Mutant \(\text{IL7R}\) collaborates with \(\text{MYC}\) to induce \(\text{T-cell acute lymphoblastic leukemia}\)

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive pediatric cancer. Amongst the wide array of driver mutations, 10% of T-ALL patients display gain-of-function mutations in the IL-7 receptor α chain (IL-7Rα, encoded by \text{IL7R})\), which occur in different molecular subtypes of this disease. However, it is still unclear whether IL-7R mutational activation is sufficient to transform T-cell precursors. Also, which genes cooperate with \text{IL7R}\) to drive leukemogenesis remain poorly defined. Here, we demonstrate that mutant \(\text{IL7R}\) alone is capable of inducing T-ALL with long-latency in stable transgenic zebrafish and transformation is associated with \(\text{MYC}\) transcriptional activation. Additionally, we find that mutant \(\text{IL7R}\) collaborates with \(\text{Myc}\) to induce early onset T-ALL in transgenic zebrafish, supporting a model where these pathways collaborate to drive leukemogenesis. T-ALLs co-expressing \(\text{IL7R}\) and \(\text{Myc}\) activate \(\text{STAT5}\) and \(\text{AKT}\) pathways, harbor reduced numbers of apoptotic cells and remake tumors in transplanted zebrafish faster than T-ALLs expressing \(\text{Myc}\) alone. Moreover, limiting-dilution cell transplantation experiments reveal that activated IL-7R signaling increases the overall frequency of leukemia propagating cells. Our work highlights a synergy between mutant \(\text{IL7R}\) and \(\text{Myc}\) in inducing T-ALL and demonstrates that mutant \(\text{IL7R}\) enriches for leukemia propagating potential.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is an aggressive and common hematological cancer of childhood. It arises from lymphoid progenitors arrested at different developmental stages, with approximately 15% of pediatric ALL cases being T-cell in origin (T-ALL) and having high risk and poor prognosis [1, 2]. Remarkable improvements in treatment outcome have led to 5-year survival rates reaching 80–90% but require the use of risk-adjusted multi-agent intensive chemotherapy that often leads to both short- and long-term severe complications [3–6]. Furthermore, a significant number of T-ALL cases still relapse and have dismal prognosis. Therefore, it is essential to gain more insight into the molecular mechanisms of T-ALL and its underlying biology to develop novel and more efficient therapeutic strategies that selectively target the leukemic cells while minimizing side effects.

The signaling axis comprised by interleukin-7 (IL-7) and its receptor, composed of IL-7Rα (encoded by \text{IL7R}) and γc (encoded by \text{IL2RG}), regulates normal T-cell development and peripheral T-cell homeostasis [7–9]. Inactivation of IL-7 or IL-7R results in severe combined immunodeficiency (SCID) [7, 10–13]. By contrast, constitutive activation of the IL-7/IL-7R axis can induce T-cell leukemogenesis [14], as evidenced by studies showing that transgenic mice overexpressing \text{Ii7}\) spontaneously develop T-cell lymphomas [15], that IL-7 induces proliferation and survival of human T-ALL cells [16–19] and that IL-7 accelerates disease progression in xenotransplant models of human T-ALL [20]. \text{IL7R}\ is also transcriptionally upregulated by Notch [21, 22], one of the most commonly mutated genes in T-ALL [23]. Moreover, somatic \text{IL7R} gain-of-function oncogenic mutations were identified in approximately 10% of T-ALL patients, including high-risk cases [24–27]. Different studies confirmed that mutant \text{IL7R}\ collaborates with \text{Cdkn2a}\ deletion, or overexpression of intracellular Notch1 or mutant \text{NRAS} [28–31], to drive T-ALL. However, the identification of the oncogenic events that cooperate with \text{IL7R}\ mutational activation in originating the disease is still limited, and, most important, whether mutant \text{IL7R}\ alone can trigger T-ALL development remains unaddressed. Finally, although there is anecdotal evidence that high IL7R expression correlates with increased leukemia propagating/stem cell activity [22], the impact of \text{IL7R} activation in regulating the overall frequency of leukemia propagating cells within T-ALL is currently not known.

