Published in final edited form as:

Leukemia. 2015 June ; 29(6): 1301–1311. doi:10.1038/leu.2015.27.

Activated Notch counteracts Ikaros tumor suppression in mouse and human T-cell acute lymphoblastic leukemia

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Abstract

Activating *NOTCH1* mutations occur in ~ 60% of human T-cell acute lymphoblastic leukemias (T-ALLs), and mutations disrupting the transcription factor IKZF1 (IKAROS) occur in ~5% of cases. To investigate the regulatory interplay between these driver genes, we have used a novel transgenic RNA interference mouse model to produce primary T-ALLs driven by reversible Ikaros knockdown. Restoring endogenous Ikaros expression in established T-ALL *in vivo* acutely represses *Notch1* and its oncogenic target genes including *Myc*, and in multiple primary leukemias causes disease regression. In contrast, leukemias expressing high levels of endogenous or engineered forms of activated intracellular Notch1 (ICN1) resembling those found in human T-ALL rapidly relapse following Ikaros restoration, indicating that ICN1 functionally antagonizes Ikaros in established disease. Furthermore, we find that *IKAROS* mRNA expression is significantly reduced in a cohort of primary human T-ALL patient samples with activating *NOTCH1/FBXW7* mutations, but is upregulated upon acute inhibition of aberrant NOTCH signaling across a panel of human T-ALL cell lines. These results demonstrate for the first time that aberrant NOTCH activity compromises IKAROS function in mouse and human T-ALL, and

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)

provide a potential explanation for the relative infrequency of *IKAROS* gene mutations in human T-ALL.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is a malignancy of T-cell progenitors, with overall survival rates of 70% in children and <40% in adults.¹ The majority of T-ALL cases harbor activating mutations in *NOTCH1*, which encodes a membrane-spanning receptor essential for lineage commitment and development of T-lymphocytes.² Mature NOTCH1 receptors comprise extracellular and transmembrane components that noncovalently associate through a heterodimerization domain (HD). Upon ligand binding, the transmembrane subunit is cleaved by the metalloproteinase ADAM10 (a disintegrin and metalloprotease 10) and subsequently by γ -secretase, releasing intracellular NOTCH1 (ICN1) from the membrane. Nuclear ICN1 forms a complex with the DNA-binding transcription factor RBP-J κ (CSL) and the coactivator MAML1 to induce Notch target genes.³

Two major classes of *NOTCH1* mutation occur in 60% of human T-ALL: point mutations that destabilize the NOTCH1 HD promoting its cleavage by γ -secretase; and disruption/deletion of the C-terminal PEST (proline, glutamic acid, serine, threonine) domain causing ICN1 protein stabilization.^{3,4} Missense mutations in FBXW7, a ubiquitin ligase implicated in ICN1 turnover, also occur in ~ 15% of T-ALL cases.^{5,6} NOTCH pathway hyperactivation induces many genes including the oncogenic transcription factor *Myc* and the transcriptional repressor *Hes1*, each required for ICN1-driven T leukemogenesis in mice.^{7–10} The frequency of NOTCH pathway activation in human T-ALL suggests several strategies for targeted therapeutic intervention.³

Activating *Notch1* mutations are also common in murine T-ALL models, and the expression of ICN1 in the hematopoietic system of mice promotes T-lineage transformation.^{11,2} In mouse, T-ALL-activating *Notch1* mutations frequently coincide with loss-of-function mutations in *Ikzf1*, which encodes the zinc-finger transcription factor Ikaros.^{12–14} Ikaros promotes hematopoietic stem cell function and directs lineage fate decisions of early hematopoietic progenitors.^{15,16} *Ikaros*^{−/−} mice lack B cells, natural killer cells and fetal T cells, and postnatally produce aberrant, clonally expanded T cells.¹⁷ Aggressive T-cell malignancies develop in mice carrying dominant-negative or hypomorphic *Ikaros* alleles,^{18–20} indicating a critical role for Ikaros in T-lineage tumor suppression. Notably, ~ 70% of T-ALLs arising in *Ikaros* germline mutant mouse models harbor *Notch1* mutations including PEST and/or HD domain mutations similar to those in human T-ALL.^{20–24}

Beverly and Capobianco¹² first suggested that Ikaros may directly antagonize Notch target gene activation, and subsequent *in vitro* studies using Ikaros-mutant murine T-ALL cell lines found that retroviral expression of the full-length Ikaros isoform Ik1 causes cell cycle arrest associated with the downregulation of canonical Notch target genes including *Hes1*.^{20,22,25,26} Ikaros binds the *Hes1* promoter in these cells, and competes with Rbpj at *Hes1* promoter sequences to inhibit Notch1-mediated reporter gene expression.^{20,26,27} In immature thymocytes, Ikaros and RBP-J κ both bind *Hes1*,²⁷ and thymocytes isolated from

young Ikaros-mutant mice show derepression of *Notch1* and selected Notch target genes including *Hes1*.^{20,22,27} Recent chromatin immunoprecipitation (ChIP) studies also demonstrate that Ikaros directly represses *Notch1* in wild-type thymocytes.^{28–30} While these studies indicate that Ikaros can repress Notch1 and its target genes in thymocytes, it remains unclear whether Ikaros loss is required to maintain oncogenic Notch pathway function in established T-ALL *in vivo*.

