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Aberrant Activation of the *GIMAP* Enhancer by Oncogenic Transcription Factors in T-cell Acute Lymphoblastic Leukemia

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Abstract

The transcription factor *TAL1/SCL* is one of the most prevalent oncogenes in T-cell acute lymphoblastic leukemia (T-ALL), a malignant disorder resulting from leukemic transformation of thymus T-cell precursors. TAL1 is normally expressed in hematopoietic stem cells (HSCs) but is silenced in immature thymocytes. We hypothesize that TAL1 contributes to leukemogenesis by activating genes that are normally repressed in immature thymocytes. Herein, we identified a novel TAL1-regulated super-enhancer controlling the *GIMAP* locus, which resides within an insulated

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Authorship contributions

W-S.L., S.H.T. and T.S. performed the experiments; C.Q.W., Z.G. and M.O. provided materials; W-S.L., P.C.T.N. and T.S. analyzed the results; V.T., M.O., and A.T.L. supervised this study; and W-S.L. and T.S. designed the research and wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

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chromosomal locus in T-ALL cells. The *GIMAP* genes are expressed in HSCs and mature T-cells but are downregulated during the immature stage of thymocyte differentiation. The *GIMAP* enhancer is activated by TAL1, RUNX1 and GATA3 in human T-ALL cells but is repressed by E-proteins. Overexpression of human *GIMAP* genes in immature thymocytes alone does not induce tumorigenesis but accelerates leukemia development in zebrafish. Our results demonstrate that aberrant activation of the *GIMAP* enhancer contributes to T-cell leukemogenesis.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) arises from the clonal expansion of transformed T-lymphoblasts caused by genetic abnormalities that induce differentiation arrest, dysregulated proliferation and aberrant cell survival.[1–3] The most frequent molecular abnormality in T-ALL is the dysregulation of transcription factor genes, including overexpression of *TAL1/SCL* and activating mutations of *NOTCH1*.[1–3] *TAL1* is normally expressed in hematopoietic stem cells (HSCs), progenitor cells and erythromegakaryocytic cells.[4] In normal HSCs, TAL1 heterodimerizes with E-proteins such as TCF3/E2A and TCF12/HEB and forms a large transcriptional complex with LMO2, LDB1 and GATA2.[5–9] TAL1 frequently co-occupies the regulatory elements with other transcription factors, including RUNX1 and the ETS family of proteins.[10, 11] Importantly, TAL1 is normally silenced in immature thymocytes,[12] whereas E-proteins are upregulated and required for thymocyte development by acting as homo- or heterodimers.[12–14] Such stage-specific regulation of TAL1 and E-proteins is essential in normal hematopoiesis.

In contrast, TAL1 is ectopically overexpressed in 40–60% of T-ALL cases as a result of chromosomal translocation, intrachromosomal rearrangement or a somatic mutation in a non-coding intergenic element.[15–19] In both human T-ALL and mouse models, *TAL1* overexpression leads to a blockage at later stages of differentiation in developing thymocytes.[12, 20, 21] We previously reported that in T-ALL cells, TAL1 coordinately regulates gene expression with GATA3, RUNX1 and MYB similar to a mechanism observed in normal HSCs.[22] In addition, TAL1 positively regulates the expression of a specific subset of genes that are negatively regulated by E-proteins.[22] These results suggested that TAL1 could activate genes that are normally repressed in immature thymocytes by counteracting E-protein function. We hypothesize that such factors would be responsible for the pathogenesis of T-ALL. Interestingly, a recent study showed that *TAL1* and its regulatory partners (*GATA3*, *RUNX1* and *MYB*) are regulated under “super-enhancers”,[23] which are clusters of enhancers that exhibit significantly high levels of histone H3 lysine 27 acetylation (H3K27Ac) and mediator bindings. Rapidly accumulating evidence demonstrates that super-enhancers are often enriched at cancer genes in various malignancies.[23–27] Hence, we further hypothesized that the critical factors involved in the T-ALL pathogenesis would be regulated by super-enhancers to sustain high expression levels.

Here, we describe a regulatory element differentially controlled by TAL1 and E-proteins in human T-ALL cells. This element is associated with a super-enhancer and is located within a cluster of genes in the same family known as the *GTPase of Immunity-Associated protein (GIMAP)*, which have been implicated in mature T-cell survival.[28–32] *GIMAP* genes and the

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enhancer are activated in normal HSCs and human T-ALL cells but not in thymocytes in immature stages. Ectopic expression of *GIMAP* genes in thymocytes accelerates T-cell leukemogenesis *in vivo*.

Materials and Methods

Cell culture

All T-ALL cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS and L-glutamine (Life Technologies). All cell lines were confirmed by DNA fingerprinting using the PowerPlex 1.2 system (Promega) in January 2013 and were regularly tested for mycoplasma contamination. 293T cells were grown in DMEM medium supplemented with 10% FBS and L-glutamine (Life Technologies).

Analysis of ChIP-Seq, gene expression and ChIA-PET datasets

The ChIP-Seq and microarray gene expression datasets were previously reported by us[22] and deposited into the NCBI GEO website (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE29181. The ChIP-Seq datasets for NOTCH1 and RBPJ by Wang *et al.*[33], CTCF by Hnisz *et al.*[34] and BRD4 have been deposited under accession numbers GSE29544, GSM1689152, GSM1689151 and GSE83777, respectively. The dataset for H3K27Ac in Jurkat cells[23] and normal thymus[35] have been deposited under accession numbers GSM1296384 and GSM1013125, respectively. All datasets were analyzed and uploaded into the UCSC genome browser, and sequences were aligned to the human genome sequence (hg19). The microarray gene expression dataset was normalized using dChip software.[36] High-confidence chromatin-chromatin interactions were retrieved from the ChIA-PET data[34] analysis using the pipeline reported by Dowen *et al.*[37] The interactions were visualized on the WashU Genome Browser.[38] Selected genes were included in Supplementary Table 1.

RNA-Seq

Total RNA was extracted from Jurkat cells by the miRNeasy extraction kit (Qiagen). Library construction and RNA-Seq analysis by the Illumina HiSeq 2000 were performed at BGI Tech Solutions (Hong Kong). Paired-end 90 bp long reads were aligned to hg19 using STAR 2.4.0i[39] with the outFilterMatchNminOverLread parameter set to 0.80 and outFilterMultimapNmax set to 1. A BEDGRAPH RNA-Seq coverage across the entire genome was calculated by Bedtools genomecov. The data have been deposited to the GEO under accession number GSE81712.

CRISPR/Cas9 knockout

Guide RNAs (gRNAs) targeting either the *GIMAP* enhancer or the whole *GIMAP* gene cluster were selected using the CRISPR Design Tool (http://crispr.mit.edu/) (Supplementary Table 2) and cloned into the lentiCRISPRvs2 vector.[40] The gRNAs and Cas9 were transduced by lentivirus infection (see Supplementary Method). Genomic DNA was isolated using the QIAamp DNA Blood Mini kit (Qiagen) followed by PCR amplification of targeted loci using specific primers (Supplementary Table 3). PCR products were directly analyzed by Sanger sequencing.

