**Fli-1 Overexpression in Hematopoietic Progenitors Deregulates T Cell Development and Induces Pre-T Cell Lymphoblastic Leukaemia/Lymphoma**

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**Abstract**

The Ets transcription factor Fli-1 is preferentially expressed in hematopoietic tissues and cells, including immature T cells, but the role of Fli-1 in T cell development has not been closely examined. To address this we retrovirally overexpressed Fli-1 in various in vitro and in vivo settings and analysed its effect on T cell development. We found that Fli-1 overexpression perturbed the DN to DP transition and inhibited CD4 development whilst enhancing CD8 development both in vitro and in vivo. Surprisingly, Fli-1 overexpression in vivo eventuated in development of pre-T cell lymphoblastic leukaemia/lymphoma (pre-T LBL). Known Fli-1 target genes such as the pro-survival Bcl-2 family members were not found to be upregulated. In contrast, we found increased NOTCH1 expression in all Fli-1 T cells and detected Notch1 mutations in all tumours. These data show a novel function for Fli-1 in T cell development and leukaemogenesis and provide a new mouse model of pre-T LBL to identify treatment options that target the Fli-1 and Notch1 signalling pathways.

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**Introduction**

The Friend Leukemia Virus Integration 1 (Fli-1) is a member of the Ets family of transcription factors [1]. Fli-1 is expressed in hematopoietic lineages and vascular endothelial cells and regulates the expression of multiple target genes involved in proliferation, differentiation and cell death. Originally, Fli-1 was discovered as a common retroviral insertion site in Friend Murine Leukemia Virus-infected cells and subsequently as a common rearrangement in human Ewing’s sarcoma resulting in an EWS/Fli1 fusion product [2]. Fli-1 is essential for embryonic development and has been shown to be required for megakaryocyte development as well as play a major role in myeloid, erythroid and natural killer (NK) cell development [1,3,4,5]. It has also been demonstrated that Fli-1 is expressed in immature T cells and concomitantly downregulated in pre-B cells [6]. Interestingly, H-2Kb-Fli-1 transgenic mice, which express high levels of FLI-1 in the thymus and spleen, die of an immunological renal disease and display increased numbers of mature B cells with reduced activation-induced cell death but no significant difference in CD4+CD8+ T cell distribution [7]. The exact role of Fli-1 in T cell development is therefore not clear.

T cell development initiates when a blood-borne foetal liver (FL) or bone marrow (BM) precursor enters the thymus [8]. These are termed double negative (DN) cells, as they do not express CD4 or CD8. DN thymocytes undergo an ordered development based on the expression of CD44 and CD25. DN1 cells (CD25⁺CD4⁺) can reconstitute the T, B, dendritic cell (DC), and NK lineages [9,10]. DN2 cells (CD25⁺CD4⁻) generate T cells, NK cells, DC and myeloid cells [10,11,12,13]. TCRβ rearrangement occurs at the DN3 stage (CD25⁺CD4⁻) and this provides irrevocable commitment to the αβ T cell lineage [14]. Further development of αβ T cells requires signalling through the pre-TCR complex [15]. The pre-TCR is responsible for initiating the DN to DP transition [15]. Finally, DN4 cells (CD25⁻CD4⁻) are rapidly dividing blasts that spontaneously become CD4⁺8⁺ (double positive; DP) [16]. The DN to DP transition is marked by enormous proliferation [17]. Therefore, exquisite control is required at this particular checkpoint or oncogenesis may arise. DP cells are subjected to a series of stringent criteria that select for either mature CD4⁺8⁻ or CD4⁻8⁺ single positive (SP) T cells that have moderate affinity for self-MHC but retain their ability to respond to foreign antigens [8].