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Here, we show that IL7R gain-of-function alone is sufficient to trigger T-cell leukemogenesis in zebrafish, a process that involves increased IL-7R-mediated signaling as well as transcriptional activation of MYC. In agreement, mutant IL-7RA also acts as a collaborating oncogene that synergizes with Myc to drive early-onset T-ALL. Notably, leukemias derived from the combination of Myc and mutant IL7R exhibit high basal IL-7R-mediated signaling activation and display higher frequency of leukemia propagating cells (LPCs) than T-ALLs derived from Myc alone.

MATERIALS AND METHODS

Transgenic DNA expression constructs
DNA constructs used to generate transgenic zebrafish have been previously described and included rag2mCherry [32], rag2eGFP [33] and rag2MyC [34]. IL7R constructs were created by PCR amplification of the human IL7R open reading frame and gateway cloning into the rag2 promoter destination vector using LR clonase II, according to manufacturer’s protocol (Life Technologies, ThermoFisher 11791020). These IL7R constructs harbor two specific mutations found in pediatric diagnostic T-ALL samples; namely IL7R<sup>mut</sup><sub>transcript</sub> c.726_727insAACCCATGC (p.L242_L243insNPC) and IL7R<sup>mut</sup><sub>protein</sub> c.731_732insTTGTTCCAC (p.T244_J245insCPT) [24]. PCR primer sequences can be found in Supplementary Table 1.

Zebrafish T-ALL models
Plasmids were linearized with NotI or XhoI and column purified. Mosaic transgenic zebrafish were generated as previously described [35, 36]. 40 ng/µL of each DNA construct was mixed with 40 ng/µL of each linearized vector, and 40 ng/µL of luciferase (luc) was added to the above mix and micro-injected into one-cell stage TuAB-strain embryos. For experiments in CG1 strain fish, the above mix was diluted 1:1. Stable transgenic rag2;IL7R<sup>mut</sup>-tdTomato CG1-strain zebrafish were created using the Tol2 transposon system [37]. Animals injected with each DNA construct were randomly selected from a pool of genetically equivalent animals. This initial group allocation as well as data collection and/or analysis were not blinded, since there were no subjective measurements in the experimental analysis. We used the minimum number of animals that allowed to perform standard deviation analysis when required and to achieve statistical significance. Animals were scored for fluorescent-labeled thymocytes at 21- and 28-days post fertilization (dpf) and followed every 7 days for disease onset and progression. Leukemic fish were defined by >50% of their body being white fluorescent. Zebrafish with fish were monitored for at least 1 year (dpf) and in some cases up to 2 years (dpf). Each experiment was repeated three times, with between two and six fish per group, depending on growth rate and disease outcome. The average latency to disease onset, disease duration, and percentage of moribund animals were calculated. A Student’s t-test was performed to determine statistical significance.

Histological and immunohistochemical analysis
Fish were sacrificed when morbund and zebrafish leukemias were harvested for further analysis. May–Grünewald Giemsa staining was performed as previously described [34] followed by imaging on the Hamamatsu NanoZoomer SQ, with the help of the Comparative Pathology Unit at MGH, Boston, USA. Sections were imaged at 40X magnification using an Olympus BX41 microscope. The ratio of positively stained cells to total cells was calculated in three separate areas of each head. A square root transformation was applied to each data point to stabilize variance and significance was calculated by Mann–Whitney test.

Quantitative real-time PCR
RNA was isolated from the cells using the Qiagen RNeasy Mini kit with on-column DNAse treatment, following the manufacturer’s instructions. Total RNA was reverse-transcribed using SuperScript<sup>™</sup> III First-Strand Synthesis SuperMix (ThermoFisher 11752050) and real-time PCR performed using Power SYBR<sup>®</sup> Green Master Mix in a ViA™ 7 real-time PCR system (both from Applied Biosystems). qRT-PCR was performed on bulk leukemias or FACS-sorted T-ALL cells (>3 genotype), PCR primer sequences are available in Supplementary Table 1. Data were normalized to beta-actin expression and fold-change was calculated using the comparative CT method <sup>ΔΔCT</sup>. Samples were run in triplicate, with error bars representing the SEM of compiled data from all replicates and experimental samples.

