2011

Cooperation between somatic Ikaros and Notch1 mutations at the inception of T-ALL

Yovina Sontani

Gavin Chapman

Peter Papathanasiou

Sally Dunwoodie

Christopher C. Goodnow

See next page for additional authors

Follow this and additional works at: https://researchonline.nd.edu.au/health_article

Part of the [Life Sciences Commons](#), and the [Medicine and Health Sciences Commons](#)

This article was originally published as:

Sontani, Y., Chapman, G., Papathanasiou, P., Dunwoodie, S., Goodnow, C. C., & Hoyne, G. F. (2011). Cooperation between somatic Ikaros and Notch1 mutations at the inception of T-ALL. *Leukaemia Research*, 35 (11), 1512-1519.

<http://doi.org/10.1016/j.leukres.2011.07.024>

This article is posted on ResearchOnline@ND at
https://researchonline.nd.edu.au/health_article/54. For more
information, please contact researchonline@nd.edu.au.

Authors

Yovina Sontani, Gavin Chapman, Peter Papathanasiou, Sally Dunwoodie, Christopher C. Goodnow, and Gerard F. Hoyne

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Cooperation between somatic Ikaros and Notch1 mutations at the inception of T-ALL

Yovina Sontani^{a,1}, Gavin Chapman^{c,d}, Peter Papathanasiou^b, Sally Dunwoodie^{c,d,e}, Christopher C. Goodnow^a, Gerard F. Hoyne^{a,*,2}

^a Immunology Department, John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia

^b Australian Phenomics Facility, Australian National University, Canberra, ACT, Australia

^c Development Biology Program, Victor Chang Cardiac Research Institute, Sydney, NSW, Australia

^d St. Vincent's Clinical School, University of New South Wales, Sydney, NSW, Australia

^e School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW, Australia

ARTICLE INFO

Article history:

Received 18 January 2011

Received in revised form 14 July 2011

Accepted 15 July 2011

Available online xxx

Keywords:

Ikaros

Leukemia

Notch

Thymocytes

T cell

ABSTRACT

To understand the interactions between *Notch1* and *Ikaros* in the evolution of T cell acute lymphoblastic leukemia (T-ALL), we traced the evolution of T-ALL in mice with an inherited Ikaros mutation, *Ikzf1*^{Plstc} which inactivates DNA binding. DNA-binding Ikaros repressed Notch1 response in transfected cell lines and in CD4⁺8⁺ (DP) thymocytes from young pre-leukemic *Ikzf1*^{Plstc} heterozygous mice. In DP thymocytes, a 50–1000 fold escalation in mRNA for Notch1 target genes *Hes1* and *Dtx1* preceded thymic lymphoma or leukemia and was closely correlated with the first detectable differentiation abnormalities, loss of heterozygosity (LOH) eliminating wild-type *Ikzf1*, and multiple missense and truncating *Notch1* mutations. These findings illuminate the early stages of leukemogenesis by demonstrating progressive exaggeration of Notch1 responsiveness at the DP thymocyte stage brought about by multiple mutations acting in concert upon the Notch1 pathway.

Crown Copyright © 2011 Published by Elsevier Ltd. All rights reserved.

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy that has been associated with recurrent mutations in a number of regulators of immature T cell differentiation, proliferation and survival including the genes encoding NOTCH1, LMO2, HOXA11, LYL1, and TAL1 [1,2]. Notch1 is a receptor that undergoes proteolytic cleavage to release its intracellular domain (ICN) as a transcription regulator, which serves a key role in normal thymocyte differentiation and is a frequent target of gain-of-function mutations in T-ALL. Notch1 was identified at a recurrent (7;9) (q34;q34.3) translocation in T-ALL that fuses the 3' portion of *NOTCH1* to the TCR- β locus, leading to expression of constitutively active forms of the Notch1 receptor (TAN1) [3]. This translocation is present in <1% of human T-ALL but several studies have provided evidence that Notch signalling in T cell leukemias and lymphomas

is often elevated even in the absence of genomic rearrangements [4,5]. Somatic mutations that alter the *NOTCH1* protein-coding sequence have been found in more than 50% of human T-ALL with diverse molecular subtypes [6]. These were found largely in the extracellular heterodimerization (HD) domain, and truncating or missense mutations in the C-terminal PEST domain that increase stability of ICN [6–10]. The extent to which these individual *NOTCH1* mutations in T-ALL confer a growth advantage or malignant transformation is unclear. T-ALL have been described with multiple *NOTCH1* mutations in *cis* altering the HD and C-terminal domains, and these have been shown to exaggerate *NOTCH1* activity in tissue culture in an additive manner and to function cooperatively for leukemogenesis *in vivo* [7,9–12]. Some T-ALL *NOTCH1* mutations are sufficient for promoting leukemogenesis while others are only sufficient for leukemogenesis in conjunction with other mutations, raising the question of how many cooperating events precede the first outgrowth of a leukemic T cell clone [13–16].

Notch1 heterodimers comprising noncovalently associated extracellular domain (NEC) and transmembrane domains (NTM) are produced by the cleavage of pro-Notch1 by a furin-like protease during transit to the cell surface [17]. Activation of Notch receptors occur upon binding of Notch Delta-Serrate-Lag2 (DSL) ligands to the EGF repeat region of the NEC leading to cleavage at S2 in the extracellular portion of NTM, creating a short-lived membrane-bound NTM which is subject to mono-ubiquitination by

* Corresponding author at: School of Health Sciences, University of Notre Dame Australia, 19 Mouat Street, Fremantle, WA 6959, Australia. Tel.: +61 8 94330236; fax: +61 8 94330210.

E-mail address: gerard.hoyne@nd.edu.au (G.F. Hoyne).