We found that Fli-1 overexpression perturbed the DN to DP transition in vitro as well as inhibited CD4 differentiation and promoted CD8 T cell development in vitro and in vivo. Interest-
Fli-1 Overexpression Induces T Cell Leukemia

A

MigR1 FTOC

Fli-1 FTOC

B

% positive

C

MigR1 OP9-DL1

Fli-1 OP9-DL1

D

% positive

E

MigR1 Thymus

Fli-1 Thymus

F

% positive

G

Fli-1 ratio vs GAPDH

Fli-1 expression
ingly, Fli-1 overexpression in vivo eventually resulted in a fatal T cell lymphoblastic leukemia/lymphoma with infiltration of leukemic cells into the thymus, spleen, lymph node, bone marrow and liver. No enhancement of pro-survival leukemic cells into the thymus, spleen, lymph node, bone marrow and liver. No enhancement of pro-survival

cell lymphoblastic leukaemia/lymphoma with infiltration of

(100 mg/kg) mice were used as donor cells and transplanted into CD45.2 lethally irradiated (2 × 550 cGy) recipients. Up to 2 million E15.5 FL or adult BM cells were precultured for 24–48 hours in 2 ml complete IMDM with 50 μM β-mercaptoethanol, 50 ng/ml SCF, 6 ng/ml IL-3, 20 ng/ml IL-6, 4 ng/ml IL-1β and 1 ng/ml IFN-γ. Subsequently, 5 × 105 FL/BM cells were centrifuged with 50% (v/v) retroviral supernatant at 1100 g for 90 min in 8 μg/ml polybrene or on Retronectin-coated plates (Takara Holdings, Inc. Shiga, Japan). One day later, lethally irradiated mice were injected intravenously with at least 2 × 105 transduced FL/BM cells. Hematopoietic and lymphoid organs from transplanted mice were analysed at 6–12 weeks post-transplant or whenever they developed disease. Results from FL and BM transplanted mice were pooled for statistical analysis.

Retroviral Transduction and Transplantation

Red cell depleted CD45.1 FL or BM cells from 5-fluorouracil-treated (100 mg/kg) mice were used as donor cells and transplanted into CD45.2 lethally irradiated (2 × 550 cGy) recipients. Up to 2 million E15.5 FL or adult BM cells were precultured for 24–48 hours in 2 ml complete IMDM with 50 μM β-mercaptoethanol, 50 ng/ml SCF, 6 ng/ml IL-3, 20 ng/ml IL-6, 4 ng/ml IL-1β and 1 ng/ml IFN-γ. Subsequently, 5 × 105 FL/BM cells were centrifuged with 50% (v/v) retroviral supernatant at 1100 g for 90 min in 8 μg/ml polybrene or on Retronectin-coated plates (Takara Holdings, Inc. Shiga, Japan). One day later, lethally irradiated mice were injected intravenously with at least 2 × 105 transduced FL/BM cells. Hematopoietic and lymphoid organs from transplanted mice were analysed at 6–12 weeks post-transplant or whenever they developed disease. Results from FL and BM transplanted mice were pooled for statistical analysis.

Foetal Thymic Organ Culture

FL reconstitution of FTOCs was performed as described previously [18]. The reconstituted foetal thymic lobes were placed on 0.8 μm polycarbonate membranes (Isopore, Millipore, Ireland) floating on 2 ml complete IMDM and analysed by flow cytometry 14 days later.

OP9-DL1 Co-cultures

E15 foetal liver (FL) cells were cultured on OP9-DL1 cells for 6 days in 5 ng/ml FLT3L and 0.25 ng/ml IL-7 [19]. FL cells were retrovirally transduced with either MigR1 control or Fli-1. Four days later the GFP+ cells were analysed for presence of DN1–4 progenitors by flow cytometry as described above.

Histology

Fresh tissues (thymus, spleen, sternum) were fixed in Bouin’s fixative overnight and then placed into 70% ethanol and embedded in paraffin. Five μm sections were cut and, after standard histiological procedure for dehydration, stained with haematoxylin and eosin.

Western Blot Analysis

Cells were lysed in RIPA buffer (25 mM Tris-Cl [pH 7.6], 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% SDS) with protease inhibitors (Complete Mini EDTA free protease inhibitor tablets, Roche Diagnostics, Castle Hill, NSW, Australia) and equal amounts (20 μg) of total protein per sample were separated via 10% SDS-PAGE and transferred to Immobilon-P (PVDF) transfer membrane (Millipore, North Ryde, NSW, Australia) for western blotting. Proteins were detected using primary antibodies against FLI-1 (sc-536, Santa Cruz, CA USA) and beta-ACTIN (A5316, Sigma-Aldrich, Castle Hill, NSW, Australia) and secondary HRP conjugated antibodies followed by visualisation using ECL reagents (Santa Cruz, CA USA).