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Cloning of constructs

The 6-kb *GIMAP* enhancer region (hg19, chr7: 150,360,481–150,366,493) was cloned into the pBSII-SK+-I-SceI zebrafish reporter plasmid[41] and the pGL4.26 *luciferase* plasmid (Promega). The *RUNX1* enhancer reporter construct[41] and the zebrafish *rag2* promoter construct[42] have been described previously. The cDNA sequence of each of the human *GIMAPs* was amplified via PCR using primers (Supplementary Table 4) and was cloned into the Rag2-I-SceI zebrafish expression vector. The cDNA of each transcription factor was cloned into the pCS2+ vector.

Zebrafish studies

Zebrafish studies were conducted in strict adherence to the recommendations of the Institutional Animal Care and Use Committee (IACUC), and all protocols were approved by the Committee at the National University of Singapore (NUS). I-SceI meganuclease-based vectors (pBSII-SK-I-SceI and Rag2-I-SceI) were used in wild-type strain to establish transgenic lines.[43] The sample size was determined based on previous similar studies reported by us.[43] At least two stable transgenic lines were generated. Each breeding was repeated at least twice. Sample randomization is not required in this study.

Isolation of hematopoietic cells from mice

All mouse experiments followed guidelines set by the National Advisory Committee for Laboratory Animal Research and the NUS IACUC. C57BL/6 mice were maintained, and bone marrow (BM) cells from 8-week-old inbred mice were flushed from the long bones with α-MEM medium supplemented with 10% FBS (Gibco). BM and thymic cells were filtered through a nylon filter (35 μm) to obtain a single-cell suspension. Flow cytometry sorting was performed using FACSAria (BD Biosciences) to isolate hematopoietic cells (see Supplementary Method).

Lentivirus infection

For lentiviral production, either the CRISPR-Cas9 plasmid or pLKO1-puro was co-transfected into 293T cells with the envelope plasmid pMD2.G and packaging plasmids pMDLg/pRRE and pRSV-REV using FuGENE 6 reagent (Roche). Viral supernatants were collected, filtered through a 0.45-μm filter (Millipore) and transduced into Jurkat cells. The infected cells were selected by puromycin (Sigma).

shRNA knockdown analysis

shRNA sequences were cloned into the lentiviral vector pLKO.1-puro (Supplementary Table 6) and transduced by lentiviral infection (see Supplementary Method). Knockdown levels were verified by qRT-PCR.

RNA extraction, cDNA and expression analysis

For analysis of gene expression in human cell lines, zebrafish and mice, total RNA was extracted using a NucleoSpin RNA kit (Macherey-Nagel) and reverse-transcribed using a QuantiTect reverse transcription kit (Qiagen). Quantitative PCR analysis was performed

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using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems) in duplicates. The primers used for qPCR are listed in Supplementary Table 5.

Luciferase assay

For Jurkat cells, the *GIMAP* enhancer reporter construct was co-transfected with the pGL4.74 *Renilla* reporter plasmid (Promega) using a Neon Transfection System (Invitrogen). *Luciferase* activity was measured using a microplate reader (Tecan) and a Dual-Luciferase Reporter Assay (Promega). To determine *luciferase* activity after knockdown, Jurkat cells that stably expressed the *GIMAP* enhancer reporter construct were selected by hygromycin treatment and were isolated into single clones. The cells were then transduced with lentivirus shRNA. *Luciferase* activity and cell viability were measured using a ONE-Glo™+Tox Luciferase Reporter and Cell Viability Assay kit (Promega).

Statistical Analysis

Two-tailed student t-tests were used to analyze differences in gene expression, luciferase activity, cell growth rate and apoptosis among groups. The standard deviations, standard error of the mean, mean, error bars and p-values are all indicated. Kaplan-Meier analysis and the Gehan-Breslow-Wilcoxon test were used to compare times to tumor onset in zebrafish study. P-values less than 0.05 were considered statistically significant.

Results

TAL1 binds at the super-enhancer site within the *GIMAP* gene cluster in T-ALL cells

We previously identified transcriptional targets directly regulated by TAL1 and its regulatory partners by chromatin immunoprecipitation-sequencing (ChIP-Seq) and microarray analysis in *TAL1*-positive T-ALL cell samples.[22] In the present study, we employed a targeted approach to identify genes and their regulatory elements that are differentially controlled by TAL1 and E-proteins. Using our previous dataset[22], we first filtered genes that were positively regulated by TAL1 and were negatively regulated by E-proteins in a T-ALL cell line (Jurkat) (Supplementary Table 1 and Supplementary Figure 1A). We next explored the Gene Expression Commons database[44] to select genes that are expressed in HSCs and are downregulated in double-negative (DN) stages of thymocytes (Supplementary Table 1 and Supplementary Figure 1B). We then selected genes that are associated with super-enhancers in T-ALL cells but not in the normal human thymus using the ChIP-Seq dataset[23] (Supplementary Table 1).

By these criteria, one element located within the gene cluster on chromosome 7q, which contains seven genes that belong to the same family (*GIMAP1*, *GIMAP2*, *GIMAP4*, *GIMAP5*, *GIMAP6*, *GIMAP7* and *GIMAP8*), was selected (Figure 1A). DNA binding of the TAL1 complex was observed between the *GIMAP2* and *GIMAP6* genes across different TAL1-positive T-ALL cell lines (CCRF-CEM, RPMI-8402 and Jurkat) (Figures 1A and 1B, orange box). Notably, this region was also occupied by NOTCH1, which is another prevalent oncogene in T-ALL, with its binding partner RBPJ/CSL. Wang *et al.* previously reported that activated NOTCH1 regulates the expression of *GIMAP* genes.[33, 45] Importantly, there was substantial binding of RNA polymerase II (RNAP2) and mediator 1 (MED1) as well as

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extensive acetylation of histone H3 lysine 27 (H3K27Ac), constituting the formation of a super-enhancer in this region (Figure 1A, red box; Supplementary Figure 1C).[23, 27] The super-enhancer was not observed in normal thymus (bottom). This result indicates that the *GIMAP* gene cluster is highly activated in T-ALL cells.

GIMAP genes are controlled by the *GIMAP* enhancer in T-ALL cells

Indeed, *GIMAP* genes were highly expressed in many T-ALL cell lines (Supplementary Figure 2A). We identified significant positive correlations in expression levels across seven *GIMAP* genes (Supplementary Figure 2B), suggesting that these genes are coordinately regulated. In fact, chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) for the Cohesin protein[34] demonstrated multiple chromatin-chromatin interactions within the cluster region in Jurkat cells (Figure 2A). Interestingly, binding of the CTCF insulator protein was observed adjacent to the *GIMAP8* and *GIMAP5* genes but not within the cluster (Figure 2A), suggesting that these are neighborhood boundaries of the gene cluster.

We next examined whether the region bound by the TAL1 complex (Figures 1 and 2A, orange box) can control the expression of *GIMAP* genes in T-ALL cells. We designed guide RNAs (gRNAs) targeting either this locus or the whole *GIMAP* gene cluster (Figure 2B, bottom) and transduced a pair of gRNAs with the *Cas9* endonuclease to enable removal of these genomic regions using CRISPR/Cas9 technology. PCR analysis showed successful deletions of the target locus (Supplementary Figure 2C). The chromatogram demonstrated a successful recombination after the cleavage (Supplementary Figure 2D). Importantly, compared to cells transduced with control gRNAs, gene expression of the *GIMAP* genes was concomitantly downregulated in samples where either the TAL1-bound region or the whole cluster was deleted (Figure 2B). Expression of the *KCNH2* gene, which is located outside of the cluster and does not interact with the TAL1-bound region (Figure 2A, right), was not affected. *GIMAP8* expression was not shown because it is not highly expressed in Jurkat cells (Figure 2A, see RNA-seq). These results demonstrate that the locus bound by the TAL1 complex possesses enhancer activity to induce the expression of the *GIMAP* genes (*GIMAP* enhancer). Of note, an additional TAL1 binding site was identified between the *GIMAP4* and *GIMAP7* genes (Figures 1A and 2A, arrowhead). However, we did not observe any chromatin-chromatin interactions between this region and *GIMAP* genes by ChIP-PET analysis (Figure 2A).