Western blot analysis and antibodies
To evaluate protein expression, cells were lysed in buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub> 10 mM NaF; 10 mM NaPyrophosphate; supplemented with protease inhibitor cocktail Complete Mini (Roche)), supplemented with 1 mM of AEBSF (Bio-Rad). Total protein was quantified using the Bradford assay (Bio-Rad). Before resolving the protein extracts, samples were resuspended in Laemmli sample buffer (Bio-Rad 1610737) and denatured for 5 min at 95 °C. Equal amounts of protein extracts were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with the following primary antibodies: p-STAT5 (Y694) (#9359), p-Akt (#473) (#9271), p-Erk (T202/Y204) (#9101), p-S6 (25235/2621) (#2211), STAT5 (#94205), Akt (#9272), Erk (#4695) and S6 (#2217) (all from Cell Signaling Technology) and beta-actin (sc-7778) from Santa Cruz Biotechnology). Immunodetection was performed by incubation with horseradish peroxidase-conjugated secondary antibodies (Promega Corporation) and developed by chemiluminescence detection using the Pierce<sup>TM</sup> ECL Western Blotting Substrate (ThermoFisher). Films exposed to the membranes were developed in a Curix60 (AGFA HealthCare), Amersham<sup>®</sup> ECL<sup>®</sup> Rainbow<sup>®</sup> Marker – Full range (GE Healthcare) was used as molecular weight reference.

Limiting dilution cell transplantation
T-ALL cells were isolated, transplanted and monitored for tumor growth as previously described [32, 36, 38]. Briefly, tumor-bearing fish were macerated in 5% FBS + 0.9X PBS, cells were strained through a 40 µm nylon mesh (BD Falcon) and isolated by FACS at several dilutions into 96-well plates [36]. Flow cytometry analysis was performed at the MGH Pathology CNY Flow Cytometry Core, MGH, Boston, USA, and at the Flow Cytometry Unit, IMM-JLA, Lisbon, Portugal. Each well contained 50 µL 5% FBS + 0.9X PBS and was supplemented with 3 x 10<sup>4</sup> whole blood cells isolated from CG1-strain zebrafish. Cells were then centrifuged, resuspended in 5 µL 5% FBS + 0.9X PBS and transplanted into the peritoneal cavity of recipient syngeneic CG1-strain zebrafish. Fish were monitored for T-ALL for up to 120 days. Leukemia-propagating cell frequency and the 95% confidence intervals were calculated using the web-based ELDA (Extreme Limiting Dilution Analysis) statistical software (http://bioinf.wehi. edu.au/software/elda/)[40].

RNAseq analysis
Leukemia cell pellets were mixed with QIAGEN RLT buffer containing 1% 2- mercaptoethanol, followed by RNA isolation using the RNAeasy Mini kit (QIAGEN). RNA samples were prepped and sequenced on the Illumina HiSeq 2000 platform as previously described [41]. RNA sequence reads were aligned to GRCh10 and the Ensembl version 85. PCR duplicates and ribosomal RNA sequences were removed [41]. Human orthologs were identified using the Beagle web interface and subsequent analysis essentially completed as previously described [41]. RNA sequencing data is available at the Sequence Read Archive (SRA) under accession number PRJNA812715.

