrafish were of T cell or B cell origin. Several lines of evidence indicated that all of the leukemias arising in this transgenic line were of T cell origin. (*i*) Each malignancy developed as a GFP-labeled lymphoma in the thymus (n = 106). (*ii*) Southern blot analysis

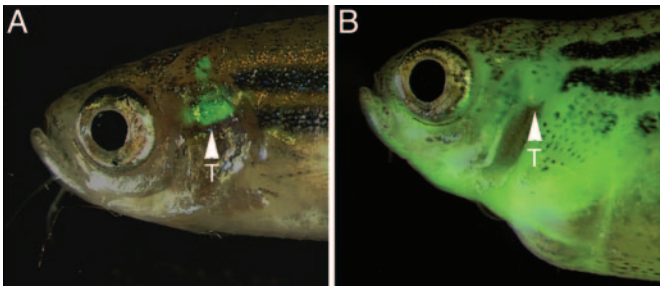

Fig. 1. Stable transgenic *rag2-EGFP-mMyc* zebrafish develop GFP-labeled thymic lymphoma, which progresses to T-ALL. Fluorescence microscopic analysis at 50 days of life showing the thymus of control *rag2-GFP* transgenic fish (*A*) and massive GFP-labeled cellular dissemination of leukemic lymphoblasts in *rag2-EGFP-mMyc* transgenic fish (*B*). Fish are oriented with anterior to the left and dorsal to the top. Arrowheads mark location of the thymus (T).

showed that Myc-induced leukemias contained oligoclonal *TCR-α* gene rearrangements (18 of 36), whereas none of the leukemias contained Ig heavy chain receptor gene (*IgM*) rearrangements (n = 21; Figs. 6 and 7 and Table 2, which are published as supporting information on the PNAS web site). (*iii*) RT-PCR analysis showed that leukemic lymphoblasts expressed high levels of the T cell-specific tyrosine kinase gene (*lck*) (22) (Fig. 2*A*) and *TCR-α* (24) (n = 30; Table 3, which is published as supporting information on the PNAS web site).

DNA flow cytometry was also used to determine clonality and to assess whether leukemias acquired chromosomal abnormalities during disease progression (Table 2; see also Fig. 8, which is published as supporting information on the PNAS web site). Ten of 63 leukemias analyzed were hyperdiploid, as indicated by clonal increases in DNA content (range = 1.01–1.45). Although most of these leukemias contained monoclonal populations of cells with increased DNA content (9 of 10), one T-ALL sample contained two distinct populations of hyperdiploid cells but failed to show *TCR-α* gene rearrangement by Southern analysis (Fig. 8*B*).

Myc-Induced Leukemias Are Transplantable. Transplantation and propagation of disease into secondary recipients is a hallmark of cancer, so we tested whether leukemic cells from *rag2-EGFP-mMyc* fish could be transplanted by i.p. injection into irradiated adult recipient fish. Lymphoblasts were isolated from primary leukemic fish, and FACS analysis confirmed that samples were highly enriched for leukemic lymphoblasts, containing 93.8 ± 1.4% (±SD) GFP-labeled leukemic cells (n = 10). GFP-labeled cells multiplied and generated leukemia in irradiated recipient fish within 1 month after injection (n = 11 independently arising leukemias). These results are similar to those reported previously for leukemia cells arising in F0 primary injected fish (15).