The *GIMAP* enhancer can be activated in normal HSCs *in vivo*

We next examined the activity of the *GIMAP* enhancer in normal hematopoietic cells using an *in vivo* reporter system. We cloned the enhancer sequence into a reporter construct that encodes the eGFP fluorescent protein with a minimal promoter sequence (Figure 3A). To analyze specific cells where the element is activated *in vivo*, we injected the construct into zebrafish embryos and analyzed the resulting eGFP expression patterns during embryogenesis. Interestingly, eGFP-positive cells were specifically detected along the posterior dorsal aorta at 2–3 days post fertilization (dpf) (Figure 3B). This region corresponded with the aorta-gonad-mesonephros (AGM), where zebrafish HSCs arise. To confirm the origin of fluorescence-positive cells, we co-introduced the *RUNX1* +23/24 enhancer (eR1) reporter construct encoding DsRed, which has been reported to be activated

in normal HSCs and progenitor cells both in zebrafish and mice.[11, 41] This analysis revealed co-localization of eGFP and DsRed in the same cells (Figure 3B, middle and bottom panels). The same findings were reproducibly observed (Supplementary Figure 3A). These results show that the TAL1-bound region near the *GIMAP2* gene locus can drive expression in HSC compartments in zebrafish. Of note, injection of the construct encoding the TAL1-bound region between the *GIMAP4* and *GIMAP7* genes showed eGFP-positive cells in circulating cells and muscles (Supplementary Figure 3B) and were not specific to HSCs.

The *GIMAP* genes are repressed in immature thymocytes

We next analyzed the activity of the *GIMAP* enhancer in the normal thymus. We co-injected a *GIMAP* enhancer reporter plasmid with the zebrafish *rag2* reporter construct, which can be activated during the immature stage of thymocyte differentiation (Figure 4A).[42] In this setting, we detected mCherry signals (by the *rag2* promoter) but not eGFP signals (by the *GIMAP* enhancer) in the thymus (Figure 4B). This result indicates that the *GIMAP* enhancer cannot be activated in immature thymocytes.

We next examined expression patterns of the orthologs of human *GIMAP* genes during mouse hematopoiesis. We analyzed the mRNA expression levels of six mouse *Gimap* genes during different stages of mouse hematopoietic cell differentiation. No ortholog of the human *GIMAP2* gene was found in mice. This analysis revealed that *Gimap1*, *Gimap5*, *Gimap6* and *Gimap8* were highly expressed in both long-term (LT) and short-term (ST)-HSCs (Figure 4C). *Gimap4, Gimap7* and *Gimap8* were also highly expressed in $CD4^+$ or $CD8^+$ single-positive (SP) T-cells. Importantly, the expression of all *Gimap* genes was downregulated during the DN stage in thymocytes, which is consistent with the result by Gene Expression Commons (Supplementary Figure 1B). Similarly, the mouse *Tal1* gene was downregulated at the DN stage (Supplementary Figure 4). In contrast, the expression of an E-protein *Tcf12/Heb* and *Ets1* was upregulated in the same cells. These results indicate that *Gimap* genes are expressed in normal HSCs and progenitor cells but are repressed in immature thymocytes where *Tal1* is silenced.

The *GIMAP* enhancer is activated by TAL1 and repressed by E-proteins in T-ALL cells

TAL1 is normally expressed together with GATA2, RUNX1 and ETS1 in HSCs and progenitor cells but is silenced during early thymocyte development.[9, 10] TAL1 expression is undetectable in normal DN4 and DP cells[12], a finding that we also confirmed. In contrast, TAL1 is overexpressed in T-ALL cells,[15–19] resulting in differentiation arrest of immature thymocytes.[18, 20] Thus, we expect that ectopic expression of TAL1 leads to aberrant expression of *GIMAP* genes via activation of the *GIMAP* enhancer. In fact, when we analyzed changes in *GIMAP* gene expression after knockdown of *TAL1* in Jurkat cells, the mRNA expression of six *GIMAP* genes was concomitantly downregulated (Figure 5A). Notably, the expression of genes outside the *GIMAP* cluster (*REPIN1, ZNF775, TMEM176B, TMEM176A* and *AOC1*; see Figures 1A and 2A) was not affected by *TAL1* knockdown. These results indicate that *GIMAP* genes located within the cluster are specifically controlled by TAL1 in T-ALL cells.

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Subsequently, we examined the functional contribution of each transcription factor involved in the TAL1 complex in regulating the *GIMAP* enhancer. Analysis of associated DNA-binding sequences revealed that E-box, GATA, RUNX and ETS motifs were observed within this region (Supplementary Figure 5A). To determine whether the *GIMAP* enhancer can be activated in human T-ALL cells, we introduced the *GIMAP* enhancer element into the *luciferase* reporter construct and transfected the construct into human T-ALL cells (Figure 5B). Compared with empty vector-transfected cells, significant inductions of *luciferase* activity were observed in *TAL1*-positive T-ALL cell lines (Jurkat and RPMI-8402) (Figure 5C), indicating that the *GIMAP* enhancer can be activated by endogenous factors in *TAL1*-positive T-ALL cells. Next, we measured *luciferase* activity after depletion of either *TAL1* or each of the regulatory partners in Jurkat cells that stably expressed the reporter construct. We transduced short-hairpin RNA (shRNA) by lentiviral infection to specifically knock down each of the target proteins (Supplementary Figure 5B). In this analysis, depletion of *TAL1*, *LMO1, GATA3, RUNX1* or *ETS1* reduced *luciferase* activity (Figure 5D) compared to control samples in which control *GFP* shRNA was transduced. This result indicates that the TAL1 complex can activate the *GIMAP* enhancer in T-ALL cells.

Notably, we found that knockdown of the E-proteins *E2A* and *HEB* increased *luciferase* activity (Figure 5D). When we examined changes in the expression of *GIMAP* genes after knockdown of each member of the TAL1 complex in Jurkat cells, the mRNA expression of all *GIMAP* genes was downregulated after depletion of *TAL1, GATA3, RUNX1* and *ETS1* but was upregulated after depletion of the E-protein *HEB* (Figure 5E). Our results indicate that the *GIMAP* genes are positively regulated by the TAL1 complex and negatively regulated by E-proteins (Figure 5F).