¹ Current address: Garvan Institute of Medical Research Sydney.

² Current address: School of Health Sciences, University of Notre Dame Australia, Fremantle, WA, Australia.

FBXW7 to promote endocytosis and cleavage by γ -secretase at the S3 site which releases the intracellular domain of NTM (ICN) [17]. The ICN then translocates into the nucleus and associates with the transcription factor CSL or RBP-Jk and Mastermind-like 1–3 [17]. This complex activates transcription of Notch target genes including *Myc*, *PTEN/PI3K*, *NFkB*, *Hes1*, *PreT α* [18]. Not only mutations in *NOTCH1* itself can lead to leukemogenesis, cooperating mutations in Notch target genes [19] and regulators (e.g. FBXW7 [20,21] and Ikaros [22–24]) have been identified.

Mice with germline mutations that inactivate the transcriptional regulator Ikaros (gene symbol *Ikzf1*) develop T-ALL exclusively [23,25] and the resulting malignancies often have activating *NOTCH1* mutations [6,23,24]. *Ikzf1* somatic mutations are also frequently found in combination with *NOTCH1* mutations in mouse retrovirus-promoted T-ALL [18] and in human B-ALL and T-ALL [26,27]. Short, dominant negative forms of Ikaros generated by alternative splicing have also been found in human T-ALL [28,29] although this has not been found in other T-ALL series [30]. Ikaros mRNA can undergo alternative splicing to yield a range of isoforms some of which lack DNA binding domains (e.g. *Ikzf1* 4–9 isoforms) and so are unable to function as transcriptional regulators but dimerize to act as dominant negative inhibitors of full length Ikaros [31]. Ikaros mutations select for the expression of these short dominant-interfering splice forms.

The relationship between Ikaros and Notch1 in thymocyte differentiation and leukemogenesis is incompletely understood. Full length Ikaros and RBP-Jk recognize the same DNA binding sequence where Ikaros can inhibit RBP-Jk binding and stimulation of transcription [22], but it is not known if Ikaros simply acts as a competitive repressor of Notch-target genes. Animal models of T-ALL provide a unique opportunity to study the cooperating events needed for the initial outgrowth of leukemic T cells, but so far studies have focussed on the malignant phase when overt leukemia and lymphadenopathy have ensued. Here, by analysing pre-leukemic stages in the evolution of T-ALL in mice with an inactivating Ikaros point mutation, we present evidence that Ikaros negatively regulates the Notch1 pathway at the DP thymocyte stage. The first detectable clonal outgrowth occurs selectively within the thymus around the DP stage of development, closely correlated with somatic loss of the remaining wild-type Ikaros allele, a 10–1000 fold increase in mRNA from Notch-response genes, and accumulation of somatic Notch1 mutations. These results indicate that multiple mutations are needed and act cooperatively to activate the Notch1 pathway before pre-leukemic thymocyte outgrowth becomes detectable.

2. Methods

2.1. Animals

Ikzf1^{Plstc/+} mice were bred with C57BL/6 J background (Jackson Laboratory) and maintained in the Australian Phenomics Facility and all procedures were approved by the Australian National University Animal Experimentation Ethics Committee.

2.2. Flow cytometry

Lymphoid tissues were prepared as single cell suspensions in ice-cold PBS/10% FCS and stained with fluorochrome labelled monoclonal antibodies prior to FACS analysis on a FACS Calibur flow cytometer and the data analysed with FlowJo (Tree Star) software.

2.3. Real time RT-PCR

DP thymocytes were sorted on FACSaria/Vantage (Becton Dickinson) and were $\geq 99\%$ pure and total RNA was isolated using Trizol reagent (1 ml per 5×10^6 cells) and reverse transcribed to cDNA using SUPERScript First Strand cDNA Synthesis kit (Invitrogen). Samples were stored at -20°C prior to use. Real-time RT-PCR was performed using the TaqMan[®] Gene Expression Assays (Applied Biosystems) on 384-well plate platform on the ABI PRISM[®] 7900HT (Applied Biosystems). Relative

quantification of mRNA level was performed using the comparative cycle threshold (CT) following the manufacturer's instruction.

2.4. Analysis of Ikaros LOH

Genomic DNA samples were prepared from sorted cells from *Ikzf1^{+/+}* and *Ikzf1^{Plstc/+}* mice and DNA was analysed for the loss of the wild type Ikaros allele by MS-PCR [32] and Amplifluor[®] PCR. End-point fluorescence intensity for the PCR products were analysed by FLUOstar OPTIMA (BMG Labtech). Positive controls included DNA from *Ikzf1^{+/+}* and *Ikzf1^{Plstc/Plstc}* embryos that was used to generate a standard curve. Using the standard curve it was possible to calculate the percentage of the wild type allele within the *Ikzf1^{Plstc/+}* samples.

2.5. Exonic sequencing for Notch 1 mutations

Mutation detection in exons 26, 27 and 34 of Notch1 was performed via PCR using primers specific for exons 26, 27 and 34 of Notch1 gene:

Exon 26 Forward (E26F)	5' AGGGACACTGGGAAACAG 3'
Exon 26 Reverse (E26R)	5' GATGGGACTGAGTGCATCCT 3'
Exon 27 Forward (E27F)	5' CCTCCAGCCCTGATTACT 3'
Exon 27 Reverse (E27R)	5' GTGCCATGTGTGTTCTCAGC 3'
Exon 34 Forward (E34F)	5' TCCCTCATGTACCTCTCTGC 3'
Exon 34 Reverse (E34R)	5' CCAACAGGGCTTGGGAAC 3'

PCR cycles included 97°C for 3 min, 94°C for 30 s, annealing at 59°C for 60 s, extension at 72°C for 90 s. PCR products (5 μl) were visualised on a 1.5% (w/v) agarose gel with $5\times$ DNA loading dye. The amplification was repeated in triplicate. The PCR products were purified using PureLink[™] PCR Purification Kit (Invitrogen) according to the manufacturer's instructions. DNA was eluted in 50 μl of water.