Zebrafish Myc-Induced T-ALLs Coexpress both *tal1/scl* and *lmo2*. Because *cMYC* expression is up-regulated in three of five molecular subgroups of human T-ALL (1, 9), including those misexpressing (*i*) *TAL1/SCL* plus *LMO1* or *LMO2*, (*ii*) *HOX11*, and (*iii*) *HOX11L2/TLX3*, we predicted that we might observe several distinct molecular subgroups of zebrafish T-ALL. However, RT-PCR (Fig. 2*A* and Table 3) and *in situ* hybridization analyses (Fig. 2 *B–E*) showed that all Myc-induced leukemias coexpress *tal1/scl* and *lmo2* (n = 20) but not *lmo1*, *hox11*, or the zebrafish orthologues of *HOX11L2/TLX3*, *tlx3a*, or *tlx3b* (26). By contrast, RT-PCR analysis revealed that normal thymocytes from *lck-GFP* and *rag2-GFP* stable transgenic fish express lower levels of *scl* and similar levels of *lmo2* when compared with Myc-induced T-ALLs (Fig. 2*A*). Finally, RNA *in situ* hybridization of paraffin-embedded sections from 70-day-old *lck-GFP* transgenic animals showed that *scl* and *lmo2* are expressed in only a subset of cortical thymocytes (Fig. 2 *F–M*).

To investigate the mechanisms by which *tal1/scl* and *lmo2* are overexpressed in zebrafish T-ALL, we asked whether expression was monoallelic or biallelic at the chromosomal level. We identified four zebrafish leukemia sample DNAs that harbored polymorphisms in the 3′ untranslated region of *scl* and three with polymorphisms in the *lmo2* gene (n = 20). RT-PCR analysis of these cDNAs revealed that in each case, *scl* and *lmo2* transcripts were up-regulated equally from both chromosomal alleles (Table 2; see also Fig. 9, which is published as supporting information on the PNAS web site), indicating that expression is biallelic and does not result from chromosomal translocations or other allele-specific deletions or mutations.

Apoptotic Pathways in Zebrafish Myc-Induced T-ALL. Myc-induced transformation in mammals collaborates with mutations that dis-

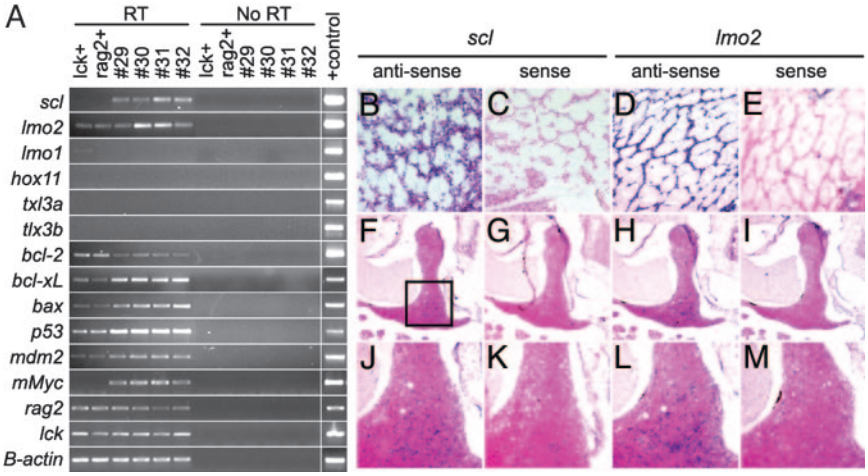

Fig. 2. Leukemic lymphoblasts express T cell markers and both *scl* and *lmo2*. (*A*) Semiquantitative RT-PCR showing that Myc-induced leukemias are arrested at a stage in which *scl* and *lmo2* are coexpressed. RT-PCR analysis of FACS-sorted, GFP-labeled thymocytes from 70-day-old transgenic *lck-GFP* (lck+) and *rag2-GFP* (rag2+) fish or leukemic lymphoblasts isolated from diseased fish (denoted by numbers). RT, reverse transcription reactions; No RT, no reverse transcription controls. (*B–M*) RNA *in situ* hybridization of paraffin-embedded sections confirms that *scl* and *lmo2* are coexpressed in lymphoblasts from *rag2-EGFP-mMyc* transgenic fish (shown here infiltrating the skeletal musculature) and that *scl* and *lmo2* are expressed in a subset of thymocytes in 70-day-old, nonleukemic, *lck-GFP* transgenic zebrafish. Images are photographed at 400× (*B–E*), 50× (*F–I*), and 200× (*J–M*). Images in *J–M* are higher-magnification images of the respective regions in *F–I* (indicated by the boxed region in *F*).