Plasmids

MigR1-GFP was a gift from Warren Pear and was used as described previously [18]. Fli-1 cDNA was cloned from 129Sv/J embryoid bodies, ligated into MigR1 and the insert confirmed by sequencing.
Southern Blot Analysis

The DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Diagnostics, Castle Hill, NSW, Australia) was used for Southern blot assay. In brief, DNA (5 µg) was digested with EcoRI, size separated on a 0.8% agarose gel, and then capillary-transferred onto positively charged Nylon membranes (Roche Diagnostics, Castle Hill, NSW, Australia). A 1.4 Kb IRES-GFP fragment was purified, labelled with digoxigenin and used as a probe according to the manufacturer’s recommendations.

TCRβ VDJ Rearrangement, Notch1 Mutation and Deletion Analysis

TCRβ VDJ rearrangements as well as Notch1 PEST mutations and 5′ deletions were examined by genomic PCR. Genomic DNA was isolated from total thymus or spleen and 100–125 ng was used per PCR reaction [20]. TCRβ VDJ rearrangements were analysed using the following primers: TCR Vbeta10 F 5′-GCGCTTCTCACCTCAGTCTTCAG and TCR Jbeta2 R 5′-TGAGAGCTGTCTCCTACTATCGATT [21]. The PEST domain (exon 34) of murine Notch1 was amplified with the primers PEST1F: 5′-TACCAGGGCCTGCCCAACAC and PEST2R: 5′-GCCTCTGGAATGTGGGTGAT [22]. Type 1 Notch1 deletions were analysed using the primers: Notch1 P1 F: 5′-CCATGGTGGAATGCCTACTT and Notch1 P2 R: 5′-CGTTTGGGTAGAAGAGATGC and control primers G3PDH F: 5′-ACCACAGTCCATGCCATCAC and G3PDH R: 5′-TCCACCACCCTGTTGCTGTA [23,24]. For sequence analysis DNA was amplified using a high fidelity polymerase. PCR products were then gel purified, sequenced and trace files were manually analysed.

Quantitative Real-time PCR

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey Nagel, Duren, Germany). First-strand cDNA synthesis was carried out with 1 µg of total RNA using random hexamers and MuMLV RT (New England Biolabs, Ipswich, MA USA). Real-time PCR was performed with a Rotor-Gene 3000 (Corbett Robotics, Brisbane, QLD, Australia) with SYBR Green for Bcl-2, Bcl-xL, and Mcl-1. SYBR Green primer sets: Bcl-2 F: 5′-CCGGGAGAACAGGGTATGATAA and Bcl-2 R: 5′-CCCACTCGTAGCCCCTCTG, Bcl-xL F: 5′-TCTACGGGAAATCGCAATGCAGCA and Bcl-xL R: 5′-AGGAACCAGCGGTTGAAGC, Mcl-1 F: 5′-TGGAGTTCTTCCACGTACAGGA and Mcl-1 R: 5′-AGCAACACCCGCAAAAGC and Gapdh F: 5′-CATGTGGTGATGTGGTTG and Gapdh R: 5′-CATGAGCTGTGGTCATGAG. For Fli-1 and Notch1 the following Taqman Gene Expression Assays were used: Fli-1 Mm00484410_m1, Notch1 Mm00435245_m1 and Gapdh: Mm99999915_g1 (Applied Biosystems). Gene expression levels were calculated relative to Gapdh and data were normalized by Fli-1 transplanted mice (n = 15). Two additional Fli-1 mice developed an erythroleukaemia and were included in an additional Kaplan-Meier curve (Fli-1 pre-T LBL-eryth crude n = 17). B. Q-PCR showing Fli-1 mRNA levels of MigR1 control (Mig) or Fli-1 thymocytes (Fli-1). C. Western Blot for Fli-1 protein of MigR1 control (Mig) or Fli-1 thymocytes (Fli-1). ACTIN = loading control. D. Total cell numbers (GFP+ and GFP−) of thymus and spleen from lethally irradiated mice reconstituted with MigR1 and Fli-1 expressing hematopoietic progenitors (mean ± SEM). E. MigR1 thymus and Fli-1 thymus, liver and lymph node cells analysed for CD4, CD8 and TCRβ expression by flow cytometry. F. Graph of GFP+ DN, immature (TCRβ+) single positive (ISP) CD8, DP, CD4 and mature TCRβhi CD8 SP thymocyte populations in MigR1 and Fli-1 leukemia mice. Data represented are mean ± SEM of 6 MigR1 mice and 13 Fli-1 mice from 3 independent transplants. doi:10.1371/journal.pone.0062346.g002