2.6. Sequencing

PCR products were gel purified using PureLink[™] Quick Gel Extraction Kit (Invitrogen) according to the manufacturer's instructions. DNA was dissolved into 50 μl of water and the concentration determined by NanoDrop (ND1000). Addition of 3' adenine to the amplified product was performed prior to cloning the product into TOPO TA Cloning[®] System (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was transformed into One Shot[®] Chemically competent *E. coli* cells (Invitrogen) with a 30 s heat shock at 42°C followed by incubation at 37°C for 1 h. 10–50 μl of each transformation was spread on a selective plate containing 100 $\mu\text{g}/\text{ml}$ ampicillin (Invitrogen) and X-gal (Invitrogen) and incubated overnight at 37°C . Approximately 12 white or light blue colonies per plate were picked and cultured on 96-well growth block (Invitrogen) containing LB media with 100 $\mu\text{g}/\text{ml}$ ampicillin. Purified plasmids of twelve clones per tumour sample were sequenced in the Biomedical Research Facility of John Curtin School of Medical Research.

2.7. Luciferase assays

Hes1-Luciferase (Hes-1-Luc) [33] and Hey1-Luc reporter gene [34] used contain *Hes1* promoter and *Hey1* promoter regions, respectively. Each promoter region contains two RBP-Jk binding sites. NIH3T3 cells were grown in DMEM containing 10% FCS. Transfections were performed in C2C12 cells using LipofectAMINE and Plus reagent (Invitrogen) according to the manufacturer's instructions. Luciferase assay transfections in 12-well trays contained 14 ng of CMV-Renilla plasmid, 350 ng of Hes1-Luc or Hey1-Luc and 350 ng of each expression plasmid (pCMX-ICN, pCMX-*Ikzf1*, pCMX-*Ikzf1^{Plstc}*) or pCAT-CMX control plasmid. Firefly and Renilla luciferase activities were assayed using the Dual-luciferase reporter system (Promega) and measured on a FLUOstar Optima Luminometer (BMG). Firefly luciferase counts were normalised against Renilla luciferase counts to account for differences in transfection efficiency.

3. Results

3.1. Expanded populations of CD8⁺CD4^{low} TCR β^{hi} thymocytes with LOH mutations

To identify the early steps leading to T-ALL, we systematically analysed the thymus of a large cohort of *Ikzf1^{Plstc/+}* mice during the pre-leukemic phase between 4 and 12 weeks of age when $\geq 90\%$ of animals remain disease free (Fig. 1A). Up to 6 weeks of age there was no difference in CD4 or CD8 differentiation in the thymus of wild type and *Ikzf1^{Plstc/+}* mice (data not shown) but by 8–12 weeks of age most *Ikzf1^{Plstc/+}* mice exhibited a larger shoulder of CD8⁺CD4^{low} cells below the normal DP thymocyte population despite normal or only slightly elevated numbers of thymocytes (Fig. 1B). These abnormal CD8⁺CD4^{low} cells in the thymus were typically

Fig. 1. Evolution of leukemia and pre-leukemic abnormalities within the thymus. (A) Percentage of healthy $Ikzf1^{+/+}$ and $Ikzf1^{Plstc/+}$ mice ($n = 49$) at the indicated ages. Animals were euthanased when they developed clinical symptoms of leukemia or thymic lymphoma including cachexia, malaise, and laboured breathing. (B) Flow cytometric staining for CD4 and CD8 on thymocytes and splenocytes from $Ikzf1^{+/+}$ (far left panel in each row) or $Ikzf1^{Plstc/+}$ mice analysed prior to clinical symptoms of leukemia at the indicated ages. Numbers above each plot show the mouse number, and the number of cells in the thymus (T) and spleen (S). Samples indicated in red correspond to those in Fig. 2B with <5%, 5–15%, or 15–25% wild-type $Ikzf1$ DNA in DP cells, respectively.

TCR β^{hi} CD5 $^{\text{hi}}$, comparable to post-positive selection CD8 SP cells in normal thymi, except that they were CD69 negative whereas most normal CD8 SP cells are CD69 $^{+}$ (Supplemental Fig. 1). The DP population itself also exhibited increased expression of TCR β , CD3 and CD5 in these individuals (Supplemental Fig. 1). Staining for spe-

cific V β regions showed monoclonal or oligoclonal expansion of TCR-V β cells (e.g. TCR-V $\beta 5^{\text{hi}}$ or TCR-V $\beta 8.3^{\text{hi}}$ cells) in the thymus of a subset of animals aged 10–12 weeks, but these expanded T cell clones were not detected in the peripheral lymphoid tissues at this age (Supplemental Fig. 2). In the majority of 8- to 12-week-old

mice with these early stage abnormalities, the number and phenotype of peripheral lymphocytes was normal, and hence these animals are referred to as “pre-leukemic”. However a minority of mice at 12 weeks of age had low frequencies of abnormal DP cells in the thymus and spleen (e.g. mouse G17.2 in Fig. 1B). Flow cytometric analysis of the peripheral DP cells in the spleen revealed that they shared an identical cellular phenotype that were typically TCR β^{hi} CD5 $^{\text{hi}}$. The similar phenotype between thymocytes and splenocytes suggests that the peripheral DP phenotype cells were thymic derived and did not arise spontaneously in the periphery (Supplemental Figs. 3 and 4).