able components of the cellular apoptotic machinery (1, 14, 27, 28). However, given the rapidity of leukemia onset in our transgenic model, we questioned whether apoptosis was suppressed in zebrafish T-ALL and, if so, whether we could document the mechanisms that deregulate cell death in these tumors. Semiquantitative RT-PCR analysis showed that each of the zebrafish T-ALLs expressed similar levels of *p53*, *mdm2*, *bcl-xL*, *bcl-2*, and *bax* RNAs ($n = 12$; Fig. 2*A* and Table 3). When compared with thymocyte controls, Myc-induced T-ALLs expressed higher levels of *bcl-xL*, *bax*, *p53*, and *mdm2*; however, *bcl-2* RNA expression was decreased, likely reflecting that T-ALLs are arrested at a stage of development marked by this gene expression profile (8).

Because human T-ALLs have either biallelic deletions of the *CDKN2A* locus (14) or, less frequently, mutational inactivation of *p53* (29, 30), we asked whether zebrafish Myc-induced leukemias harbor abnormalities in orthologues of these loci. The *p16* copy number was not decreased in these tumors as detected by Southern blot analysis ($n = 21$) (Table 2; see also Fig. 10, which is published as supporting information on the PNAS web site). Because >90% of mutations in *p53* occur within exons 4–8 (the region that encodes the DNA-binding domain) (31, 32), we analyzed zebrafish T-ALL samples for mutations in *p53* in these corresponding exons. We sequenced genomic DNA from zebrafish T-ALL leukemia cells and failed to identify any mutations in exons 4–9 of the *p53* genomic locus ($n = 12$; Table 2).

Cre/lox Conditional Transgenic Strategies in the Zebrafish. T-ALL develops in 100% of stable transgenic *rag2-EGFP-mMyc* fish and progresses to widespread disease before reproductive maturity, necessitating harvesting sperm from diseased males to maintain the transgenic line by *in vitro* fertilization (IVF) (15). Because IVF procedures are cumbersome and not amenable to forward genetic approaches, we sought to develop a conditional transgenic approach, allowing identification and maintenance of zebrafish lines that did not develop leukemia until the investigator selectively induced T cell-specific expression of the Myc oncogene. For this purpose, we developed Cre/lox-mediated transgenic approaches, which have been reported in mice (33, 34) and *Xenopus* (35, 36) but not zebrafish.

We created a vector in which the CMV promoter drives the ubiquitous expression of a dsRED2 transgene that is followed by multiple transcription stop sites and flanked by loxP sites. The EGFP coding sequence was cloned downstream of this cassette (*CMV-loxP-dsRED2-loxP-EGFP* vector; Fig. 3*A*). Transient injection of the *CMV-loxP-dsRED2-loxP-EGFP* construct into embryos without *Cre* recombinase results in dsRED2 fluorescence and no EGFP expression (50 ng/μl; Fig. 3 *C* and *D*). By contrast, coinjection of the *CMV-loxP-dsRED2-loxP-EGFP* plasmid (50 ng/μl) with *Cre* RNA (25 ng/μl) resulted in the excision of the *dsRED2* allele and juxtaposition of the *EGFP* transgene next to the *CMV* promoter, leading to embryos that express EGFP and no red fluorescence (Fig. 3 *F* and *G*). Excision was extremely efficient in embryos injected with 25 ng/μl *Cre* RNA because single cells with red fluorescence were observed in <5% of injected zebrafish embryos ($n = 100$).