Although *Notch1* activation and *Ikaros* disruption often co-occur in murine T-ALL, in human adult T-ALL, 60% of which harbor *NOTCH1/FBXW7* mutations, genetic *IKZF1* abnormalities only occur at ~ 5% frequency.^{31,32} Interestingly, while a recent pediatric T-ALL study identified *IKZF1* mutations in 13% of the ‘early T-cell precursor’ T-ALL subtype, with 50% of these *IKZF1*-mutant cases also harboring activating *NOTCH1* mutations, in a non-early T-cell precursor T-ALL cohort *NOTCH1* mutation was common (43%), but *IKZF1* lesions were rare (2%).³³ This divergence may reflect different requirements for compromised IKZF1 function in different human T-ALL subtypes and in different species, and also raises the possibility that IKZF1 is functionally compromised by alternative mechanisms in human T-ALL.

Here, we describe a novel RNA interference (RNAi)-based mouse model allowing inducible re-expression of endogenous Ikaros in T-ALL *in vivo*. We show that spontaneous or engineered *Notch1* activation can override the effects of inducible Ikaros restoration in established T-ALL, indicating that ICN1 interferes with Ikaros tumor-suppressive functions. Furthermore, we find that the expression of IKZF1 is reduced in primary human T-ALL, in part, due to aberrant NOTCH pathway activation.

MATERIALS AND METHODS

Transgenic mice

TREtight-GFP-Ikaros.4056 transgenic mice were generated using previously described protocols.³⁴ Genotyping protocols are in Supplementary Methods. Doxycycline (Dox) (Sigma-Aldrich, St Louis, MO, USA) was administered in the diet at 600 mg/kg food (Specialty Feeds, Glen Forrest, WA, Australia). All mouse experiments were approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

Cell culture and western blotting

Culture of OP9-DL1 stromal feeder cells, retroviral transduction of fetal liver cells and western blotting protocols and antibodies are described in Supplementary Methods.

Leukemia transplantation

Culture, retroviral transduction and transplantation of leukemia cells is described in Supplementary Methods.

Flow cytometry and blood analysis

Blood was collected from the retro-orbital plexus of mice, or by tail prick, and parameters were measured with an Advia 2120 hematological analyzer (Bayer, Leverkusen, Germany). Flow cytometry analysis is described in Supplementary Methods.

RNA-seq

Total RNA was extracted from sorted GFP^{+/int}CD4⁺CD8⁺ leukemia cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and sequenced on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). Reads were aligned to the mm10 genome using subread³⁵ and analyzed using edgeR,³⁶ limma³⁷ and voom³⁸ as detailed in Supplementary Methods. The RNA-seq data are available as Gene Expression Omnibus series GSE64928.

Bio-ChIP sequencing

Double-positive (DP) thymocytes were enriched by CD8 MACS (magnetic-activated cell sorting) from the thymus of *Ikzf1^{ihCd2/ihCd2}Rosa26^{BirA/BirA}* mice and used for chromatin precipitation by streptavidin pulldown as recently described³⁹ and as outlined in Supplementary Methods.

RESULTS

Ikaros knockdown in transgenic mice causes T-cell leukemia

Human leukemia-associated genetic alterations in *IKZF1* often reduce rather than ablate its function.^{33,40,41} To model this in mice, we generated retroviral vectors encoding different microRNA-based short hairpin RNAs (shRNAs) that suppressed Ikaros protein expression in a T-cell line (Figure 1a). In an *in vitro* T-lineage differentiation system involving culture of primary fetal liver hematopoietic stem and progenitor cells on an OP9 stromal cell feeder layer expressing the Notch ligand Delta-like-1,⁴² retroviral expression of the Ikaros.2709 or Ikaros.4056 shRNAs (both targeting the 3'-untranslated region of Ikaros common to all mRNA isoforms; Supplementary Figure S1) delayed progression through the CD4⁺CD8⁺ 'double-negative' (DN) stages of T-cell development (Figure 1b). This differentiation block was readily overcome by ectopic coexpression of the full-length Ikaros isoform Ik1 (Figure 1b), suggesting minimal shRNA off-target effects. Reconstituting the hematopoietic system of lethally irradiated recipient mice with primary fetal liver cells infected with LMP vectors stably expressing the Ikaros.2709 or Ikaros.4056 shRNAs resulted in rapid development of a lethal, disseminated, transplantable, GFP⁺, CD4⁺CD8⁺ (DP) leukemia (Supplementary Figure S1). These results demonstrate that shRNA-mediated suppression of Ikaros in primary hematopoietic cells retards T-lineage differentiation and promotes leukemogenesis.