The indication above that abnormal leukemic clones evolve initially within the thymus was investigated further by testing thymocytes for somatic *Ikzf1* loss of heterozygosity (LOH). DP thymocytes were sorted from individual mice in a 6- to 12-week-old cohort, genomic DNA prepared, and a quantitative fluorescent SNP assay used to measure the amount of *Ikzf1* $^{+/+}$ and *Ikzf1* $^{\text{Plstc}/+}$ DNA in each sample (Fig. 2A). This revealed that DP thymocytes in the majority of the *Ikzf1* $^{\text{Plstc}/+}$ mice retained the wild-type *Ikzf1* allele in heterozygous state up to 6 weeks of age but this allele accounted for less than 25% of *Ikzf1* DNA or became undetectable in a progressively increasing proportion of 8- to 12-week-old mice (Fig. 2B). The extent of *Ikzf1* LOH in DP cells of individual mice correlated with the extent of flow cytometric abnormalities (Fig. 2C). Somatic loss of wild-type Ikaros in DP thymocytes occurred in animals that did not have thymic lymphoma nor peripheral leukemia. For example, the wild type Ikaros allele was less than 10% or undetectable in DP cells from G17.23 and G16.62, respectively (Fig. 2B), yet total thymocyte numbers in these individuals were only twice normal (Fig. 1B) and the expanded thymic clone of TCR-V β 8.3 $^{+}$ or TCR-V β 5 $^{+}$ T cells was undetectable in peripheral tissues (Supplemental Fig. 2).

3.2. Somatic loss of LOH correlates with a large increase in Notch target gene expression in DP thymocytes

In parallel with the flow cytometric and LOH analyses of the cohort of *Ikzf1* $^{\text{Plstc}/+}$ mice above, mRNA was prepared from the same samples of sorted DP cells and used to measure expression of Notch target genes and other developmental genes by real-time PCR. We focussed first on *Hes1* which was of particular functional relevance because its product is known to suppress CD4 expression [35] and hence might explain the CD8 $^{+}$ CD4 $^{\text{low}}$ phenotype of the earliest detectable abnormal thymocytes. Compared to control DP cells sorted in parallel from wild-type mice, *Hes1* mRNA was modestly elevated in 6- to 8-week-old mice with no evidence of an abnormal CD4–CD8 profile, but became dramatically increased to 100–1000 times the normal DP cell levels in DP cells from 10- to 12-week-old *Ikzf1* $^{\text{Plstc}/+}$ mice (Fig. 3A and Supplemental Fig. 5). Increased *Hes1* mRNA was inversely correlated with loss of wild-type *Ikzf1* in DP thymocytes (Fig. 3A, $r = -0.77$), and with increased percentage of CD8 $^{+}$ CD4 $^{\text{low}}$ thymocytes measured flow cytometrically (Fig. 3B, $r = 0.82$). Thus, while the heterozygous Ikaros point mutation did not result in any appreciable increase in expression of the Notch1 target gene *Hes1* in DP thymocytes that retained 50% wild-type *Ikzf1* DNA, the proportion of thymocytes that had lost the wild-type allele was closely correlated with dramatically exaggerated *Hes1* expression.

Ikaros LOH in DP thymocytes was closely correlated ($r = -0.79$) with a 10–100 fold increase in *Dtx1* expression (Fig. 3C). Expression of *pre-Ta*, which is both developmentally regulated and Notch1 regulated [36], was increased in most samples with LOH but less well correlated (Fig. 3C, $r = -0.58$). There was little correlation between *Ikzf1* LOH and mRNA expression of *Rag1* gene, which is not known to be a direct Notch1 target (Fig. 3C, $r = -0.24$).

The evidence above that the remaining wild type Ikaros allele in *Ikzf1* $^{\text{Plstc}/+}$ DP cells served as a critical negative regulator for

Fig. 2. Correlation between loss of Ikaros heterozygosity and appearance of abnormal flow cytometric subsets in thymi of *Ikzf1* $^{\text{Plstc}/+}$ mice. (A) Quantitative fluorescent SNP assay to measure the percentage of wild-type and *Ikzf1* $^{\text{Plstc}}$ alleles. DNA standards were prepared by mixing different proportions of DNA from *Ikzf1* $^{+/+}$ and *Ikzf1* $^{\text{Plstc}/+}$ embryos. (B) Percent wild-type *Ikzf1* DNA in sorted DP cells from individual *Ikzf1* $^{+/+}$ and *Ikzf1* $^{\text{Plstc}/+}$ mice at the indicated weeks of age. (Red) <5% wild type *Ikzf1* remaining, (blue) 5–15% wild type *Ikzf1* remaining, (purple) 15–25% wild type *Ikzf1* remaining, (black circle) >25% wild type *Ikzf1* remaining, (filled squares) wild type *Ikzf1*. (C) Relationship between % wild-type *Ikzf1* DNA in DP cells and flow cytometric evidence of abnormal thymocyte differentiation as reflected by the ratio of CD8 $^{+}$ CD4 $^{\text{low}}$ cells to DP cells. Pearson's correlation coefficient (r); P value depicts the significance of linear regression as calculated by Fisher's exact test on GraphPad Prism. Age of mice indicated by colour: black, 4 weeks; green, 6 weeks; purple, 8 weeks; blue, 10 weeks; red, 12 weeks.