Overexpression of human *GIMAP* genes in immature thymocytes promotes T-cell leukemogenesis

Because *GIMAP* genes are normally repressed during the DN stage in thymocytes (Figure 4C) but are overexpressed in human T-ALL cells, we postulated that ectopic expression of *GIMAP* genes in immature thymocytes could contribute to leukemogenesis. Hence, we examined the tumorigenic ability of *GIMAP* genes *in vivo*. Prior to this analysis, we first confirmed cell growth in a human T-ALL cell line where either the *GIMAP* enhancer or the whole *GIMAP* gene cluster had been deleted (Figure 2). These conditions slightly reduced the growth of T-ALL cells (Supplementary Figure 6A). Individual gene knockdown using validated shRNAs (Supplementary Figure 6B) demonstrated that inhibition of *GIMAP5* and *GIMAP7* reduced cell growth (Supplementary Figure 6C). Analysis with annexin staining also revealed that knockdown of *GIMAP5* and *GIMAP7* induced apoptotic cell death (Supplementary Figure 6D). Although the inhibitory effect on growth after the loss of GIMAP proteins is relatively weak *in vitro*, these results supported previous findings that demonstrated a protective role of *Gimap5* against cell death.[46, 47] Based on these results, we selected *GIMAP5* and *GIMAP7* genes for the transgenic study.

We used the zebrafish *rag2* promoter to specifically overexpress multiple human *GIMAP* genes in immature thymocytes to recapitulate human T-ALL. In this setting, overexpression of *GIMAP5 and GIMAP7* either individually or in combination did not induce T-ALL in

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zebrafish (data not shown), suggesting that *GIMAP* genes alone do not possess sufficient oncogenic potential to initiate leukemia. Hence, we next overexpressed the *GIMAP5* and *GIMAP7* genes in immature thymocytes together with the *MYC* T-ALL oncogene. Strikingly, transgenic zebrafish that showed concomitant overexpression of the *GIMAP5* and *GIMAP7* genes significantly accelerated tumor onset compared to the *MYC* single transgenic fish (Figures 6A and B). These results indicate that ectopic expression of *GIMAP* genes promotes T-cell leukemogenesis *in vivo* when overexpressed with other oncogenes.

Discussion

In the present study, we report that *GIMAP* genes are highly expressed in human T-ALL cells as well as in normal mouse HSCs. Expression of the *GIMAP* genes is differentially controlled by TAL1 and E-proteins via the *GIMAP* enhancer. Ectopic overexpression of human *GIMAP5* and *GIMAP7* genes in the zebrafish thymus accelerated tumor development in the presence of *MYC*. Our results demonstrate that aberrant activation of the *GIMAP* enhancer contributes to T-cell leukemogenesis.

The GIMAP family of proteins has been implicated in lymphocyte development. Studies with knockout mice show that these proteins are involved in the development and survival of mature T-lymphocytes.[28, 32, 48] One research group reported that mouse *Gimap1*, *Gimap4*, *Gimap5* and *Gimap6* are downregulated in DP cells and that their expression is dramatically increased in $CD4^+$ or $CD8^+$ SP T-cells,[28, 48, 49] which was consistent with our results. In general, the vast majority of thymocytes undergo apoptosis by positive selection during the transition from DP to SP cells, and only selected T-cells acquire the ability to survive.[50] These findings suggest a possible role of GIMAPs in the positive selection of T-cells. In addition, we have now shown that *GIMAP* genes are highly expressed in HSCs and progenitor cells. This suggests that *GIMAP* genes could be physiological targets of TAL1 in normal HSCs.

Notably, the roles of *GIMAPs* in survival-or-death determinations vary among the family proteins. For example, *GIMAP4* has been reported to accelerate T-cell death, whereas *GIMAP5* exhibits a protective role against cell death through association with the Bcl-2 protein family.[28, 30, 46, 51] *Gimap5* knockout mice show a dramatic decrease in T-cell numbers.[52] *GIMAP1* is also vital for the development/survival of mature T-lymphocytes.[49] Additionally, GIMAP2 can heterodimerize with GIMAP7 protein to activate GIMAP7 function in lymphocytes.[53, 54] These studies indicate that multiple GIMAP proteins cooperate to maintain T-cell survival. Further investigation is necessary to elucidate the roles of GIMAP proteins during T-cell leukemogenesis.

Interestingly, the *GIMAP* genes have also been reported to be downstream targets of the *NOTCH1* oncogene in T-ALL.[31, 33] ChIP-Seq data demonstrate binding of NOTCH1 and its partner RBPJ/CSL at the *GIMAP* enhancer in T-ALL cells.[33] Notch1 upregulates the *GIMAP* family genes, and pharmacological inhibition of the Notch signaling pathway perturbs T-ALL cell proliferation.[31] Hence, *GIMAP* can be positively regulated by two major T-ALL oncogenes, namely, TAL1 and NOTCH1. In normal T-cell development, NOTCH1 is downregulated during the transition from the DN3 to the DN4 stage,[55] when

GIMAP genes are silenced. Activating mutations of *NOTCH1* are frequently found in *TAL1*-positive human T-ALL as well as in transgenic mouse models, leading to increased *MYC* expression.[56, 57] Hence, progressive increases in *GIMAP* gene expression may contribute to the oncogenic collaboration among TAL1, NOTCH1 and MYC in T-ALL.