To investigate tumor-suppressive mechanisms of Ikaros in T-cell leukemia, we used a reversible RNAi strategy to restore Ikaros expression in leukemias driven by its knockdown. Tetracycline-regulated RNAi requires three components: a tetracycline response element (TRE) promoter controlling shRNA expression; a tetracycline transactivator; and Dox, which reversibly regulates transactivator function. We used an established strategy^{34,43} to generate transgenic mice where a TRE promoter targeted to the *type I collagen* (*Col1a1*) locus controls coexpression of GFP and the Ikaros.4056 shRNA. We crossed these mice

(designated TRE-GFP-shIkaros) to Vav-tTA transgenic mice,⁴⁴ which express the tTA (tetracycline-off; active without Dox) transactivator across the hematopoietic system (Supplementary Figure S2). Vav-tTA;TRE-GFP-shIkaros bitransgenic mice succumbed with a median latency of 6 months to GFP⁺ DP T-cell leukemia (Figure 1c) similar to leukemias from previously described germline Ikaros-mutant mice^{18,19} and reminiscent of the cortical/mature subtype of human T-ALL.⁴⁵

Inducible Ikaros restoration in T-ALL *in vivo*

To restore endogenous Ikaros expression in T-ALL *in vivo*, we transplanted primary leukemia cells from three different Vav-tTA; TRE-GFP-shIkaros mice (designated as ALL65, ALL101 and ALL211) into separate cohorts of immunocompromised, T-cell-deficient *Rag1*^{-/-} recipient mice. Upon development of leukemia indicated by splenomegaly and lymphocytosis, a subset of mice were administered Dox supplemented food (Supplementary Figure S2). GFP fluorescence intensity of leukemia cells (expressing surface CD4 and/or CD8 unlike host cells) fell steadily upon Dox treatment of leukemic mice and endogenous Ikaros protein expression in flow-sorted leukemia cells correspondingly increased to reach a plateau following 3 days on Dox (Figures 1d and e).

To identify Ikaros-regulated genes in T-ALL *in vivo*, we compared RNA-seq expression profiles of leukemia cells isolated from untreated (GFP^{high}) and 3-day Dox-treated (GFP^{mid}) mice. Analysis of triplicate untreated and Dox-treated mice bearing ALL101 identified *Ikaros* as the most significantly upregulated gene in the transcriptome, associated with significant induction/repression of thousands of genes at 5% false discovery rate (Supplementary Figure S3 and Supplementary Table S1). In ALL65, ALL101 and ALL211, Dox treatment induced endogenous *Ikaros* expression by 5.5-, 9.8- and 10.8-fold, respectively (Figure 2a). Transcriptional changes upon dynamic Ikaros restoration in the three different primary leukemias were well correlated (Figure 2a), revealing 563 'Ikaros-activated' and 299 'Ikaros-repressed' genes common to all T-ALLs (5% false discovery rate; Figure 2b and Supplementary Tables S2 and 3).

Ikaros restoration in T-ALL preferentially perturbs Ikaros-bound genes We reasoned that genes with expression that strongly positively or negatively correlated with Ikaros in T-ALL may be under its direct regulation. Our recent Bio-ChIP-seq analysis of genome-wide Ikaros binding in murine DP thymocytes identified 7740 Ikaros-bound peaks corresponding to 4989 'bound' genes.³⁹ Combining this data with restoration RNA-seq data for the three primary T-ALLs revealed that Ikaros is far more likely to bind genes that are expressed in leukemia cells than those that are inactive (37% vs 5%, Fisher's exact test $P < 10^{-15}$; Figure 2c). Furthermore, differentially expressed genes were significantly enriched for Ikaros binding compared with other expressed genes, with 54% of 563 Ikaros-activated genes and 45% of 299 Ikaros-repressed genes bound (Fisher's exact test $P < 10^{-15}$ and $P < 0.005$, respectively; Figure 2c and Supplementary Tables S2 and 3).

The transcriptional response to Ikaros restoration in T-ALL mimics Notch1 inhibition

Notch1 and several canonical Notch target genes including *Ptcra* and *Igf1r* were notable among direct Ikaros-repressed genes in all three T-ALLs (Figures 2a, b and d and

Supplementary Table S3), consistent with recent studies indicating that Ikaros directly represses *Notch1* in murine thymocytes.^{28–30} Gene set analysis demonstrated that the global Ikaros-restoration expression profiles of ALL65, ALL101 and ALL211 were highly correlated with a published expression signature derived from a murine T-ALL cell line treated with a γ -secretase inhibitor (GSI) that inhibits Notch signaling⁷ (Figure 2e and Supplementary Table S4). Furthermore, Ikaros restoration globally suppressed a set of genes recently identified as bound by Rbpj and activated by Notch signaling in T-cell leukemia³⁰ (Figure 2f). These observations indicate that the ongoing Ikaros suppression is critical for maintaining Notch/Rbpj target gene expression and Notch pathway activity in established T-ALL *in vivo* (see below).