Applying this strategy to our transgenic models of T cell malignancy, the *rag2-EGFP-mMyc* transgene was modified by inserting the *loxP-dsRED2-loxP* cassette between the *rag2* promoter and the *EGFP-mMyc* oncogene (Fig. 4*A*), and two stable transgenic lines were generated (G7 and G16). In the absence of Cre-mediated recombination, *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic fish exhibited high levels dsRED2 expression in the developing thymocytes but failed to express the *EGFP-mMyc* transgene or develop lymphoma or leukemia (Fig. 4*B*). After the injection of *Cre* RNA into G7 and G16 one-cell embryos, the *dsRED2* allele was excised in both lines (Fig. 4*C*); however, only transgenic zebrafish from the G7 line developed T-ALL. Most leukemias arising in the G7 line expressed both dsRED2 and EGFP-mMyc (Fig. 4*D*),

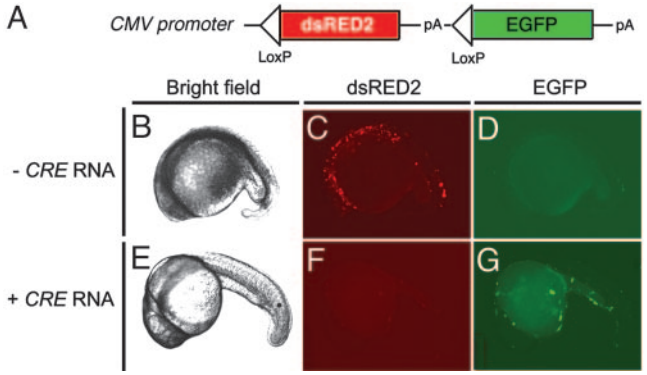

Fig. 3. Cre-mediated recombination in transiently injected embryos. (*A*) Diagram of the *CMV-loxP-dsRED2-loxP-EGFP* construct. (*B–G*) One-cell-stage embryos were injected with the *CMV-loxP-dsRED2-loxP-EGFP* vector in the absence of *Cre* RNA (− *Cre* RNA) (*B–D*) or with *Cre* RNA (+ *Cre* RNA, 25 ng/μl) (*E–G*). Shown are bright-field (*B* and *E*), red fluorescence (dsRED2) (*C* and *F*), and green fluorescence (EGFP) images (*D* and *G*) of embryos at 26 h postfertilization. Anterior is to the left, and dorsal is toward the top.

suggesting that Cre recombination was incomplete in embryos injected with 25 ng/μl *Cre* RNA. Some of the G7 line leukemias exhibited complete recombination and expressed only the *EGFP-mMyc* transgene, indicated by green fluorescence without any