Notch1-induced gene expression was corroborated in cell lines bearing luciferase reporters controlled by promoter elements from two different Notch target genes, *Hes1* and *Hey1*. Co-transfection of DNA encoding the wild-type Ikaros full-length isoform (Ik1) inhibited transcriptional induction by DNA encoding the intracellular domain of Notch1 (ICN). By contrast, co-transfection of DNA encoding Ik1 bearing the H191R mutation was unable to repress activation of the *Hes1* or *Hey1* reporter constructs (Fig. 4). These results are consistent with other evidence that Ikaros negatively regulates Notch1 target gene expression [22,23,35,37–39]. Since the H191R point mutation disrupts DNA binding but leaves expression of otherwise normal full-length Ikaros isoforms in DP cells, these results collectively establish that the inhibitory effect of Ikaros on transcription of Notch1 targets in DP cells requires the DNA recognition domain and cannot be supplied through other parts of the full-length Ikaros protein.

Fig. 3. Correlation between *Ikzf1* LOH and increased mRNA for Notch target genes in DP thymocytes of *Ikzf1*^{Plstc/+} mice. (A) Relationship between % wild-type *Ikzf1* DNA and mRNA from the Notch target gene *Hes1* in DP cells. Individual mouse numbers are shown, colour-coded by age: black, 4 weeks; green, 6 weeks; purple, 8 weeks; blue, 10 weeks; red, 12 weeks. *r* represents Pearson's correlation coefficient and *P* value depicts the significance of linear regression as calculated by Fisher's exact test. (B) Relationship between flow cytometric evidence of abnormal thymocyte differentiation and *Hes1* mRNA in DP cells. *r* represents Pearson's correlation coefficient and *P* value depicts the significance of linear regression as calculated by Fisher's exact test. (C) Relationship between % wild-type *Ikzf1* DNA and mRNA for *Dtx1*, *Ikzf1*, *Rag1*, and *pre-Ta*. Individual mouse numbers are shown, colour-coded by age: black, 4 weeks; green, 6 weeks; purple, 8 weeks; blue, 10 weeks; red, 12 weeks. *r* represents Pearson's correlation coefficient. The x axis for *Dtx1* expression in DP cells is represented as Log₁₀ values while all other graphs are shown on a linear scale.

Fig. 4. Ikaros H191R encoded by *Ikzf1*^{Plstc} cannot repress Notch1C-induced transcription. The *Hes1* or *Hey1* promoter was coupled to a luciferase reporter (*Hes1-luc*, *Hey1-luc*) that was stably transfected into C2C12 cells. The C2C12 cells were transiently transfected with plasmids encoding constitutively active Notch1 receptor (ICN) with or without plasmids containing wild type *Ikzf1* or *Ikzf1*^{Plstc} H191R allele. Error bars represent the standard deviation of the mean luciferase activity. Data is shown as mean $\pm$ SD (***p* < 0.001).

3.3. Notch1 missense and PEST truncation mutations accompany Ikaros LOH at the earliest detectable stages of abnormal thymopoiesis

In parallel with analysing Ikaros LOH and Notch1-target gene expression in the cohort of pre-leukemic *Ikzf1*^{Plstc/+} mice, we PCR amplified, cloned and sequenced *Notch1* exons 26 and 27 encoding the HD domain and exon 34 encoding the C-terminal PEST domain from genomic DNA of the sorted DP cells (Table 1 and Supplemental Figs. 6 and 7). In DP cells from 12 *Ikzf1*^{Plstc/+} mice aged between 4 and 8 weeks of age with no thymic flow cytometric abnormalities, 3 independent insertion/deletions were detected in exon 34 that truncate ICN (2 in 4-week-old mouse G17.59; 1 in 8-week-old G17.1). These two animals also had little or no increase in *Hes1* mRNA in DP cells and retained a wild-type *Ikzf1* allele in the majority of cells (Fig. 3A). ICN truncating mutations were found at a higher rate (9 mutations in 7 of 11 mice tested) in DP cells from mice with flow cytometrically abnormal thymocytes, despite many lacking detectable leukemia (e.g. G17.23). Of the flow cytometrically abnormal samples with ICN-truncating mutations, all that were tested also had complete or near-complete *Ikzf1* LOH and 50–1000 fold increased *Hes1* mRNA (Fig. 3A, samples G17.2, G17.23, G17.27, G16.64, G15.32). No insertion/deletion truncating mutations were found in exons 26 and 27. Missense mutations in Notch1 exons 26, 27 and 34 were detected in DP cells from thymi with normal or abnormal flow cytometry (Table 1, Supplemental Figs. 6 and 7), including several such as L1668P (L1679P in human NOTCH1) that are repeatedly found in human T-ALL and are well established to destabilize the HD domain negative regulatory region [10]. While a comparable rate of nucleotide substitutions was found in thymic samples with normal or abnormal flow cytometry, the replacement-to-silent ratio was consistent with selection for replacements in DP cells from thymi with abnormal flow cytometric profiles (R/S = 6) whereas there was no evidence for selection in samples without flow cytometric abnormalities (R/S = 1.4). Because the different exons were amplified separately, we cannot determine if the HD missense mutations were combined in *cis* with ICN truncating mutations as has been observed in human T-ALL. Thus, while potentially activating *Notch1* mutations precede the first flow cytometrically detectable thymocyte abnormalities in preleukemic animals, the transition to detectably abnormal thymopoiesis is accompanied by strong selection for activating *Notch1*

Table 1
Number of mutation exons 26, 27 and 34 of the *Notch1* gene observed in individual clones from *Ikzf1^{Plstc/+}* mice.