Variable responses of different primary T-ALLs to sustained Ikaros restoration

To examine whether Ikaros suppression is required for T-ALL maintenance *in vivo*, cohorts of leukemic recipient mice were subjected to sustained Dox treatment. Dox treatment of recipient mice bearing ALL65 or ALL211 markedly reduced spleen size and leukemia burden, significantly prolonging survival (Figure 3a and Supplementary Figure S5). Regression of these two leukemias was stable for up to 3 weeks (described further below); however, Dox-treated mice eventually relapsed with T-ALL similar to the original disease but lacking GFP expression. Given that Vav-tTA;TRE-GFP-shIkaros leukemias likely harbor multiple oncogenic mutations that by definition collaborate with Ikaros suppression to drive transformation, we hypothesized that there would be significant selective pressure on antecedent T-ALL cells to inactivate Ikaros by alternative mechanisms following shRNA shut-off. Indeed, western blot analysis of multiple relapsed leukemias from independent Dox-treated mice bearing ALL65 and ALL211 revealed that most expressed high levels of an Ikaros species not evident during the early stages of Ikaros restoration, and of size corresponding to a dominant-negative isoform (Ik-DN; Figure 3b and Supplementary Figure S6). Additionally, Hes1 mRNA expression was similar in untreated and relapsed T-ALL (Supplementary Figure S6). These data suggest that while restoring Ikaros expression initially leads to repression of Notch1 and its target genes followed by T-ALL regression, subsequent expression of Ik-DN in surviving leukemia cells can reactivate the Notch pathway and drive leukemia relapse. These results emphasize the specificity of *in vivo* RNAi for inducible control of endogenous gene expression, and establish that ongoing Ikaros suppression is critical for ALL65 and ALL211 maintenance.

Notably, and in contrast to mice harboring ALL65 and ALL211, mice transplanted with ALL101 showed disease progression despite Dox treatment. Leukemia clearance was not observed and recipient mice succumbed to disease within 7–10 days (Figure 3a and Supplementary Figure S5). This was surprising given that Ikaros protein restoration was similarly robust in ALL65 and ALL101 following 3 days of Dox treatment (Figure 1e and Supplementary Table S5), and suggested an intrinsic resistance of ALL101 to Ikaros restoration.

Ikaros-resistant ALL101 expresses abundant mutant ICN1 protein

To investigate the molecular basis of the divergent responses of different primary leukemias to sustained Ikaros restoration, we examined whether they harbored different pre-existing

activating mutations in *Notch1*, previously shown to cooperate with Ikaros deficiency during T leukemogenesis. RNA-seq of ALL65 identified a heterozygous P401S mutation not previously associated with T-ALL and unlikely to affect Notch1 function, but also revealed a heterozygous N2385T mutation in a region of the C-terminal PEST degron recurrently mutated in human T-ALL⁴ (Figure 4a and Supplementary Figure S7). RNA-seq of ALL101 revealed two activating point mutations: a missense mutation (L1668P) in the Notch1 HD precisely homologous to the recurrent NOTCH1 L1678P mutation in human T-ALL;⁴ and a nonsense mutation (S2398X) that deletes the C-terminal PEST degron (Figure 4a and Supplementary Figure S7). cDNA sequencing revealed that the L1668P and S2398X mutations were *in trans* (Supplementary Figure S7), which was surprising given that HD and PEST domain mutations invariably occur *in cis* in human T-ALL.⁴ ALL211 harbored a missense mutation (Y1706S) affecting a highly conserved tyrosine residue in the Notch1 HD domain, and a PEST-truncating S2446fsX1 mutation (Figure 4a and Supplementary Figure S7).

Although all three primary T-ALLs harbored *Notch1* coding region mutations predicted to increase ICN1 production and/or stability, western blotting revealed considerable differences in the abundance of γ -secretase-cleaved, active ICN1 in each tumor. Full-length ICN1 was readily detected in ALL65 consistent with Notch1 PEST degron disruption, and truncated ICN1 of predicted size was detected in ALL211 but at low levels (Figure 4b). In contrast, truncated ICN1 was highly abundant in ALL101, and a full-length ICN1 species likely resulting from cleaved Notch1 L1668P-mutant protein was also detected at low levels (Figure 4b).

Ikaros restoration expression profiles differ between primary T-ALLs

Although the extent of Ikaros mRNA and protein restoration was remarkably similar in each primary leukemia (Figures 4c and d), we observed several notable differences in the baseline expression and Ikaros restoration response of *Notch1* and particular Notch target genes between tumors. In untreated leukemias, Notch1 mRNA expression generally correlated with ICN1 protein levels, with transcript levels in ALL101 >2-fold higher than ALL65 and >7-fold higher than ALL211 (Figure 4c). While we observed >2-fold repression of Notch1 mRNA following 3 days of Dox treatment in all three leukemias, protein expression of full-length ICN1 in ALL65 and PEST-deleted ICN1 in ALL101 remained unchanged at this time point (Figures 4b and d). Despite this, expression of multiple Notch target genes including *Myc*, *Hes1* and *Igflr* was consistently reduced, and published gene sets associated with Myc activation were downregulated (Figure 4c and Supplementary Figure S8). Taken together with the fact that *Myc*, *Hes1*, *Ptcra* and *Igflr* are bound by Ikaros in normal DP thymocytes (Figure 2d and Supplementary Figure S8), our data suggest that Ikaros restoration can directly repress these critical Notch target genes in established T-ALL in the absence of appreciable changes in ICN1 abundance. Repression of *Myc* by Ikaros has been reported previously in pre-B cells,⁴⁶ and our observations suggest that this may also be an important direct mechanism of T-ALL suppression by Ikaros (see Discussion). Intriguingly, while Ikaros bound the *Myc* gene body in DP thymocytes, no binding was observed at a recently described Notch1-dependent enhancer ~ 1.3 Mb downstream of *Myc*^{47,48} (Supplementary Figure S8).