Figure 2. Fli-1 overexpression induces pre-T LBL in vivo. BM or FL cells were transduced with MigR1 or Fli-1 and used to transplant lethally irradiated mice. A. Kaplan-Meier survival curve of MigR1 (n = 8) versus
Figure 3. *Fli-1*-induced pre-T LBL perturbs thymic, splenic and bone marrow architecture. Representative tissue sections (Haematoxylin & Eosin stain) of MigR1 and *Fli-1* mice. MigR1 sections show normal organ architecture, whereas *Fli-1* sections show thymus and spleen architecture effaced by small infiltrating malignant lymphocytes also present in the *Fli-1* sternum. A. Thymus (x10 magnification). B. Spleen (x10 magnification). C. Sternum (x40 magnification).
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Figure 4. Fli-1 pre-T LBL can be transplanted to secondary recipients, is not due to retroviral insertion site and is oligoclonal. A. Splenic cells from a mouse with Fli-1 leukaemia were injected into 4 sublethally irradiated recipients, which subsequently developed disease around 3 weeks post-transplant. Three additional transplants into 11 unirradiated mice gave similar results. Single cell suspensions from spleen and liver were analysed for CD4, CD8 and TCRβ expression by flow cytometry. Data representative of 4 independent transplants. B. Southern blot of total thymocyte (thy) genomic DNA from mice transplanted with Fli-1 transduced bone marrow showing multiple different retroviral integration sites in Fli-1 leukaemia. EcoRI digested DNA was probed with a DIG-labelled IRES-GFP fragment. Lane 1: λ Hind III marker, 2–6: primary Fli-1 thymi as indicated, 7: secondary from Fli-1 #622, 8: mouse #625 Radiation-Induced Thymic Lymphoma (RITL). Data representative of three independent experiments. C. TCRβ VDJ rearrangement as assessed by PCR showing normal VDJ rearrangement in MigR1 control mice (7 bands: Vβ-Jβ21–28) and oligoclonal rearrangement in Fli-1 mice (1–4 bands). Lanes 1 & 24: Marker, 2–23: MigR1 control (Mig) or primary and secondary (2) Fli-1 thymocytes (Thy) or spleen cells (Spl) as indicated. 23: Fli-1 #627 is TCRβ null. doi:10.1371/journal.pone.0062346.g004

dividing each expression value by the median of gene expression of MigR1 control mice.

Statistics

Student t-tests were normally distributed whilst Mann-Whitney U tests were performed on data that was not normally distributed. These decisions were made using Instat 3.0 software (GraphPad Software, LaJolla, CA, USA). The Kaplan-Meier curve was generated using Prism (GraphPad Software, LaJolla, CA, USA) and significance for survival curve differences were calculated using the Log-rank test. Significance was determined as p<0.05 or less.