TCR gene rearrangements and clonality analysis
The detection of TCR rearrangements on RNA-Seq raw data was performed with the MIXCR tool [42]. At the time of analysis, the zebrafish TRB locus germline sequences were unavailable at the IMGT database. For the alignment, reference sequences were therefore obtained from Meeker et al., 2010 [43], and manually curated for the basic anchor points (FR1 begin, CDR3 begin, and V end – see [42]) and added to the latest version of the Danio rerio TCR libraries (https://github.com/repseqio/library-imgt/releases) in the json format, with the assistance of the repseqio tool (https://github.com/repseqio/repseqio, doi:10.5281/zenodo.804526). Alignment was performed for the entire library, but only TCR clonotype informative rearrangements are shown. In order to assess clonal diversity, the relative frequency of each clonotype should be accounted for alongside the clonotypic richness [44]. We have calculated Shannon equitability index, as a measure of clonotype evenness, on a scale from 0 to 1, where 1 represents balanced distribution of clonotypes frequencies, and lower values are indicative of the presence of clonal expansions. MIXCR
output tables with clonotype counts were further manipulated and eqitability calculated using custom shell and R scripts. TCR rearrangement analysis plots were generated with the R software.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, CA, USA). Differences between groups were calculated using a two-tailed Student’s t test when parametric test assumptions were met. Otherwise, Mann–Whitney tests were performed. Differences in survival curves were analyzed using the Gehan-Breslow-Wilcoxon statistic. Each IL7R downstream pathway and found that those samples showed that mutant IL7R mutant leukemias. Principal component analysis (PCA) showed that mutant IL7R-driven T-ALLs comprise a transcriptionally distinct subgroup that segregates away from Myc-induced T- or B-ALLs, as well as from their normal counterparts (Fig. 2A). As expected, RNAseq analysis confirmed qPCR results and showed that mutant IL7R-derived leukemias were bona fide T-ALLs (Fig. 2B). Furthermore, analyses of TCR-β gene rearrangements showed that healthy thymocytes displayed a high number of clonotypes with very high equitability values (indicative of normal, highly polyclonal distribution of T cell clones), whereas mutant IL7R leukemias displayed few clonotypes with low equitability (Fig. 2C and Supplementary Table 2). This indicates that T-ALLs arising in zebrafish with stable expression of mutant IL7R were (oligo)clonal. As expected, mutant IL7R-driven leukemias exhibited elevated IL-7R-mediated signaling, indicated by high expression of common STAT5 downstream target genes (Fig. 2B).

**RESULTS**

**Mutant IL7R alone is capable of inducing T-ALL in zebrafish**

Somatic IL7R gain-of-function oncogenic mutations can be found in approximately 10% of T-ALL patients. Here, we used a stable transgenic approach to explore the capacity of mutant IL-7Rα to trigger T-ALL development. In these experiments all thymic T cells have the potential for transformation over time. Using the Tol2 transposon system [37], we first generated a stable transgenic CG1-strain zebrafish line (rag2:IL7Rmut2-tdTomato) expressing an IL7R mutation (p.T244_I245insCPT) previously identified in a T-ALL patient [24]. This type of IL7R gain-of-function mutations, named type 1a [13], are insertions or insertion-deletions in exon 6 leading to the introduction of a juxtamembrane-to-transmembrane region of the receptor that promotes IL-7Rα homodimerization and consequent constitutive signaling [24]. Although none of the mosaic F0 founder fish developed tumors (n > 100 animals followed for 1.5 years), 9 (47%) out of 19 transgenic F1 offspring developed leukemia with a mean latency of 20 weeks (range of 17 to 27 weeks; Fig. 1A, B). As expected, control stable transgenic rag2:RFP animals did not develop disease within their lifespan (Fig. 1B). To characterize mutant IL7R-driven leukemias deeper, we next confirmed the lymphoblast morphology of leukemic cells (Fig. 1C) and their T-cell phenotype, as confirmed by qRT-PCR analysis (Fig. S1A). Notably, mutant IL7R T-ALLs displayed reduced numbers of apoptotic cells (Figs. 1C and S1B). STATS, PI3K/Akt/mTOR and MEK/Erk pathways are activated by IL-7R-mediated signaling in healthy lymphocytes and leukemia cells [9, 14, 17, 18, 24, 25, 45–47]. In agreement, we observed hyperactivation of IL-7R-mediated signaling in mutant IL7R leukemias, as evidenced by upregulation of phosphorylation levels of STAT5, Akt and S6 (a downstream target of PI3K/Akt/mTOR pathway), and Erk 1 and 2 (Fig. 1D). Interestingly, we observed some heterogeneity in the levels of hyperactivation of each IL7R downstream pathway and found that those samples with milder STAT5 activation tended to display higher relative levels of Erk phosphorylation. Altogether, our data indicate that IL7R mutational activation alone can drive T-ALL in zebrafish.
Leukemias had fewer apoptotic cells than leukemias driven by IL7Rmut transgenic approach to create zebra accelerated the time to leukemia onset, we next used a mosaic Mosaic transgenic fi (identi T-ALL in transgenic zebra Mutant IL7R collaborates with Myc to induce early-onset T-ALL. These analyses showed that human transcriptional differences between signaling activation of Myc, an oncogenic driver in human T-ALL [48, 49].