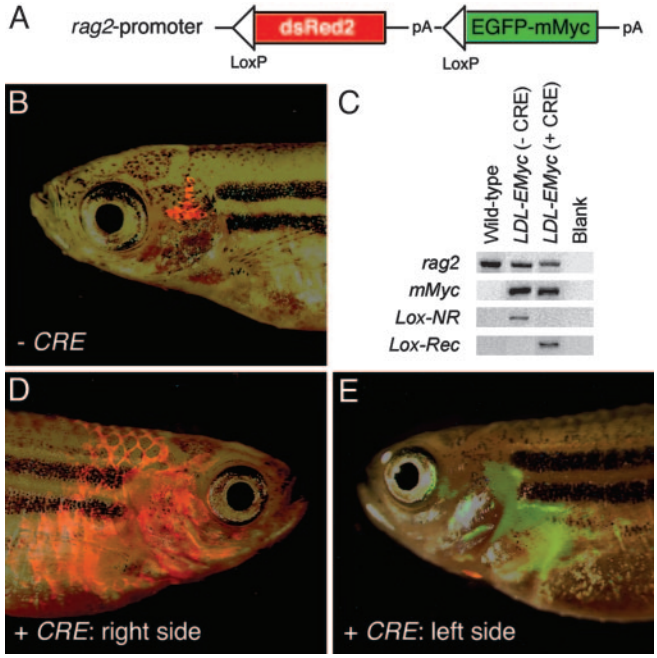

Fig. 4. *Cre* RNA injection into stable transgenic *rag2-loxP-dsRED2-loxP-EGFP-mMyc* fish leads to transgene recombination and rapid onset of Myc-induced T-ALL. (*A*) Diagram of *rag2-loxP-dsRED2-loxP-EGFP-mMyc* construct. (*B*) Thymocytes from a 73-day-old *rag2-lox-dsRED2-EGFP-mMyc* transgenic fish are red-fluorescent-labeled in the absence of *Cre* expression. (*C*) PCR of genomic DNA isolated from blood cells of WT control or transgenic *rag2-loxP-dsRED2-loxP-EGFP-mMyc* (*LDL-EMyc*) fish. *rag2* primers amplify genomic DNA, *mMyc* primers amplify the *mMyc* transgene, and Lox primers amplify either a 1.7-kb nonrecombined fragment (*Lox-NR*) or a 0.4-kb fragment, which results when Cre recombination has occurred (*Lox-Rec*). (*D* and *E*) One-cell-stage *rag2-loxP-dsRED2-loxP-EGFP-mMyc* embryos were injected with the *Cre* RNA (25 ng/μl) and grown to 51 days of development, at which time they had both GFP- and dsRED2-labeled (*D*) or GFP-positive alone (*E*) leukemias. The same fish is shown, right (*D*) and left (*E*) side. Images are composites of dsRED2 and GFP fluorescence and bright-field images.

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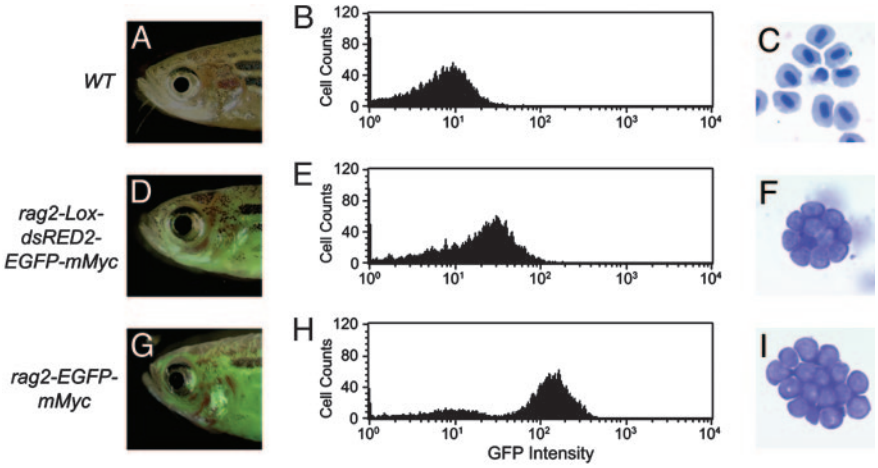

Fig. 5. Leukemias developing in *Cre*-injected *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic fish are transplantable, express low levels of GFP fluorescence, and have typical lymphoblast morphology. WT control (*A–C*) and irradiated WT fish transplanted with lymphoblasts from *rag2-loxP-dsRED2-loxP-EGFP-mMyc* (*D–F*) or *rag2-EGFP-mMyc* transgenic (*G–I*) fish. (*A*, *D*, and *G*) Fluorescent microscopic analysis with fish oriented with anterior to the left and dorsal to the top. Images are a composite of GFP fluorescence and bright-field images. (*B*, E, and *H*) FACS analysis based on GFP fluorescence. (*C*, *F*, and *I*) Giemsa/May–Grunwald staining of blood cells obtained from cytospin analysis. Original images were photographed at 1,000×.

detectable red fluorescence (Fig. 4*E*). Heterozygous *rag2-loxP-dsRED2-loxP-EGFP-mMyc* fish (G7 line) were bred to AB WT fish and injected with Cre RNA at the one-cell stage of development. In total, 12 of 186 CRE-injected progeny developed disease in the G7 line by 151 ± 61 days (range = 52–192 days; $n = 5$).