Our analysis also identified unique molecular features of ALL101 that may contribute to its 'resistance' to Ikaros restoration. Despite significant Notch1 mRNA repression in ALL101 following 3 days of Dox, its Notch1 expression remained higher than in untreated ALL65 or ALL211 (Figure 4c). Furthermore, even after 7 days of Dox treatment, ICN1 protein expression in ALL101 was maintained at levels exceeding those in untreated ALL211 (Figure 4d). Intriguingly, expression of *Ptcra*, encoding a component of the pre-TCR complex that synergises with Notch signaling to drive the survival and proliferation of immature T cells,⁴⁹ was on average 190-fold higher in ALL101 relative to the Ikaros-responsive leukemias ALL65 and ALL211 (Figure 4c).

***Notch1* activation overrides Ikaros restoration to promote T-ALL relapse**

Given that mice bearing ALL101 (abundant ICN1) showed disease progression despite Ikaros restoration, whereas those bearing ALL65 and ALL211 (lower ICN1 expression) underwent sustained remission, we hypothesized that ICN1 may override the tumor-suppressive effects of Ikaros re-expression. To test this directly, we retrovirally transduced ALL65 or ALL211 leukemia cells at low efficiency with a vector that coexpresses ICN1 and a red fluorescent protein (RFP) marker, or a control vector expressing RFP alone (Figure 5a). Mice transplanted with these cells developed leukemias comprising a mixture of untransduced (GFP⁺) and transduced (GFP⁺RFP⁺) cells. In all cases, Dox treatment of leukemic mice shut off shRNA-linked GFP expression in leukemia cells as expected. To assess the effects of ICN1 expression on leukemia progression, we monitored the relative proportion of RFP⁺ cells within each leukemia by peripheral blood flow cytometry. As predicted, Dox treatment of mice harboring ALL65 or ALL211 transduced with a control RFP-only virus underwent sustained remission, but eventually relapsed consistent with the initial characterization of these leukemias (Figures 5b–d and Supplementary Figure S5). In contrast, ICN1-IRES-RFP expression caused a rapid emergence of RFP⁺ leukemia cells upon Ikaros restoration, and mice rapidly succumbed to leukemia that was completely RFP⁺ (Figures 5b–d and Supplementary Figure S5). Similar effects were seen using an RFP vector that stably coexpresses the Ikaros.2709 shRNA, confirming that ALL65/ALL211 regression is specifically driven by Ikaros re-expression (Figures 5b–d and Supplementary Figure S5). All mice bearing ALL101 progressed on Dox, consistent with our earlier results (Figure 5d and Supplementary Figure S5). These results suggest that high-level, sustained expression of active ICN1 (endogenously in ALL101, or retrovirally in ALL65/ALL211) overrides the effects of endogenous Ikaros expression to drive T-ALL relapse. Notably, ICN1 expression did not compromise endogenous Ikaros protein re-expression in ALL65 after 4 days of Dox treatment (Figure 5e), suggesting that ICN1 may predominantly interfere with Ikaros protein function in this context.

Activated NOTCH1 signaling represses IKAROS in human T-ALL cells

Although *NOTCH1* mutations occur in ~ 60% of human T-ALLs, genetic mutation/deletion of *IKAROS* only occurs in ~ 5% of cases.^{31,32} Given that RNAi-based *Ikaros* knockdown together with spontaneous *Notch1* mutations drive T-ALL in mice, we hypothesized that reduced *IKAROS* mRNA expression may be a recurrent feature of human T-ALL cases with NOTCH pathway activation. We examined RNA-seq data from 10 pediatric T-ALL primary samples (nine harboring *NOTCH1* or *FBXW7* mutations and one with high-level NOTCH

target gene expression) alongside control primary human thymus samples that mainly comprise CD4⁺CD8⁺ thymocytes (TT and IA, unpublished). Notably, *IKAROS* ranked among the top 200 genes with reduced expression in T-ALL samples relative to normal thymocytes (average 70% lower expression, Student's *t*-test $P < 10^{-4}$) (Figure 6a). As expected, expression of the canonical NOTCH target genes *HES1* and *DTX1* showed the opposite trend, with elevated expression in T-ALL relative to normal thymocytes (Figure 6a).