Mutant IL7R collaborates with Myc to induce early-onset T-ALL in transgenic zebrafish To address the ability of mutant IL7R to cooperate with Myc to accelerate the time to leukemia onset, we next used a mosaic transgenic approach to create zebrafish T-ALLs [35, 38, 39, 50, 51]. Specifically, one-cell stage Tu/AB-strain embryos were micro-injected with rag2:Myc + rag2:mCherry alone or in combination with rag2:mut2 or another type 1a IL7R gain-of-function mutation (mut1, p.L242.L243insNPC) found in a different T-ALL patient [24]. Mosaic transgenic fish with mCherry-positive thymus were identified at 21 days post fertilization (dpf) and followed for disease onset and progression. Leukemia was defined as >50% of the fish body being overtaken by fluorescent-labeled cells [38, 39]. These analyses showed that human IL7R mutations collaborated with Myc (Fig. 3A, B) to accelerate leukemia onset significantly.

Leukemia cells had similar shape and size, with comparable lymphoblast morphology, irrespectively of whether they originated in mosaic Myc or Myc + IL7Rmut zebrafish (Fig. 3C). As expected, leukemias expressed T-cell specific markers (Lck, CD4, CD8, and TCRα and β), but not B-cell specific genes (e.g. Pax5, CD79a or IgM), indicating they were of T-cell origin (Fig. S1C). Immunohistochemistry analysis showed Myc + IL7Rmut expressing leukemias had fewer apoptotic cells than leukemias driven by Myc alone, as assessed by TUNEL on section (Fig. 3C, S1D). Myc + IL7Rmut leukemias also displayed upregulation of IL-7R-mediated signaling (Fig. 3D). Taken together, our results confirm that mutant IL7R, and consequently IL-7R signaling pathway activation, can collaborate with Myc to induce early-onset T-ALL.

Leukemias arising from the combination of IL7R mutation and Myc overexpression display high basal IL-7R-mediated signaling activation We next performed RNAseq on primary T-ALLs to identify potential transcriptional differences between Myc and Myc + IL7Rmut induced leukemias. As expected from the qRT-PCR results (Fig. S1C), both Myc and Myc + IL7Rmut expressing leukemias were bona fide T-ALL, as determined by multiple markers of T- and B-cell development (Fig. 4A). Importantly, we observed a unique transcriptional profile in Myc + IL7Rmut expressing T-ALLs, including activation of key STATS downstream target genes, such as cish or serpinc1 (Fig. 4A and Supplementary Table 3). This reflects high basal IL-7R-mediated signaling activation, as observed also by STATS and S6 kinase phosphorylation (Fig. 3D). In agreement, transcriptome data integration together with GO enrichment analysis highlighted the enrichment for the IL-2/STAT5 signaling hallmark gene set in Myc + IL7Rmut derived leukemias, as well as for protein phosphorylation (Fig. 4B). Analysis of productive TCR-β gene rearrangements showed that Myc + IL7Rmut T-ALLs displayed higher numbers of clonotypes, with similar representation (given by the Shannon equitability index, which accounts for the relative frequency of each clonotype), than leukemias derived from Myc alone (Fig. 4C). This implicates that whereas Myc T-ALLs tend to be mono or oligoclonal, Myc + IL7Rmut T-ALLs tend to be polyclonal (Fig. 4C and Supplementary Table 2). A higher degree of leukemia polyclonality indicates stronger oncogenic potential and transformation of larger pools of initiating cells [31, 45], which is in accordance with the fact that mutant IL7R accelerated disease onset in Myc-induced T-ALLs (Fig. 3).
Fig. 3 Mutant IL7R collaborates with Myc to accelerate T-ALL onset. a Tu/AB-strain fish injected at the one-cell stage with either rag2|Myc alone or with rag2|IL7Rmut1 or rag2|IL7Rmut2. Animals were also co-injected with rag2|mCherry to visualize leukemia onset and progression. Representative images of transgenic mosaic zebrafish at 28 dpf; Panels are merged fluorescent and brightfield images; Scale bar, 1 mm.