Results

Fli-1 Overexpression Perturbs T cell Development in vitro and in vivo

As Fli-1 is expressed throughout T cell development it was hypothesized that retroviral Fli-1 overexpression could potentially perturb T cell differentiation. In order to test this, we transduced foetal liver (FL) with control MigR1-GFP retrovirus or Fli-1-GFP retrovirus and reconstituted foetal thymic lobes. Fourteen days later, thymocytes were analysed by flow cytometry using antibodies to CD4 and CD8. It can be clearly seen that the Fli-1 foetal thymic organ cultures (FTOC) demonstrated a significant inhibition of the DN to DP transition as evidenced by an increased percentage of DN and a decreased percentage of DP cells (Figure 1A-B). There was also a significant reduction in the percentage of the CD4+ SP population in Fli-1 FTOCs. Finally, an expansion of the percentage of immature (TCRβ–/–/–) CD8+ SP (ISP) cells was found in Fli-1 FTOCs.

As Fli-1 expression resulted in a retardation of the DN to DP transition we hypothesized that it may alter normal DN1–DN4 differentiation. Therefore, we cultured FL transduced with control MigR1 or Fli-1 on OP9-DL1 cells for 8 days. Indeed, Fli-1 significantly retarded development at the DN3 stage (Figure 1C–D). Additionally, Fli-1 also significantly enhanced the differentiation of DN1 cells (Figure 1C-D).

In vivo validation of the FTOC results was performed by analysing lethally irradiated mice transplanted with FL or bone marrow (BM) cells transduced with control MigR1 or Fli-1 retrovirus. After 10–12 weeks, thymocytes from MigR1 and Fli-1 transplanted mice were assessed for CD4 and CD8 expression by flow cytometry. There was no difference in total (GFP+ and GFP–) thymocyte numbers between the control MigR1 and Fli-1 overexpressing thymi (Figure S1). However, it was evident that Fli-1 mice had a decreased percentage of CD4+ SP and a significantly increased percentage of CD8+ SP (Figure 1E-F). These results are consistent with the previous Fli-1 FTOC data and demonstrate an acute effect of Fli-1 overexpression on T cell development, which ultimately leads to an expansion of the percentage of CD8+ T cells and a reduction in the percentage of CD4+ T cells.

This led us to analyse Fli-1 expression by QRT-PCR in normal DN1–4, ISP CD8, DP, CD4 SP and CD8 SP thymocytes (Figure 1G). The highest expression of Fli-1 in immature thymocytes was in DN1 and DN2 whilst DN3 and DN4 had the lowest expression. As thymocytes matured, the levels of Fli-1 increased such that DP, CD4 SP and CD8 SP had the highest levels of Fli-1. As expected, Fli-1 overexpression seemed to mainly affect those T cell subpopulations that normally have low levels of Fli-1.

Given this data and that Fli-1 activation had previously been shown to induce erythroleukaemia in BALB/c mice, it was hypothesized that Fli-1 may induce T cell oncogenesis in C57BL/6 mice [1].

As Fli-1 overexpression severely perturbed T cell development after 10–12 weeks in vivo we monitored Fli-1-transplanted mice for leukaemia or lymphoma induction over an extended period. As hypothesized, Fli-1 reconstituted mice presented with enlarged thymus, spleen and lymph nodes with a median onset of 111 days (Figure 2A-Kaplan-Meier Curve). Control MigR1 mice did not develop disease over the study period. However, two additional mice developed an erythroleukaemia (Figure 2A-grey curve). To confirm Fli-1 overexpression we performed quantitative real-time PCR (Q-PCR) for mRNA expression on all mice (only six shown) and Western blot analysis for FL-1 protein expression on two control MigR1 thymi and four Fli-1 thymi that developed disease. This clearly demonstrated increased Fli1 mRNA and FL-1 protein expression in Fli-1 thymi compared to the control MigR1 (Figure 2B-C).

Total cell numbers (GFP+ and GFP–) in Fli-1 thymus and spleen were significantly higher than those of the MigR1 controls (Figure 2D). The Fli-1 leukaemia phenotype was very similar to the preleukaemic phenotype with reduced CD4+, CD4+8+ and expanded CD8+ cells evident (Figure 2E). The CD4, CD8 and TCRβ phenotype of cells in the Fli-1 thymus, liver and lymph node suggested that the disease was a T cell lymphoblastic leukaemia/lymphoma (pre-T LBL) [25]. However, there were two Fli-1-transplanted mice that had no surface TCRβ expression (Figure S2). Fli-1 spleen and bone marrow were comparably abnormal (Figure S3).