These results raised the possibility that hyperactive NOTCH signaling contributes to reduced *IKAROS* expression in *NOTCH1/FBXW7*-mutated T-ALL. To address this, we mined data from previous microarray analysis of transcriptional changes associated with GSI-based inhibition of NOTCH signaling across a panel of human T-ALL cell lines with prototypical *NOTCH1* mutations.⁵⁰ Remarkably, acute NOTCH pathway inhibition was associated with robust induction of *IKAROS* transcription across multiple T-ALL cell lines regardless of their GSI sensitivity (Figure 6b), and expression of the canonical NOTCH target genes *HES1* and *DTX1* was negatively correlated with *IKAROS* expression in these experiments (Figure 6c). We confirmed that this effect was reproducible and specific to *NOTCH1*-mutant T-ALL cell lines (Figure 6d and Supplementary Figure S9). Conversely, further augmenting NOTCH signaling in these cell lines through retroviral ICN1 expression caused *IKAROS* mRNA repression (Figure 6e). These results indicate that aberrant NOTCH pathway activation may contribute to reduced *IKAROS* expression in human T-ALL.

DISCUSSION

In this study, we have used a novel, inducible shRNA-based transgenic mouse model to dynamically restore endogenous Ikaros expression in three independent T-cell leukemias driven by its knockdown *in vivo*. This elicited remarkably concordant global transcriptional changes, most notably potent suppression of the T-ALL proto-oncogene *Notch1* and several of its critical target genes. Gene set testing confirmed that Ikaros restoration in T-ALL *in vivo* causes global gene expression changes previously associated with inhibition of Notch signaling in T-ALL cells. Building on previous work in cultured T-ALL cell lines derived from Ikaros-mutant mice,^{20,22,25–27} we find that the Notch pathway remains acutely sensitive to endogenous Ikaros-mediated repression in established T-ALL *in vivo*. It is particularly notable that genes including *Myc*, *Hes1* and *Igf1r*, encoding critical oncogenic mediators of activated Notch1 in T-ALL,^{7–10,51} can be potently repressed upon Ikaros restoration in T-ALL without apparent changes in active ICN1 protein levels. Furthermore, dynamic Ikaros restoration in ALL65 and ALL211 caused marked *Myc* repression within just 3 days, and sustained leukemia remission. Our data therefore demonstrate that Ikaros loss promotes T-ALL maintenance by derepressing Notch pathway activity at multiple levels.

Despite highly concordant early transcriptional responses to dynamic Ikaros restoration in three independent primary T-ALLs, their phenotypic response to sustained Ikaros restoration was remarkably divergent. We show that high levels of active ICN1—arising either spontaneously (in ALL101) or through enforced expression—render T-ALL cells relatively impervious to the effects of Ikaros restoration. The mechanism whereby ICN1 overrides the

tumor-suppressive effects of Ikaros remains unclear; however, it may involve direct competition with Ikaros at critical target genes such as *Myc*. Indeed, the pattern of Ikaros binding to the *Myc* gene body we observe in DP thymocytes resembles ICN1 binding of *Myc* in T-ALL cells.⁵² Intriguingly, IKAROS was recently identified in the ICN1 interactome of human T-ALL cells,⁵³ suggesting that ICN1 may also directly interfere with IKAROS protein function.

We demonstrate for the first time that *IKAROS* expression is significantly reduced in primary human T-ALL, notable given that gene-level *IKAROS* mutation/deletion is infrequent in this leukemia.³¹ Given that we made this observation in a T-ALL patient cohort with prototypical *NOTCH1/FBXW7* mutations, it is intriguing that acute inhibition of aberrant NOTCH signaling in a panel of human T-ALL cell lines with similar *NOTCH1* mutations causes transcriptional upregulation of IKAROS. Consistent with this, additional ICN1 expression in these T-ALL cell lines caused further IKAROS repression. These results suggest that an important consequence of mutational NOTCH pathway activation in human T-ALL may be repression of IKZF1, which allows unfettered expression of oncogenic NOTCH1 target genes. Our experiments in mice establish that suppression of Ikaros mRNA can disable its T-ALL suppressor functions, suggesting that a reduction in *IKAROS* mRNA expression could be similarly pathogenic in human T-ALL. As NOTCH1 is primarily a transcriptional activator, repression of IKZF1 by activated NOTCH signaling is likely to be indirect.

Not all murine T-ALLs with *Notch1* mutations have *Ikaros* gene mutations,^{12,24} and it is plausible that *Notch1* activation in murine T-ALL may similarly repress Ikaros expression. Indeed, a previous study listed *Ikaros* among a limited number of genes that are induced following Notch pathway inhibition by either GSI treatment or DN-MAML expression in murine T-ALL cells harboring oncogenic *Notch1* mutations.⁷ This previously unremarked observation bears striking resemblance to our findings in human T-ALL cell lines, suggesting an evolutionarily conserved mechanism whereby Notch pathway activation represses Ikaros expression/function during T-ALL pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mathew Salzone, Melanie Salzone, M Dayton, E Lanera, G Dabrowski, P Kennedy, K Stoev, C Smith, L Wilkins, S Brown and WEHI Bioservices staff for mouse work; W Alexander and E Major for ES cell and mouse resources; R Lane, J Corbin and A Keniry for technical assistance; E Viney and J Sarkis at the Australian Phenomics Network Transgenic RNAi service; M Everest and M Tinning at the Australian Genome Research Facility; and W Shi for assistance with exactSNP. We also thank the Children's Oncology Group for primary human T-ALL samples, S Lowe and J Zuber for vectors and D Largaespada for Vav-tTA mice, and also S Lowe, J Zuber, D Izon, N Kershaw and members of the Dickins laboratory for advice and discussions. This work was supported by the National Health and Medical Research Council of Australia Project Grants 575535 and 1024599, Program Grant 490037, Senior Research Fellowship (GKS), Career Development Fellowship (RAD) and Early Career Fellowship (LC). IA was supported by the National Institutes of Health (1RO1CA133379, 1RO1CA105129, 1RO1CA149655, 5RO1CA173636 and 5RO1CA169784), the William Lawrence and Blanche Hughes Foundation, The Leukemia & Lymphoma Society, The V Foundation for Cancer Research and the St Baldrick's Foundation. The work was also funded by Australian Government NHMRC IRIISS, an Australian Research Council Future Fellowship (SLN), Boehringer Ingelheim (MB), an ERC Advanced Grant (291740-LymphoControl) from the