DP sample	Thymus flow cytometry	Exon 34 #clones seq'd	Exon 34 truncating mutations	Exon 34 mutations	Exon 26 #clones seq'd	Exon 26 mutations	Exon 27 #clones seq'd	Exon 27 mutations	Replacement mutations	Silent mutations
G17.55	Normal	7	0	1	1	0	12	0	0	1
G17.57	Normal	10	0	5	4	0	11	0	3	2
G17.59	Normal	11	1	5	1	0	10	0	3	2
G18.13	Normal	5	0	0	2	0	10	0	0	0
G18.14	Normal	9	0	0	4	1	5	1	1	1
G18.15	Normal	6	0	0	7	0	11	0	0	0
G18.31	Normal	9	0	0	6	1	5	0	0	1
G18.33	Normal	0	0	0	2	0	11	0	0	0
G17.1	Normal	11	1	2	6	3	1	2	4	3
G17.3	Normal	11	0	5	6	2	9	0	4	3
G17.19	Normal	12	0	2	9	2	8	0	3	1
G17.25	Normal	12	0	3	11	1	8	0	3	1
Totals		103		23	59	10	101	3	21	15
Sequenced nt		148,320			23,541		16,362			
Mutations per 100,000 nt sequenced				16		42		18		
Total exonic mutations per 100,000 nt										25
Replacement/silent ratio										1.4
G17.2	Abnormal	11	1	4	7	0	5	1	4	1
G17.23	Abnormal	11	1	2	8	0	6	0	2	0
G17.24	Abnormal	8	0	1	10	1	7	0	2	0
G17.27	Abnormal	7	1	2	11	1	4	0	2	1
G16.62	Abnormal	1	0	0	11	0	7	0	0	0
G16.64	Abnormal	5	1	2	10	0	6	0	2	0
G16.65	Abnormal	0	0	0	9	1	6	1	1	1
G20.109	Abnormal	7	0	1	1	1	4	0	2	0
G20.91	Abnormal	7	1	4	1	0	7	0	4	0
G20.92	Abnormal	4	0	1	2	0	7	1	2	0
G20.47	Abnormal	0	0	0	2	0	6	1	1	0
G20.58	Abnormal	5	2	2	5	1	7	0	2	1
G20.29	Abnormal	0	0	1	2	1	6	1	3	0
Totals		66		20	79	6	78	5	27	4
Sequenced nt		103,680			31,521		12,636			
Mutations per 100,000 nt sequenced				19		19		40		
Total exonic mutations per 100,000 nt										26
Replacement/silent ratio										6.75

somatic mutations in addition to somatic mutations that eliminate the remaining wild-type Ikaros allele.

4. Discussion

In this study we sought to understand the relationship between Ikaros and Notch1 signalling for normal T cell differentiation and the initial stages of neoplastic growth. We confirm and extend earlier evidence [22,23] that Ikaros acts as a critical inhibitor of Notch1-induced gene expression at the DP thymocyte differentiation stage and we show that somatic *Ikzf1* LOH mutations in thymocytes are accompanied by a dramatic escalation in expression of Notch1 target genes. Potentially activating mutations in Notch1 itself can be detected even earlier, and these are strongly enriched concurrent with *Ikzf1* LOH and flow cytometric abnormalities in thymocyte growth or differentiation. These results extend the view emerging from human T-ALL and associated experimental studies [10,11] that multiple mutations acting in concert upon the Notch pathway are needed to exaggerate Notch1 transcriptional activity above a threshold that can promote neoplastic T cell growth *in vivo*.

The overriding view of Ikaros function in T cell development is that it functions to repress Notch signalling in DP cells due to Ikaros' ability to bind to 5' regulatory elements within the *Hes1* and *Dtx1* promoters [23,24,35]. Somatic mutations in the HD domain of Notch 1 occur in ~40–50% of T-ALLs and Notch receptors that bear HD mutations can be expressed at the cell surface and are still responsive to ligands, but they can also signal in a ligand independent manner [10,40]. The cell autonomous activation of Notch signals could contribute to the induction or the progression of T-ALL in several ways. We observed HD mutations in *Ikzf1*^{Plstc/+} mice as young as 4 weeks of age when T cell development was normal, indicating that individual somatically acquired Notch 1 mutations are not sufficient to drive T cell leukemia or disrupt thymopoiesis. Similar HD mutations and mutations that lead to truncation of the Notch1 intracellular domain have been identified in other animal models bearing *Ikaros* mutations suggesting that Notch1 is a frequent target of mutations in these T cell derived tumors [23,24]. This is consistent with the conclusions from retroviral introduction of Notch1 HD or truncating mutations into bone marrow reconstituted mice [11].

The systematic analysis here of young animals that had few or no abnormal T cells circulating in their spleen, lymph nodes or bone marrow provides a view of the early evolution of T-ALL. Previous studies of other Ikaros mutant animals have focussed on the malignant phase when overt leukemia and lymphadenopathy has ensued. LOH of the remaining wild type *Ikzf1* allele was observed and was suggested to be a late stage event in progression to T cell leukemia when large numbers of abnormal T cells accumulated extrathymically in peripheral tissues [23–25]. However, in the current study *Ikzf1* LOH was detected in *Ikzf1*^{Plstc/+} mice between 6 and 8 weeks of age and this was strongly correlated with a 100–1000 fold increase in Notch target gene expression and the first detectably flow cytometric changes in thymocyte development. At this stage, abnormal T cells were only detectable within the thymus and not in the peripheral tissues. The most significant changes occurred in *Hes1* and *Dtx1* expression and these two target genes were also observed to be increased in studies of tumors derived from *Ikzf1*^{Plstc/+} and *Ikaros*^{L/L} mice [23,24]. This contrasts to the report by Mantha et al. who did not detect LOH of the wild type allele of *Ikzf1* in *Ikzf1*^{Plstc/+} mice before 12 weeks of age. Dramatically exaggerated Notch signalling in DP cells can arrest their development [41] and may explain why leukemias in *Ikzf1*^{Plstc/+} mice are generally of a DP or CD8⁺CD4^{int} cell immature T cell phenotype.