Importantly, we showed that E-proteins (E2A and HEB) negatively regulate *GIMAP* enhancer activity in T-ALL cells. E-proteins form a heterodimer with TAL1 but can also form homodimers by themselves to regulate gene expression.[14] E-proteins are normally required for thymocyte development in a stage-specific manner.[12, 13] E-proteins are upregulated during thymocyte development, and their expression levels are highest during the DN and DP stages in thymocytes as reported by other researchers[12] and by us (Supplementary Figure 4), in which *TAL1* is silenced and *GIMAP* genes are downregulated (Figure 4). These findings indicate that *GIMAP* genes are repressed by E-proteins in normal immature thymocytes but can be reactivated by ectopic expression of TAL1 and/or by inhibition of the E-protein dimers. E-proteins have been implicated as tumor suppressors in T-ALL, and a heterozygous loss of either *E2a* or *Heb* markedly accelerates the onset of T-ALL in *Tal1*-transgenic mice.[21] Therefore, we hypothesize that overexpression of TAL1 and loss of E-proteins synergistically upregulate GIMAPs (Figure 5F), thereby accelerating T-cell leukemogenesis. Taken together, our study provides a novel mechanism by which TAL1 contributes to T-cell leukemogenesis through induction of the *GIMAP* enhancer activity in developing thymocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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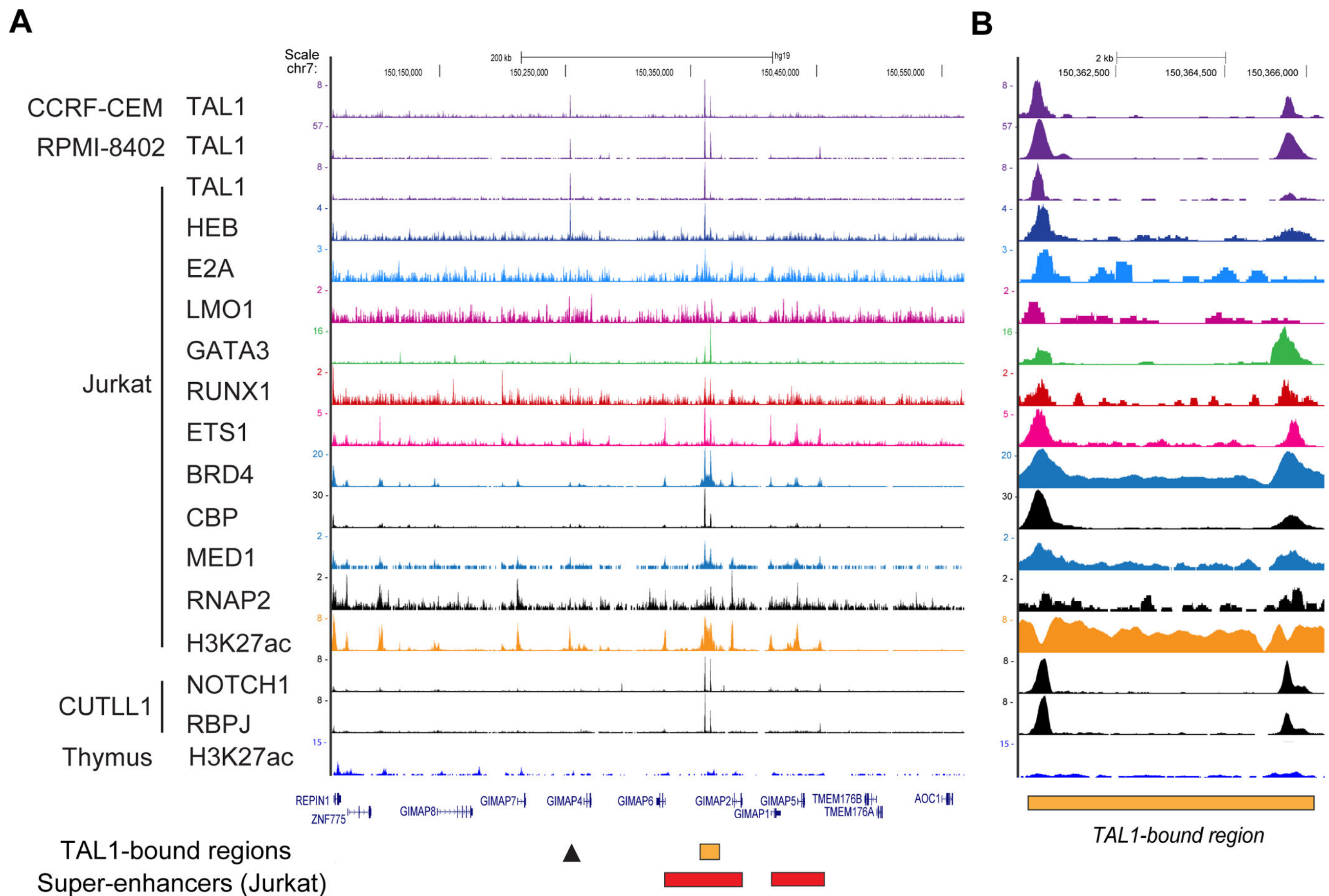

Figure 1. The TAL1-bound super-enhancer in the *GIMAP* gene cluster

(A, B) The ChIP-Seq gene tracks represent binding of transcription factors at the *GIMAP* gene cluster in T-ALL cell samples. Binding of transcription factors (TAL1, HEB, E2A, LMO1, GATA3, RUNX1, ETS1, BRD4, CBP, MED1, NOTCH1, RPBJ and RNA polymerase 2 (RNAP2)) in four T-ALL cell lines (CCRF-CEM, RPMI-8402, Jurkat and CUTLL1) as well as histone H3 acetylation at lysine 27 (H3K27ac) in Jurkat cells and normal thymus are shown. The x-axis indicates the linear sequence of genomic DNA, and the y-axis indicates the total number of mapped reads. The black horizontal bar indicates the genomic scale in kilobases (kb). Blue boxes in the gene map represent exons, and arrows indicate the location and direction of the transcriptional start site. The TAL1-bound region between the *GIMAP6* and *GIMAP2* genes is indicated as an orange box. The arrowhead shows an additional TAL1 binding site. The super-enhancers are shown as red boxes.

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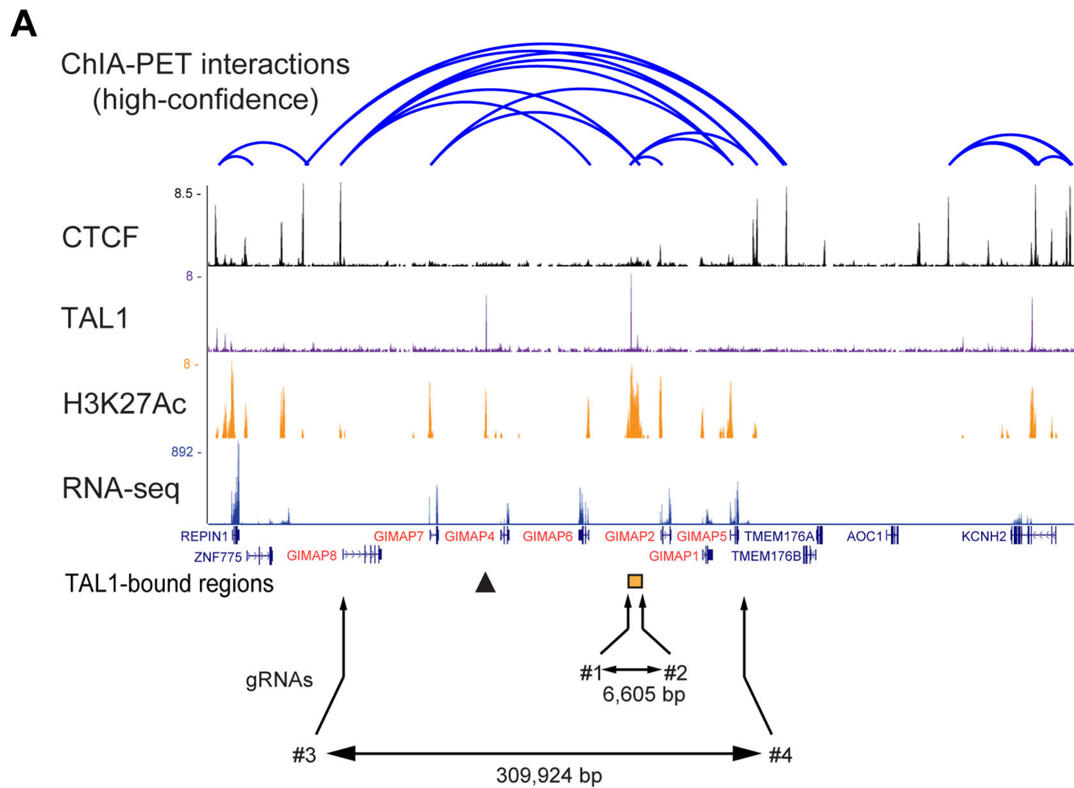