European Community's Seventh Framework Program (MB), the Leukaemia Foundation of Australia (scholarship to MW, fellowship to MDM), a Sylvia and Charles Viertel Charitable Foundation Fellowship (RAD), Victorian State Government OIS grants and a Victorian Endowment for Science, Knowledge and Innovation (VESKI) Fellowship (RAD).

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Figure 1.

Reversible Ikaros knockdown promotes T-cell leukemogenesis. **(a)** Western blot of Ikaros expression in 2Q T hybridoma cells stably transduced with LMP vectors expressing Ikaros shRNAs or a control shRNA targeting *Renilla* luciferase (Ren.713). The larger species corresponds to the Ikaros isoform Ik1, and the lower species the Ik2/3 isoforms. Actin is a loading control. **(b)** CD44 and CD25 flow cytometry profiles showing T-lineage differentiation (proceeding from the CD44⁺CD25⁺ (DN1) stage anti clockwise to the CD44⁺CD25⁺ (DN4) stage) of fetal liver cells co-infected with LMP-Cherry vectors expressing control Ren.713 or Ikaros shRNAs along with control MSCV-IRES-GFP or MSCV-Ik1-IRES-GFP vectors. Lin⁺CD4⁺CD8⁺GFP⁺Cherry⁺-gated cells were analyzed following 12 days of culture on OP9-DL1 monolayers. **(c)** Kaplan–Meier survival curve for Vav-tTA;TRE-GFP-shIkaros ($n = 41$) and control Vav-tTA;TRE-GFP-shLuc ($n = 11$) bitransgenic mice. All 11 moribund mice examined harbored GFP⁺ DP T-cell leukemia. **(d)** Flow cytometry profile of GFP expression of splenocytes from representative leukemic recipient mice following transplant with Vav-tTA;TRE-GFP-shIkaros leukemia ALL101. Dox was administered at leukemia onset. **(e)** Western blot analysis of Ikaros expression in ALL65 and ALL101 cells isolated from representative leukemic mice that were untreated (ut) or Dox treated as indicated.

Figure 2.

Inducible Ikaros restoration in T-ALL *in vivo*. **(a)** Scatterplots of RNA-seq differential expression (log2 fold change) upon Ikaros restoration (comparing 3 days Dox with untreated) in T-ALL cells harvested from mice transplanted with different primary T-ALLs, comparing ALL65 with ALL101 (left; Pearson's $r = 0.54$), ALL65 with ALL211 (middle; $r = 0.52$), and ALL101 with ALL211 (right; $r = 0.52$). *Ikzf1/Ikaros* and *Notch1* are indicated. **(b)** MA plot of average RNA-seq expression differences upon Ikaros restoration (comparing 3 days Dox with untreated) in T-ALL from combined analysis of ALL65, ALL101 and

ALL211. Genes with significantly increased (red) or decreased (blue) expression upon Ikaros restoration are indicated (false discovery rate (FDR) <0.05). *Ikzf1/Ikaros*, *Notch1* and the Notch target genes *Myc*, *Igf1r*, *Hes1* and *Ptcra* are indicated. (c) Pie charts showing the proportion of genes bound by Ikaros by ChIP-seq in DP thymocytes (shaded) within the indicated expression categories identified by combined analysis of Ikaros restoration in ALL65, ALL101 and ALL211. The total number of genes in each category is indicated. Enrichment *P*-values are relative to all other expressed genes. (d) Ikaros binding at the *Notch1*, *Igf1r* and *Ptcra* loci in DP thymocytes. Gray bars below the Bio-ChIP-seq track indicate significant Ikaros-binding ($P < 10^{-10}$). The Y axis indicates the number of mapped sequence reads. (e) Gene set analysis barcode plot, with RNA-seq differential gene expression from combined analysis of Ikaros restoration in ALL65, ALL101 and ALL211 *in vivo* shown as a shaded rectangle with genes horizontally ranked by moderated t-statistic. Genes upregulated upon Ikaros restoration are shaded pink ($z > 1$) and downregulated genes are shaded blue ($z < 1$). Overlaid are a previously described set of genes induced (red bars) or repressed (blue bars) upon Notch inhibition in a murine T-ALL cell line.⁷ Red and blue traces above and below the barcode represent relative enrichment. *P*-value was computed by the roast method⁵⁴ using both up- and downregulated genes. (f) Gene set analysis barcode plot as for (e) but with blue bars indicating 81 Rbpj-bound, Notch-activated genes recently identified in a murine T-cell leukemia cell line.³⁰

Figure 3.