T-ALLs in Cre-Injected Fish Are Similar to Those from *rag2-EGFP-mMyc* Leukemias. Fluorescent microscopic analysis revealed that *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic zebrafish injected with *Cre* RNA at the one-cell stage develop thymic leukemias as adults, indicating that these tumors are of T cell origin. Additionally, fluorescent leukemia cells can be transplanted into irradiated recipients (Fig. 5 *D* and *G*; $n = 3$) and have lymphoblast morphology as determined by Giemsa/May–Grunwald staining (Fig. 5 *F* and *I*; $n = 2$). Taken together, these results indicate that leukemias arising in the conditional *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic line (G7) are similar to those observed in the *rag2-EGFP-mMyc* stable transgenic line.

Discussion

We have previously shown that transgenic zebrafish develop Myc-induced T cell lineage leukemia (15); however, a detailed molecular characterization of these tumors has not been described. Leukemias developing in *rag2-EGFP-mMyc* transgenic fish are remarkably similar to those found in human patients with T-ALL. For example, zebrafish Myc-induced T-ALLs arise after a defined latency period and have clonal *TCR-α* gene rearrangements, suggesting that additional mutations are required for malignant transformation of the T cell. Additionally, these zebrafish T-ALLs coexpress both *scl* and *lmo2*, resembling the most common and most treatment-resistant molecular subtype of this disease in humans (1, 11). Both *scl* and *lmo2* are biallelically activated in zebrafish Myc-induced T-ALL, in a pattern similar to that of a subset of patients who have overexpression of *SCL* and *LMO2* in leukemic lymphoblasts, indicating that malignant transformation likely results from the disruption of upstream regulatory mechanisms that normally turn off the expression of these transcription factors during double-negative thymocyte development (8).

Although zebrafish Myc-induced T-ALLs are similar to human leukemias, there are also key differences. First, zebrafish Myc-induced T-ALLs resemble only one subclass of human T-ALLs, those that coexpress *SCL* and *LMO2*. Remarkably, we never observe expression of *hox11/tlx1* or the *hox11L2/tlx3* family members in Myc-induced leukemias in the zebrafish (26). It is possible that timing of transgene expression during thymocyte development affects the subtype of T-ALL that is observed. For example, *rag2* expression is tightly regulated during T cell development and is only induced in cells undergoing active *TCR-α* and *TCR-β* gene rearrangement (37, 38), providing two waves of Myc transgene expression during thymocyte development. Another possibility is that the mechanisms that regulate HOX11- and HOX11L2-induced transformation in humans are not found in the zebrafish. *rag2-hox11* and *rag2-tlx3* transgenic zebrafish will need to be developed to determine whether overexpression of a hox11 family member can synergize with Myc in the genesis of zebrafish T-ALL.

Human and murine leukemias escape cell death by inactivation of multiple gene products involved in regulating the apoptotic pathways. For example, leukemias developing in *Emu-Myc* mice harbor mutations that curb apoptosis, including the loss of p19(*ARF*), mutation of *p53*, or up-regulation of Mdm2 (27), and most human T-ALLs have biallelic deletion of the *CDKN2A* locus, which encodes the *P14*(*ARF*) gene (14). Because of these findings, we expected to identify abnormalities that down-regulate apoptotic pathways in our zebrafish leukemias; however, we did not find variable expression levels of *mdm2*, *bcl-xL*, *bcl-2*, *p53*, or *bax*, nor did we find deletions in the *p16* gene locus or mutations in the *p53* DNA-binding domain. Thus, we conclude that either suppression of apoptosis is mediated by a currently unidentified mechanism in zebrafish T-ALLs or Myc-induced transformation in zebrafish lymphoid cells does not require an associated mutational inactivation of the apoptotic machinery. Given that teleost fishes apparently lack an *ARF* gene (39), it is likely that overexpression of myc may not activate the cell death machinery through the p53 pathway in zebrafish, as has been documented in mammalian cells (40). Further experiments using *rag2-EGFP-bcl-2* transgenic (41) and *p53*-deficient fish (25) will likely resolve whether suppression of apoptosis is required for malignant transformation of the T cell in zebrafish.