Fli-1 transplanted mice had significant percentage increases in both TCRβ–/– ISP CD8 and mature TCRβhi CD8 SP cells (Figure 2E). Additionally, the size of the Fli-1 ISP CD8 cells in thymus, liver and lymph nodes was much smaller than control MigR1 ISP CD8s which are blasts; suggesting that Fli-1 ISP CD8 cells were not leukaemic blasts (Figure 2E). Fli-1 mice also had significant decreases in DP and CD4 SP cells, consistent with the FTOC data (Figs. 1A-B). Taken together, these results implied that Fli-1 overexpression could induce a T cell malignancy resembling pre-T LBL and strongly suggest that Fli-1 can act as a T cell oncogene.
Fli-1 Overexpression Induces T Cell Leukemia

**Bcl-2**

S=spleen
T=thymus
L=Liver

**Bcl-xL**

**Mcl-1**
Fli-1-induced Pre-T LBL Perturbs Thymic, Splenic and Bone Marrow Architecture

Histology sections of MigR1 and Fli-1 thymus, spleen, liver and sternum (BM) revealed a significant infiltration of small cells in all tissues of Fli-1 transplanted mice (Figure 5). Specifically, the Fli-1 thymus had lost its normal cortical-medullary demarcation and was completely permeated with malignant T cells (Figure 3A). The Fli-1 spleen had no B cell follicles whilst the MigR1 control spleen did (Figure 3B). Finally, the bone marrow of the Fli-1 sternum was filled with uniform-sized cells in comparison to the more sparse heterogeneity of the bone marrow cells from the MigR1 control (Figure 3C). These data are consistent with the flow cytometry results demonstrating that Fli-1 transplanted mice had leukemic cells throughout the thymus, spleen, lymph node, liver and bone marrow.

Fli-1 Pre-T LBL can be Transplanted to Secondary Recipients, is Integration Site Independent and Oligoclonal

To ascertain if Fli-1 pre-T LBL could be transferred to secondary recipients, 3×10⁶ spleen cells from a Fli-1 mouse were injected intravenously into sublethally irradiated recipients. All mice became hunched, moribund and had ruffled fur after 20–21 days. Therefore, spleens and livers from these mice were analysed for expression of CD4, CD8 and TCRβ by flow cytometry. All 4 mice demonstrated a similar CD4⁺/CD8⁺ population and CD4⁺/CD8⁺ SP population as the primary mouse (Figure 4A). These data reveal that the Fli-1-induced pre-T LBL is transplantable.

To preclude that site specific integration effects could be responsible for the observed pre-T LBL development, a genomic Southern blot of both primary and secondary pre-T LBLs was undertaken with a GFP specific probe. The results unequivocally demonstrate that each individual pre-T LBL had different multiple integration sites and as such the observed pre-T LBL is due to aberrant FLI-1 expression (Figure 4B). Additionally, we sequenced integration sites in 5 tumours and could find no association with known oncogenes (Table S1).

Interestingly, analysis of TCRβ rearrangements revealed that most Fli-1 pre-T LBL possessed one dominant clone but were oligoclonal (Figure 4C). The exceptions to this were those that did not express TCRβ (Figure 4C, lanes 16 & 23). As expected, identical TCRβ rearrangements were found in the thymus and spleen of the same Fli-1 transplanted mice (Figure 4C, lanes 18–19) and in primary and secondary Fli-1 transplanted mice (Figure 4C, lanes 3–6).

No Increase in Pro-survival Bcl-2 Family mRNAs in Fli-1 Pre-T LBL

Fli-1 has been demonstrated to upregulate Bcl-2 in erythroleukaemia [26]. Therefore, we analysed primary and secondary Fli-1 leukemias and MigR1 controls for Bcl-2, Bcl-XL and Mcl-1 mRNA expression by Q-PCR (Figure 5). Surprisingly, all Fli-1 pre-T LBLs, whether primary, secondary or grown in vitro had lower levels of Bcl-2, Bcl-XL and Mcl-1 mRNA than the MigR1 control (Figure 5). These data show that overexpression of the pro-survival Bcl-2 family members is unlikely to be involved in the induction of Fli-1 pre-T LBL.