Ikaros restoration causes regression of ALL65 and ALL211 but not ALL101. **(a)** Flow cytometry analysis showing the proportion of leukemia cells (expressing CD4 and/or CD8) in the peripheral blood of representative recipient mice bearing ALL65 (upper panels), ALL101 (middle panels) or ALL211 (lower panels), either untreated (left panels) or during Dox treatment (middle and right panels). **(b)** Western blot analysis of Ikaros expression in ALL65 (upper panels) and ALL211 (lower panels) leukemia cells isolated from several independent leukemic mice that were either untreated (d0), or had relapsed following Dox treatment of indicated duration. Ik-DN indicates truncated species arising specifically at relapse, corresponding to DN isoforms (e.g. Ik6). ICN1 expression is also shown, and actin is a loading control.

Figure 4. ALL101 expresses abundant, truncated ICN1. **(a)** Schematic of the Notch1 protein showing mutations identified in ALL65, ALL101 and ALL211. **(b)** Western blot analysis of ICN1 expression in T-ALL cells harvested from mice transplanted with different primary leukemias and Dox treated as indicated. The Val1744 antibody recognizes an epitope on ICN1 formed following γ -secretase-mediated cleavage of Notch1. Full-length ICN1 (predicted size 87 kDa) is evident in ALL65, whereas ALL101 and ALL211 express truncated ICN1 (predicted sizes 72 and 77 kDa, respectively). Actin is a loading control. **(c)**

Expression of *Ikzf1/Ikaros*, *Notch1* and the Notch1 target genes *Myc*, *Hes1*, *Ptcra* and *Igf1r* in different primary T-ALLs upon Ikaros restoration (RNA-seq RPKM). ALL101 results are expressed as mean $\pm$ s.e.m., $n = 3$ mice per condition. **(d)** Western blot analysis of ICN1 and Ikaros expression in T-ALL cells harvested from mice transplanted with different primary leukemias and Dox treated as indicated. The ALL101/ALL211 panels are cropped from the same blot to show relative ICN1 abundance in each leukemia.

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Figure 5.

ICN1 expression renders ALL65 and ALL211 resistant to Ikaros restoration. **(a)** Strategy for determining the effects of ectopic ICN1 expression on Ikaros restoration in T-ALL. **(b)** Flow cytometry analysis showing the proportion of CD4⁺CD8⁺ leukemia cells in the peripheral blood of representative mice transplanted with ALL65 cells infected with RFP-linked shRen. 713, shIk.2709 or ICN1, either at leukemia onset (upper panels) or following Dox treatment as indicated (lower panels). **(c)** RFP fluorescence profile in leukemia cells from representative leukemic mice as described in **(b)**. **(d)** Time-course analysis of peripheral leukemia burden in mice transplanted with ALL65 (upper), ALL101 (middle) and ALL211

(lower) leukemia cells transduced with the indicated vectors and Dox treated upon leukemia development. Each line indicates an individual recipient mouse. Leukemia burden exceeding ~ 80% of peripheral white blood cells was generally associated with massively elevated lymphocyte counts and morbidity. **(e)** Western blot analysis of Ikaros expression in leukemia cells isolated from leukemic recipient mice as described in **(b)**, following 4 days of Dox treatment. Cells were sorted based on RFP expression as indicated.

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Figure 6.

Reduced IKAROS expression in primary human T-ALLs with NOTCH pathway activation. (a) Expression of *IKZF1*, *HES1* and *DTX1* (RNA-seq FKPM) in 10 primary T-ALL samples harboring *NOTCH1* or *FBXW7* mutations relative to two normal human thymus samples. Student's *t*-test $P = 0.00002$, 0.154 and 0.152 , respectively. (b) Heatmap of microarray-based differential gene expression (log FC) following treatment of human T-ALL cell lines with activating *NOTCH1* mutations with the GSI Compound E (CompE, 500 nM) or vehicle control (dimethyl sulfoxide (DMSO)) for 24 h as indicated. Data were derived from GEO

accession GSE5716.⁵⁰ P12, P12-ICHIKAWA. (c) Scatterplots of expression values median centered by cell line for the human T-ALL cell line data described in (b), showing correlations between *IKZF1*, *HES1* and *DTX1*. (d) Reverse transcription quantitative-PCR (RT-qPCR) analysis of *IKZF1* expression following GSI treatment of human T-ALL cell lines, comparing LOUCY (*NOTCH1*-wild-type) to CUTLL1 and HBP-ALL (activating *NOTCH1* mutations). Mean $\pm$ s.e.m., $n = 3$ independent treatments. (e) RT-qPCR analysis of *IKZF1* expression in human T-ALL cell lines transduced with empty MSCV-IRES-GFP (MIG) or MIG-ICN1. Mean $\pm$ s.e.m., $n = 3$ independent transductions.

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