b Kaplan–Meier analysis (Gehan-Breslow-Wilcoxon test). Number of animals analyzed per genotype is shown in parenthesis. Red dots denote fish that developed leukemia from rag2|Myc + rag2|IL7Rmut1 injected fish, whereas black dots show leukemias developing in rag2|Myc + rag2|IL7Rmut2 fish. c May–Grünewald and Wright-Giemsa stained cytospins showing lymphoblast morphology (n ≥ 2 leukemias/genotype analyzed); Scale bar, 50 µm. Histological analysis of primary T-ALLs (n ≥ 3 leukemias/genotype analyzed); Hematoxylin and eosin-stained sections juxtaposed to immunohistochemistry for TUNEL; Arrowheads denote examples of positively stained cells; Scale bar, 10 µm. Percent positive cells ± SEM are shown within each image panel. Asterisks denote significant differences between Myc and Myc + IL7Rmut leukemias as assessed by Student’s t test. d Immunoblot analysis of phosphorylated protein levels in bulk leukemias or FACS-sorted T-ALL cells (n ≥ 3/genotype).

Fig. 4 Myc + IL7Rmut induced leukemias display IL-7R-mediated signaling upregulation and are polyclonal. a Heatmap representation showing expression of well-known T- and B-cell associated genes, as well as common STAT5 target genes (adj. P < 0.05). b Transcriptome data integration and gene set enrichment analysis show a significant enrichment of the IL-2/STAT5 signaling hallmark gene set in Myc + IL7Rmut derived leukemias when compared with Myc derived leukemias. c TCR-β gene rearrangements in Myc + IL7Rmut vs Myc derived T-ALLs. Shown as dotplots and boxplots are the number of clonotypes of the TRB locus and the equitability value per sample, both based on productive rearrangements.
DISCUSSION

IL7R type 1a gain-of-function mutations introduce a de novo cysteine in the IL-7Ra juxtamembrane-to-extracellular domain, leading to disulfide bridge formation that promotes receptor homodimerization and consequent constitutive downstream signaling [13, 24, 25]. Whether these mutations are sufficient to drive T-ALL remains unclear. Also, the repertoire of oncogenic hits that collaborate to promote T-ALL development in the context of IL7R activation is still limited. The mutant allele frequency of IL7R mutations in some ALL patients is compatible with IL7R activation being an early event in the natural history of the disease [45, 52]. In accordence, our experiments with the stable zebrafish line indicate that mutant IL7R alone can be sufficient to trigger T-ALL. Evidently, the relatively low penetrance and long latency of tumor development in this model, together with the clonal nature of the leukemias, indicate that other cooperating hits are required for full transformation and T-ALL establishment. The identification of these hits warrants further investigation. This notwithstanding, our analyses showed that mutant IL7R leads to endogenous Myc activation, a key player in T-ALL development [34, 53-56], highlighting the importance of the interplay between the two oncogenes in the genesis of this malignancy. In human T-ALL, MYC is transcriptionally activated by Notch1 [55, 57], and a majority of T-ALL patients (50–60%) present with NOTCH1 gain of function mutations [23]. IL7R gain-of-function mutations frequently co-occur with NOTCH1 mutations, and NOTCH1 mutations tend to be more common in IL7R mutant patients (75–90%) than in the general T-ALL population [23, 24, 58]. Moreover, adult T-ALL patients with IL-7R pathway mutations (which associate with Notch pathway mutations) are slow-responders that benefit from post-induction chemotherapy but not from hematopoietic stem cell transplantation [59]. Overall, the cooperative effect we discovered in zebrafish between IL7R and MYC appears to reflect an interaction that is of biological and clinical relevance in human T-ALL.