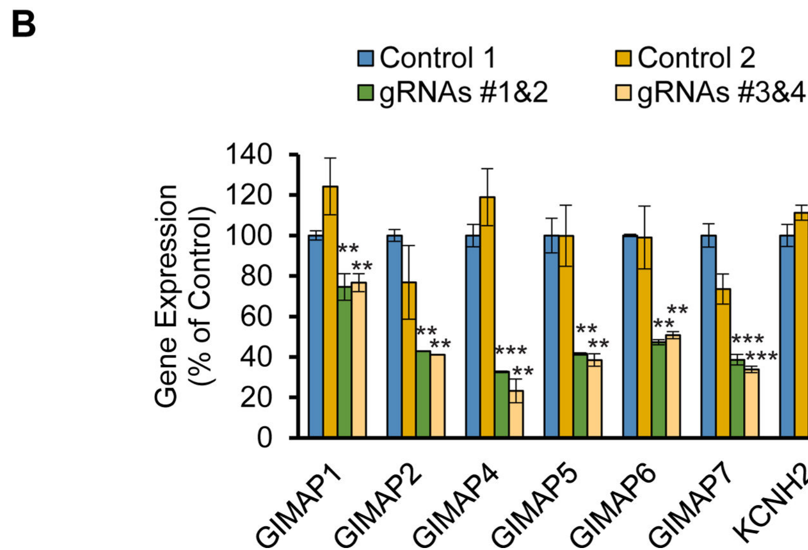

Figure 2. *GIMAP* genes are controlled by the *GIMAP* enhancer in T-ALL cells

(A) ChIA-PET chromatin-chromatin interactions and CTCF bindings in Jurkat cells were analyzed using the dataset reported by Hnisz *et al.* (Science, 2016). The ChIP-Seq gene tracks represent bindings of TAL1 and CTCF as well as H3K27Ac in Jurkat cells. The mRNA expressions were analyzed by RNA-Seq. Combinations of gRNAs (#1 and #2; #3 and #4) were used to knock out the TAL1-bound region (orange box) and the entire *GIMAP* gene cluster, respectively. The sizes of the deleted regions are shown. The TAL1-bound region between the *GIMAP6* and *GIMAP2* genes is indicated as an orange box. The

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arrowhead shows an additional TAL1 binding site. **(B)** Expression levels of each *GIMAP* gene in the knockout clones were measured by quantitative RT-qPCR (qRT-PCR) analysis. Expression values were normalized to that of *GAPDH* and are shown as the percentage of control values, which were transduced by gRNA targeting of the *eGFP* gene. The values are presented as the means ± SD of two samples. The *KCNH2* gene resides to the right of the *GIMAP* gene cluster and serves as a negative control. *p<0.05, **p<0.01, ***p<0.001 by a two-sample, two-tailed t-test compared to the expression in control 1.

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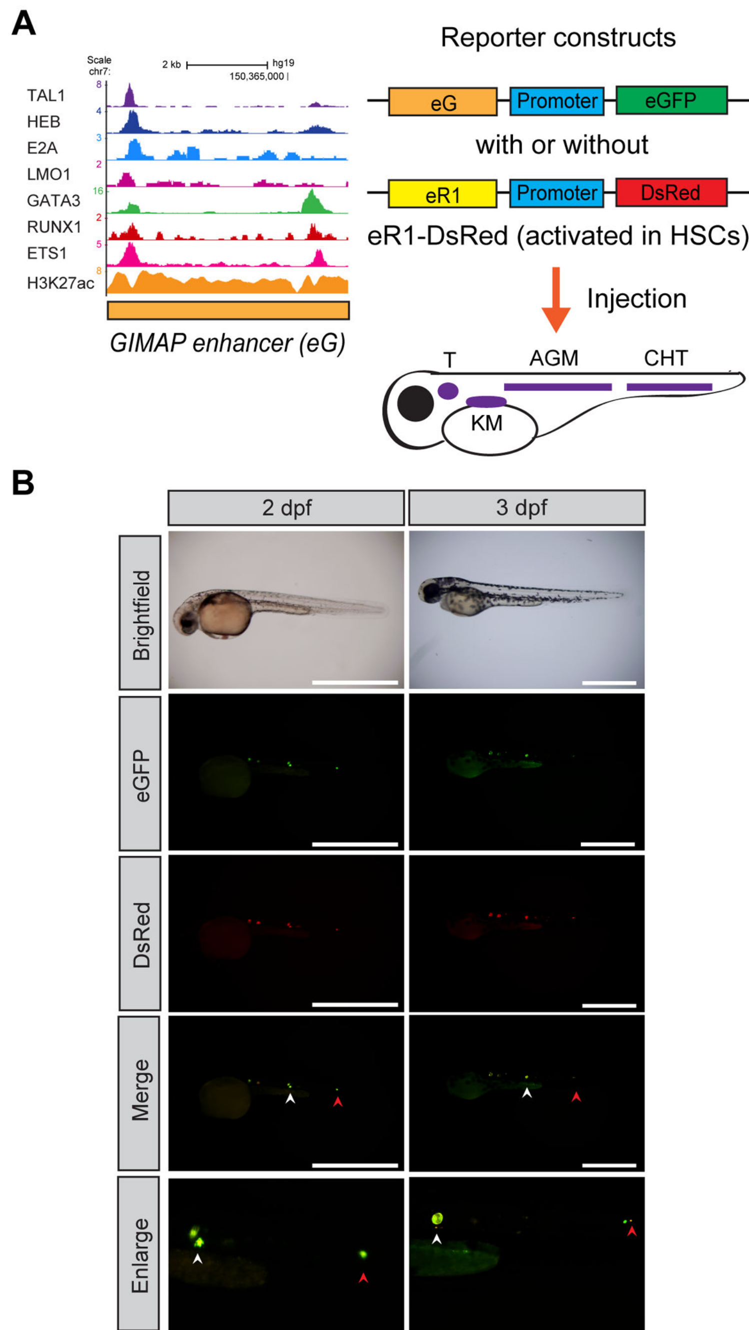

Figure 3. The *GIMAP* enhancer is activated in normal zebrafish HSCs

(A) Schematic representation of the experimental design. The *GIMAP* enhancer (eG) was introduced into the reporter construct carrying a minimal promoter sequence (promoter) and an *eGFP* gene. The *RUNX1* stem cell enhancer (eR1) was cloned into the reporter construct encoding the *DsRed* gene. The reporter constructs were injected into a single-cell zebrafish embryo. The cartoon displays regions of zebrafish hematopoietic tissues. The kidney marrow (KM), aorta-gonad-mesonephros (AGM), caudal hematopoietic tissue (CHT), and thymus (T) are indicated. **(B)** Activation of the reporter constructs in early hematopoiesis in

zebrafish. Fluorescent images show one representative zebrafish embryo (n=16) injected with reporter constructs at 2 and 3 dpf. The AGM and CHT are indicated by white and red arrowheads, respectively. Scale bar, 1 mm.