A cooperative role between Ikaros and Notch mutations and pre-TCR signalling in T cell transformation and leukemia has been highlighted [6,8,10]. Consistent with these results T-ALL is greatly suppressed in *Rag1*-deficient *Ikzf1*^{Plstc/+} mice where the TCR-β chain cannot be rearranged to assemble pre-TCR (unpublished data). Notch1 and Notch3 activate *pTa* gene expression cells in a way that is repressed by DNA-binding Ikaros isoforms [23,42]. Indeed, *Ikzf1* LOH was correlated with a 2–8 fold increase in *pTa* mRNA in DP cells from *Ikzf1*^{Plstc/+} mice similar to that reported by Dumortier et al. in tumors derived from *Ikaros*^{L/L} mice [23]. Notch1 induces Notch3, and constitutively active Notch3 signalling alters *Ikzf1* mRNA splicing to yield short non-DNA binding isoforms that relieve Ikaros repression of Notch-induced *pTa* [42]. Thus there appear to be two alternative pathways for somatically inactivating Ikaros' repressive function in the evolution of T-ALL, either by somatic loss of the wild-type *Ikzf1* as occur here or by accumulation of other mutations in *NOTCH1*, *FBXW7*, etc. that exaggerate NOTCH signalling sufficiently to suppress full-length Ikaros mRNA and protein.

The data presented here emphasize the need for multiple mutations dysregulating Notch-signalling during the inception of T cell leukemia *in vivo*. The earliest stages of abnormal thymic differentiation were accompanied by somatic mutations in the HD and PEST domains of the *Notch1* gene that are known to exaggerate ICN production, induce expression of *Myc* [11] and Cyclin-D1 (26) and inhibit the expression of tumour suppressor genes (e.g. p19ARF – p53) or growth regulators such as *p27kip1* and *Nur77* [43]. Loss of DNA-binding Ikaros appears to be a critical collaborator in this inception phase, relieving the ICN-RBPJk-MAML transcriptional complex from repressive effects of Ikaros [23,42] and correlating closely with a dramatic increase in Notch target gene expression in pre-leukemic DP cells. This multi-step increase in Notch signalling may be required to achieve a threshold for inducing *Myc*, repressing p19Arf, and initiating neoplastic DP cell growth *in vivo*, as suggested by Chiang et al. [11]. Mutant Notch1 receptors can still bind and respond to Notch ligands and this may be important in the engraftment of the mutant cells into the bone marrow and secondary lymphoid tissues [44]. Given that multiple steps appear necessary for the initiation of leukemic growth, T-ALL cells may be particularly sensitive to agents that target even one of these steps to lower Notch signalling by intermediate amounts.

Conflict of interest

The authors declare that they have no competing financial support or relationships that may pose conflict of interest with the studies presented in this paper.

Acknowledgements

We thank Belinda Whittle and the staff of the Australian Phenomics Facility for advice in developing the LOH assay and for genotyping. We thank Holly Burke for help with curating the *Ikzf1*^{Plstc/+} mouse colony.

Funding support: We gratefully acknowledge research support by an ARC Federation Fellowship (CG), NHMRC Program Grant 427620 (CG), and JDRF/NHMRC Special Program Grant 219167 and JDRF Program Grant 7-2006-327 (CG & GH).

Contributions: CCG and GFH are equal senior authors; YS performed the majority of studies; GC performed studies shown in Fig. 4; PP performed studies in Fig. 1 and also in unpublished studies related to the manuscript; SLD helped design studies shown in Fig. 4 and contributed to writing and CCG and GFH helped to plan and direct experiments and writing of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.leukres.2011.07.024](https://doi.org/10.1016/j.leukres.2011.07.024)