Previous studies showed that retroviral expression of IL7R mutants in murine T-cell or hematopoietic precursors can collaborate with Cdkn2a deletion or with overexpression of mutant NRAS (G13D) or intracellular Notch1 to induce T-ALL [28–31]. However, these models require transduction of progenitors in vitro and subsequent transplant into recipient mice, which may limit their physiological relevance. In the present studies, we showed that IL7R mutation collaborates with Myc in accelerating T-ALL onset and decreasing apoptosis of leukemia cells. Since, contrary to Myc, mutant IL7R is not sufficient to drive T-ALL in mosaic zebrafish, our results may hint at the possibility that IL7R mutation is a late event in T-ALL development which occurs after MYC activation and cooperates with it by preventing apoptosis. This agrees with the fact that IL7R mutations in T-ALL patients are often subclonal [58].

Previous studies suggested that IL-7R-mediated signaling may enrich for LPC potential in T-ALL [22, 60]. Here, we provided the first direct evidence, comparing Myc versus Myc + IL7Rmut leukemias, that IL7R mutational activation increases LPC frequency. Whether this ability is restricted to the collaboration between Myc and IL-7Ra or whether it extends to other oncogenic scenarios should be investigated in the future. IL7R gain-of-function mutations occur not only in T-ALL but also, with lower frequency, in B-ALL patients [25, 61]. Using conditional mutant IL7R knock-in mice crossed with CD2-Cre animals to produce progeny in which recombination occurs at the common lymphoid precursor stage, we recently demonstrated that IL-7R activation in lymphoid progenitors leads to the development of B-ALL rather than T-ALL [45]. In these mice, physiological IL7R transcriptional regulation is preserved. In contrast, lymphoid-restricted, forced expression of wild type mouse or human IL7R drives T-ALL in transgenic mice [14]. Our current studies indicate that ectopic expression of wild type IL-7Ra is not sufficient to promote leukemia development in zebrafish, whereas gain-of-function mutations lead to the development of T-ALL, but not B-ALL. While the exact causes for these differences remain to be determined it seems evident that the ability of IL7R to act as an oncogene in B or T lymphoid progenitors will depend not only on IL7R mutational status but also on how IL-7Ra expression is regulated. Characterizing these mechanisms and how they impact the sensitivity of particular lymphoid precursors to transformation will be of major importance for the understanding of how IL7R participates in human leukemia development.

T-ALL cases with IL7R mutation may benefit from targeted therapeutics against JAK, MEK/Erk pathway, PI3K/Akt/mTOR pathway or BCL2 [14, 19, 62]. Given that IL7R-mediated signaling can confer resistance to glucocorticoids [62, 63] and IL7R mutations associate with very poor prognosis upon relapse [27], targeted therapies may be critical to circumvent resistance to conventional therapy and prevent relapse [62, 63]. Our findings are aligned with this possibility. The relevance of the crosstalk between IL7R and
MYC activation in the genesis of T-ALL suggests that therapeutic combinations involving bromodomain inhibitors, which can downregulate both MYC and IL7R [64], may benefit T-ALL cases with IL7R mutation and especially those with refractory or relapsed disease [65]. Also, upregulation of Pim1 in T-ALL cells from stable zebrafish is in agreement with previous studies proposing the use of Pim inhibitors in IL7R-dependent T-ALL, including in relapsed cases [17, 66]. Interestingly, we observe the upregulation of Lck in IL7R mutant leukemias, suggesting the possibility that Src-family kinase inhibitors may be of relevance in cases with IL7R mutation. In addition, our zebrafish models may be leveraged for the identification of new players in IL7R-dependent T-ALL and for the fast, economical pre-clinical testing of new targeted therapies against T-ALL.

Overall, our studies demonstrate that IL7R mutation is sufficient to trigger T-ALL development in zebrafish, while also cooperating with Myc to accelerate disease onset and increase leukemia propagating cell frequency. They also highlight the potential of zebrafish as a powerful model system to identify cooperating hits in the context of IL7R-mediated leukemia development and to dissect the underlying molecular mechanisms of how such co-occurring oncogenic hits cooperate in T-ALL development.

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AUTHOR CONTRIBUTIONS
MLO designed and performed experiments, analyzed and interpreted data; AV and EGG performed experiments and histological analyses; SI performed bioinformatics analyses; CP and VMV performed the analysis of TCR gene rearrangements; JTB and DML jointly contributed the study; MLO, DML and JTB wrote the manuscript. All authors critically read and contributed to the final version of the manuscript.

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COMPETING INTERESTS
The authors declare no competing interests.

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