Leukemias developed in 100% of stable transgenic *rag2-EGFP-mMyc* fish, which is optimal for performing genetic screens designed to uncover mutations that enhance or suppress leukemogenesis. However, the transgenic *rag2-EGFP-mMyc* zebrafish line has been difficult to maintain, because these fish develop disease before reaching full reproductive maturity. To resolve this problem, we developed conditional transgenic zebrafish by using Cre/lox technology (33–36). Two transgenic zebrafish lines were identified

that contained the *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgene. In the absence of *Cre* RNA expression, both lines had strong expression of dsRED2 within the developing T cells, and no fish developed disease, indicating that the mMyc oncogene was not expressed in the absence of Cre recombination. After injection of *Cre* RNA into one-cell-stage embryos, both transgenic lines exhibited recombination at the loxP sites; however, only the G7 stable line produced offspring that developed T-ALL. Lack of leukemia onset in the second transgenic line (G16) reinforces the need to develop multiple transgenic lines and suggests that positional effects of integration and/or concatamer orientation may significantly affect Cre-mediated excision and subsequent expression of the second transgene.

Because only 13% of Cre-injected transgenic fish developed disease, it is likely that injection of *Cre* RNA results in suboptimal recombination and mosaic activation of the *EGFP-mMyc* transgene after recombination. This interpretation is supported by the fact that most leukemias arising in *Cre*-injected *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic fish are both dsRED2- and GFP-labeled, indicating that leukemic clones had partial Cre-mediated recombination at the locus containing transgene concatamers. Similarly, individual fish had a leukemic clone that expressed both dsRED2 and GFP arising in one thymus and a second leukemia clone that expressed only GFP in the other thymus, indicating that Cre recombination occurs in a mosaic fashion within hematopoietic progenitors of individual fish. Similar results have been observed in *Xenopus* (36). For example, transient injection of *Cre* RNA into stable transgenic frogs harboring a *CMV-loxP-ECFP-loxP-EYFP* transgene resulted in mosaic expression of the second ORF. Furthermore, in some animals, neither blue (ECFP) nor yellow (EYFP) fluorescence was detected in transgenic frogs after *Cre* RNA injection, leading the researchers to conclude that the copy number of the reporter had been reduced by recombination without generating an active EYFP expression cassette (36). By contrast, breeding these *CMV-loxP-ECFP-loxP-EYFP* transgenic frogs to stable transgenic *Cre*-expressing animals resulted in ≈100% of doubly transgenic offspring having homogenous expression of the second *EYFP* ORF. Thus, developing transgenic zebrafish lines that specifically express Cre in the developing T cells will likely aid in the establishment of more penetrant models of disease.

Although zebrafish models of T-ALL exhibit key differences when compared with the human disease, analysis of the conserved mechanisms underlying transformation will likely lead to insights into the pathogenesis of human disease. For example, the molecular mechanisms responsible for regulating biallelic activation of *scl* and *lmo2* are unknown, and the downstream targets of Myc that are responsible for oncogenic transformation and genomic instability have yet to be identified. Because the zebrafish affords the unique opportunity to perform forward genetic screens, it should be possible to dissect the pathways that regulate Myc-induced disease in a genetically tractable vertebrate. The proven feasibility of Cre/lox-mediated strategies in the zebrafish will aid the development of models of leukemia, lymphoma, and other cancers, which will provide opportunities for both genetic and chemical modifier screens designed to identify suppressors and enhancers in carcinogenesis. For example, a dominant modifier genetic screen could be conducted by breeding *N*-ethyl-*N*-nitrosourea-mutagenized fish with homozygous *rag2-loxP-dsRED2-loxP-EGFP-mMyc* fish and then analyzing the progeny for the time of leukemia onset after CRE recombination and expression of the Myc transgene. Mutations that modify the time of leukemia onset could be either enhancers that result in more rapid onset of leukemia due to the inactivation of one allele of a tumor suppressor gene or suppressors that delay or prevent the onset of Myc-induced transformation. Finally, use of the Cre/lox technology in the zebrafish will provide tools for assessing cell lineage commitment and plasticity of stem cells and generating conditional knockouts in developing embryos.

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