References

- [1] Aster JC, Pear WS, Blacklow SC. Notch signaling in leukemia. *Annu Rev Pathol* 2008;3:587–613.
- [2] Grabher C, von Boehmer H, Look T. Notch 1 activation in the molecular pathogenesis of T-cell acute leukaemia. *Nat Rev Cancer* 2006;6:347–59.
- [3] Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 1991;66:649–61.
- [4] Chiaranante R, Calzavara E, Balordi F, Sabbadini M, Capello D, Gaidano G, et al. Differential regulation of Notch signal transduction in leukaemia and lymphoma cells in culture. *J Cell Biochem* 2003;88:569–77.
- [5] Bellavia D, Campese DF, Checquolo S, Balestri A, Biondi A, Cazzaniga G, et al. Combined expression of pTalpha and notch3 in T cell leukaemia identifies the requirement for preTCR for leukemogenesis. *Proc Natl Acad Sci USA* 2002;99:3788–93.
- [6] O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH, et al. Activating Notch1 mutations in mouse models of T-ALL. *Blood* 2006;107:781–5.
- [7] Eguchi-Ishimae M, Eguchi M, Kempfski H, Greaves M. NOTCH1 mutation can be an early, prenatal genetic event in T-ALL. *Blood* 2008;111:376–8.
- [8] Malecki MJ, Sanchez-Irizarry C, Mitchell JL, Histen G, Xu ML, Aster JC, et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. *Mol Cell Biol* 2006;26:4642–51.
- [9] Mansour MR, Duke V, Foroni L, Patel B, Allen CG, Ancliff PJ, et al. Notch-1 mutations are secondary events in some patients with T-cell acute lymphoblastic leukemia. *Clin Cancer Res* 2007;13:6964–9.
- [10] Weng A, Ferrando A, Lee W, Morris J, Silverman L, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 2004;306:269–71.
- [11] Chiang MY, Xu L, Shestova O, Histen G, L'Heureux S, Romany C, et al. Leukemia-associated NOTCH1 alleles are weak tumor initiators but accelerate K-ras-initiated leukemia. *J Clin Invest* 2008;118:3181–94.
- [12] Kindler T, Cornejo MG, Scholl C, Liu J, Leeman DS, Haydu JE, et al. K-Ras^{G12D}-induced T-cell lymphoblastic lymphoma/leukemias harbor *Notch1* mutations and are sensitive to γ -secretase inhibitors. *Blood* 2008;112:3373–82.
- [13] Campese AF, Garbe AI, Zhang F, Grassi F, Screpanti I, von Boehmer H. Notch1-dependent lymphomagenesis is assisted by but does not essentially require pre-TCR signaling. *Blood* 2006;108:305–10.
- [14] Ferrando A, Look T. Gene expression profiling in T-cell acute lymphoblastic leukemia. *Semin Hematol* 2003;40:274–80.
- [15] Howe SJ, Mansour MR, Schwarzwald K, Bartholomae C, Hubank M, Kempfski H, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* 2008;118:3143–50.
- [16] Leong KG, Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. *Blood* 2006;107:2223–33.
- [17] Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 2009;137:216–33.
- [18] Uren AG, Kool J, Matentzoglou K, de Ridder J, Mattison J, van Uiter M, et al. Large-scale mutagenesis in p19(ARF)- and p53-deficient mice identifies cancer genes and their collaborative networks. *Cell* 2008;133:727–41.
- [19] Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med* 2007;13:1203–10.
- [20] O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, et al. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* 2007;204:1813–24.
- [21] Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, et al. The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J Exp Med* 2007;204:1825–35.
- [22] Beverly LJ, Capobianco AJ. Perturbation of Ikaros isoform selection by MLV integration is a cooperative event in Notch1C-induced T cell leukemogenesis. *Cancer Cell* 2003;3:551–64.
- [23] Dumortier A, Jeannot R, Kirstetter P, Kleinmann E, Sellars M, dos Santos NR, et al. Notch activation is an early and critical event during T-Cell leukemogenesis in Ikaros deficient mice. *Mol Cell Biol* 2006;26:209–20.
- [24] Mantha S, Ward M, McCafferty J, Herron A, Palomero T, Ferrando A, et al. Activating Notch1 mutations are an early event in T-cell malignancy of Ikaros point mutant Plastic/+ mice. *Leuk Res* 2007;31:321.
- [25] Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. *Cell* 1995;83:289–99.
- [26] Maser RS, Choudhury B, Campbell PJ, Feng B, Wong KK, Protopopov A, et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature* 2007;447:966–71.
- [27] Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* 2008;453:110–4.
- [28] Nakayama H, Ishimaru F, Avitahl N, Sezaki N, Fujii N, Nakase K, et al. Decreases in Ikaros activity correlate with blast crisis in patients with chronic myelogenous leukemia. *Cancer Res* 1999;59:3931–4.
- [29] Sun L, Crotty ML, Sensel M, Sather H, Navara C, Nachman J, et al. Expression of dominant-negative Ikaros isoforms in T-cell acute lymphoblastic leukemia. *Clin Cancer Res* 1999;5:2112–20.
- [30] Marçais A, Jeannot R, Hernandez L, Soulier J, Sigaux F, Chan S, et al. Genetic inactivation of Ikaros is a rare event in human T-ALL. *Leuk Res* 2010;34:426–9.
- [31] Georgopoulos K. Haematopoietic cell-fate decisions, chromatin regulation and Ikaros. *Nat Rev Immunol* 2002;2:162–74.
- [32] Rust S, Funke H, Assmann G. Mutagenically separated PCR (MS-PCR): a highly specific one step procedure for easy mutation detection. *Nucleic Acids Res* 1993;21:3623–9.
- [33] Jarriault S, Le Bail O, Hirsinger E, Pourquie O, Logeat F, Strong CF, et al. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol* 1998;18:7423–31.
- [34] Maier MM, Gessler M. Comparative analysis of the human and mouse Hey1 promoter: Hey genes are new Notch target genes. *Biochem Biophys Res Commun* 2000;275:652–60.
- [35] Kathrein KL, Chari S, Winandy S. Ikaros directly represses the notch target gene *Hes1* in a leukemia T cell line: implications for CD4 regulation. *J Biol Chem* 2008;283:10476–84.
- [36] Reizis B, Leder P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes Dev* 2002;16:295–300.
- [37] Beverly LJ, Capobianco AJ. Targeting promiscuous signaling pathways in cancer: another Notch in the bedpost. *Trends Mol Med* 2004;10:591–8.
- [38] Kleinmann E, Geimer Le Lay AS, Sellars M, Kastner P, Chan S. Ikaros represses the transcriptional response to Notch signaling in T-cell development. *Mol Cell Biol* 2008;28:7465–75.
- [39] Chari S, Winandy S. Ikaros regulates Notch target gene expression in developing thymocytes. *J Immunol* 2008;181:6265–74.
- [40] Chiang MY, Xu ML, Histen G, Shestova O, Roy M, Nam Y, et al. Identification of a conserved negative regulatory sequence that influences the leukemogenic activity of NOTCH1. *Mol Cell Biol* 2006;26:6261–71.
- [41] Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* 1999;11:299–308.
- [42] Bellavia D, Mercarozzi M, Campese AF, Grazioli P, Talora C, Frati L, et al. Notch 3 and the Notch3-upregulated RNA binding protein HuD regulate Ikaros alternative splicing. *EMBO J* 2007;26:1670–80.
- [43] Murata K, Hattori M, Hirai N, Shinozuka Y, Hirata H, Kageyama R, et al. Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. *Mol Cell Biol* 2005;25:4262–75.
- [44] Gordon WR, Roy M, Vardar-Ulu D, Garfinkel M, Mansour MR, Aster JC, et al. Structure of the Notch1-negative regulatory region: implications for normal activation and pathogenic signaling in T-ALL. *Blood* 2009;113:4381–90.