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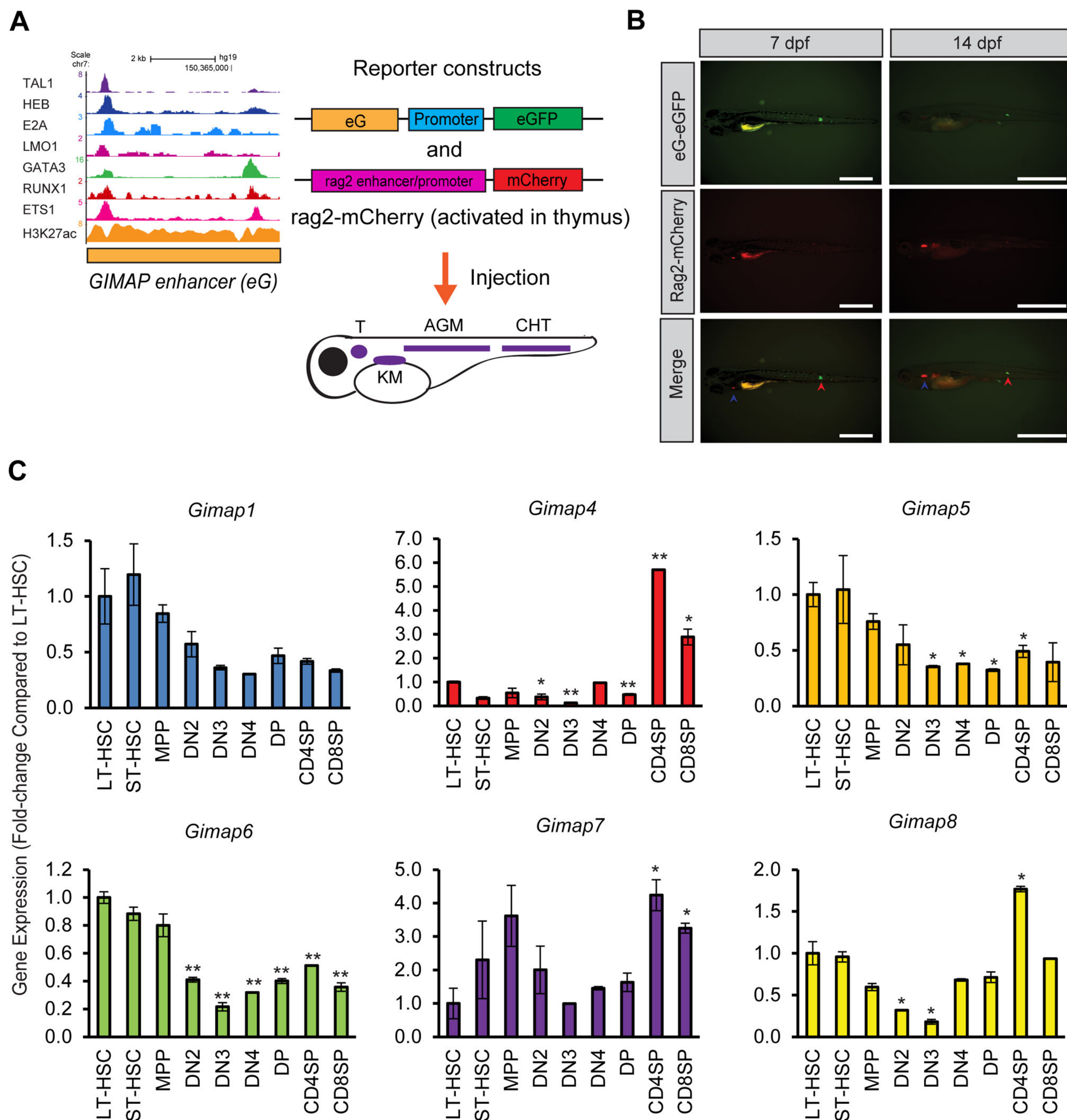

Figure 4. The *GIMAP* enhancer is repressed in immature thymocytes

(A) Schematic representation of the experimental design. The *GIMAP* enhancer (eG) was introduced into the reporter construct carrying a minimal promoter sequence (promoter) and an *eGFP* gene. The zebrafish *rag2 promoter* was cloned into the reporter construct encoding a *mCherry* gene. The reporter constructs were injected into a single-cell zebrafish embryo. See Figure 3A for details. **(B)** The *GIMAP* enhancer is not activated in immature zebrafish thymocytes. Fluorescent images show one representative zebrafish (n=4) injected with reporter constructs at 7 and 14 dpf. The CHT and thymus are indicated by red and blue

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arrowheads, respectively. Scale bar, 1 mm. **(C)** mRNA expression levels of *Gimap* genes in mouse hematopoietic cells. Total RNA was harvested from mouse hematopoietic cells at different stages: long-term HSC (LT-HSC), short-term HSC (ST-HSC), multi-potent progenitor (MPP), double-negative 2 (DN2), DN3, DN4, double-positive (DP), CD4-single-positive (CD4SP) and CD8-single-positive (CD8SP). Expression levels in DN1 cells could not be analyzed due to the lack of a sufficient sample size. The mRNA expression levels of six *Gimap* genes (*Gimap1, Gimap4, Gimap5, Gimap6, Gimap7,* and *Gimap8*) as well as β-actin were measured by quantitative RT-PCR analysis. The expression values were normalized to that of β-actin and were shown as fold-changes compared to LT-HSCs. The values are presented as the means ± SD of two samples. *$p<0.05$, **$p<0.01$ by a two-sample, two-tailed t-test compared to the expression in LT-HSC.

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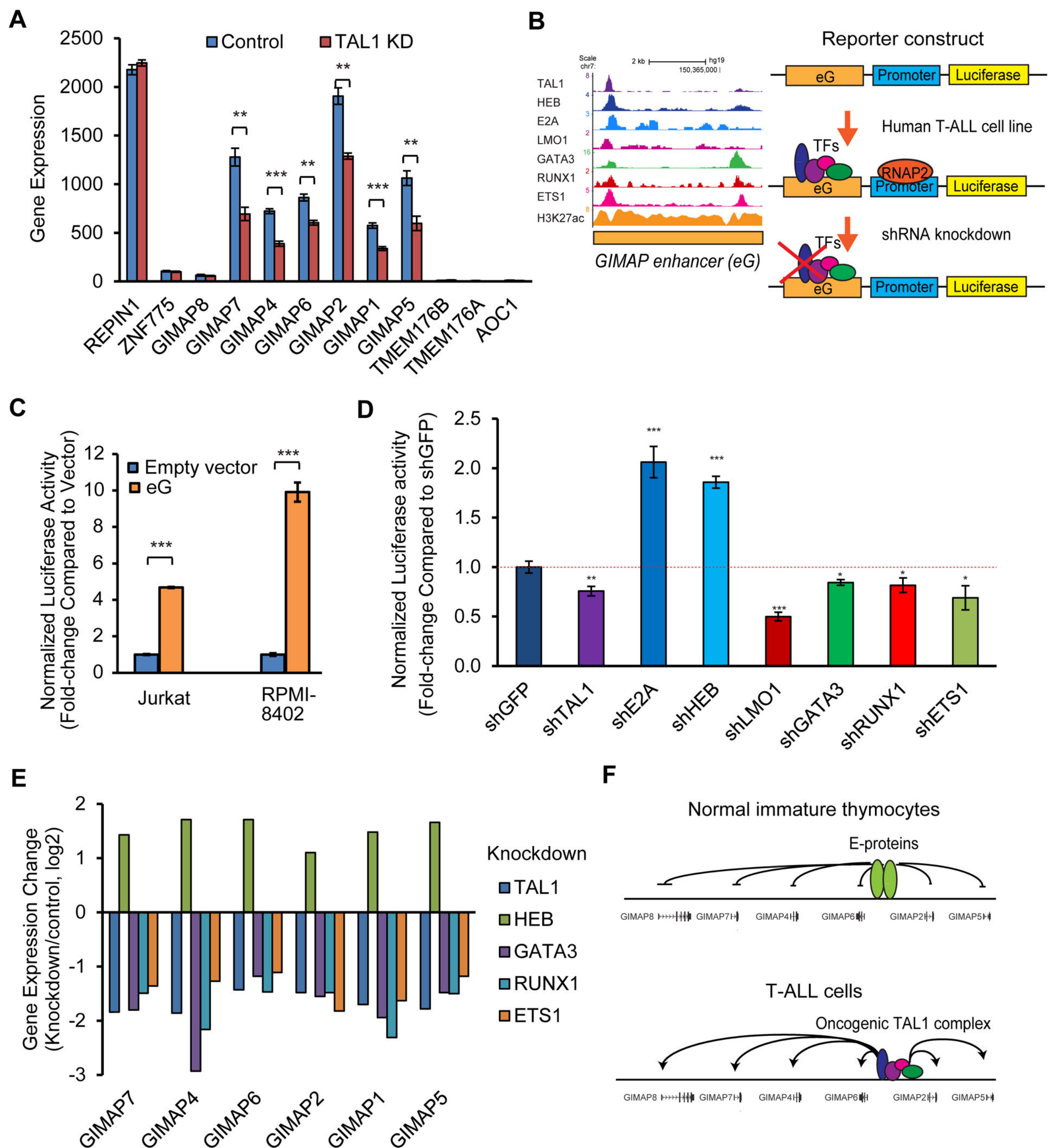

Figure 5. The *GIMAP* enhancer is activated by TAL1 and is repressed by E-proteins in T-ALL cells

(A) Expression changes for *GIMAP* genes after *TAL1* knockdown in Jurkat cells. Global gene expression was analyzed using microarrays in four control samples and four *TAL1* knockdown samples as previously described.[22] The expression values were normalized by dChip software and are shown as the means ± standard deviations (SDs) of four samples; $**p<0.01$, $***p<0.001$ by a two-sample, two-tailed t-test. **(B)** Schematic representation of the experimental design. The *GIMAP* enhancer (eG) was introduced into the reporter

construct carrying a minimal promoter sequence (promoter) and a *luciferase* gene. The *luciferase* reporter construct was transfected into human T-ALL cells. *Luciferase* activity was measured in T-ALL cells after knockdown of the transcription factor (TF). **(C)** Induction of *GIMAP* enhancer activity in *TAL1*-positive T-ALL cells. The *GIMAP* enhancer reporter construct was co-transfected with the *renilla* plasmid (internal control for transfection efficiency) into two *TAL1*-positive T-ALL cell lines (Jurkat and RPMI-8402). An empty *luciferase* vector was used as a control. L*uciferase* activity values were normalized to *renilla* activity and are shown as fold-changes compared to the empty vector. The values are presented as the means ± SDs of biological triplicates. ***p<0.001 by a two-sample, two-tailed t-test. **(D)** Inhibition of *GIMAP* enhancer activity by knockdown of transcription factors in T-ALL cells. Jurkat cells that stably expressed the *GIMAP* enhancer reporter construct were established and were transduced with shRNA targeting a transcription factor using lentivirus infection. *Luciferase* activity and cell viability were measured after 5 days of virus infection. *Luciferase* activity values were normalized to cell viability and are shown as fold-changes compared to the control sample, which was transduced with shGFP. The values are presented as the means ± SDs of biological triplicates; *p<0.05, **p<0.01, ***p<0.001 by a two-sample, two-tailed t-test. **(E)** Expression changes of *GIMAP* genes after knockdown of transcription factors in Jurkat cells (see the Figure 5A legend for details). The mean fold-change is shown as log 2 of knockdown over control cells. **(F)** Proposed model of *GIMAP* gene activation in normal and T-ALL cells. E-protein dimers negatively regulate *GIMAP* gene expression in normal immature thymocytes. The oncogenic TAL1 complex positively regulates *GIMAP* gene expression in T-ALL cells. Arrows indicate cases of gene activation.

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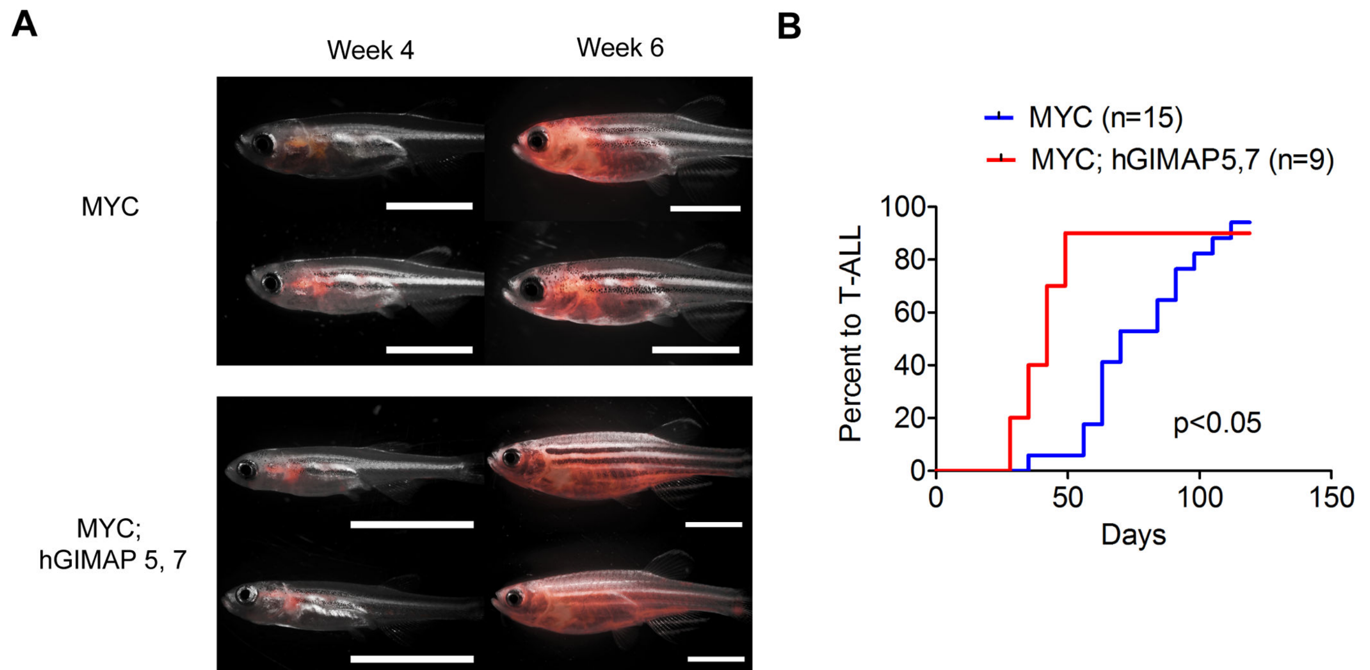

Figure 6. Overexpression of *GIMAP* genes accelerates T-ALL onset *in vivo*

(A) Tumor development in transgenic zebrafish. Tumor cells expressing fluorescent proteins (eGFP and mCherry) in *rag2::Myc-EGFP; rag2::mCherry (MYC)* (n=15) and *rag2::Myc-EGFP; rag2::hGIMAP5; rag2::hGIMAP7; rag2::mCherry* (*MYC; hGIMAP5,7*) (n=9) transgenic fish were analyzed at 4 and 6 weeks using epi-fluorescence stereomicroscopy. Two representative zebrafish for each group are shown. Panels show the merged fluorescent and bright-field images. Scale bar, 5 mm. **(B)** The tumor onset of T-ALL in *MYC*-transgenic fish (n=15) and *MYC; GIMAP5, 7* transgenic fish (n=9) were analyzed via the Kaplan-Meier method. P<0.05 by the Gehan-Breslow-Wilcoxon test.