Intracellular NOTCH1 is Upregulated in Fli-1 Pre-T LBL

NOTCH1 plays a central role in T cell development and NOTCH1 mutations have now been detected not only in around 55% of all human T-ALL but also in a high fraction of murine pre-T LBL [22,23,24,27,28]. Therefore, we chose to analyse tissues from both MigR1 control and Fli-1 tumour cells for the presence of intracellular NOTCH1 protein by flow cytometry. Firstly, the amount of intracellular NOTCH1 staining seen in the Fli-1 thymus was significantly greater than the MigR1 control (Figure 6A) (p<0.05). All Fli-1 tissues examined expressed more intracellular NOTCH1 than the corresponding MigR1 control (Figure 6A). Additionally, there was at least a doubling of intracellular NOTCH1 in all preleukaemic Fli-1 thymi tested at 6 weeks post-transplant compared to MigR1 control thymi (Figure 6B).

Until recently mutations in the PEST domain were thought to be the most common Notch1 mutation in murine pre-T LBL [27]. Since then it has been discovered that 5’ Notch1 deletions, resulting in ligand-independent activation, are far more frequent [23,24]. When we sequenced the PEST domain of Fli-1 tumours, only four out of eight primary samples had mutations (Table S2). We therefore analysed MigR1 control and Fli-1 tumour cells for the presence of 5’ Notch1 deletions. It was found that all primary and secondary Fli-1 pre-T LBL as well as a radiation-induced thymic lymphoma contained type 1 5’ Notch1 deletions [23] (Figure 6C). Fli-1 transduced T cell precursors and FDC-P1 cells and Mxi1 transduced myeloid cells were all negative (Figure 6C). Additionally, sequencing of the flanking regions of the 5’ Notch1 deletions showed evidence of different RAG dependent type 1 rearrangements in each pre-T LBL including one rearrangement at a novel RSS site (Table S3) [23].

The presence of these 5’ Notch1 deletions also explains the observed upregulation of Notch1 on the mRNA level (Figure 6D), since they confer ligand independent transcriptional activation from internal sites in or close to exon 25. However, preleukaemic Fli-1 thymi also showed Notch1 mRNA upregulation (Figure 6E) in the absence of 5’ Notch1 deletions (Figure 6F), suggesting other mechanisms of Notch1 activation might play a role in the early stages of Fli-1 induced pre-T LBL.

Taken together, these results demonstrate that Fli-1 overexpression induced a pre-T LBL in mice. This malignancy was associated with upregulation of Notch1 mRNA and protein before frank leukaemia was observed. 5’ Notch1 deletions were found in all Fli-1 leukemic cells.

Discussion

Fli-1 was initially identified as an insertion site in erythroleukaemia in BALB/c mice and subsequently shown to be required for the development of megakaryocytes [1,3]. Fli-1 has also been found to control myeloid and B cell development [4,29]. However, the data presented here for the first time, demonstrate that Fli-1 also plays a role in T cell development. Fli-1 overexpression leads to a block in the DN3 to DN4 transition with a subsequent inhibition of the DN to DP transition. The Fli-1-induced DN3 arrest is consistent with endogenous Fli-1 downregulation at the DN2 stage as enforced Fli-1 expression in DN3 would increase Fli-1 levels and inhibit progression to DN4.
Fli-1 overexpression in vivo eventually causes a pre-T LBL in C57BL/6 mice, outcompeting all other lineages. Usually, it is thought an acceleration of the DN to DP transition is leukemogenic as in the case of activated Ras or Barss deficient cells [30,31,32]. However, a delay of the DN to DP transition as seen in Lek and Scl/Lmo transgenic and E2A deficient mice can also give rise to T cell lymphoma [33,34,35]. Clearly, any deregulation of this expansionary phase of T cell development driven by the pre-TCR can have dire consequences.

The detection of atypical small TCRb ISP CD8 in Fli-1 thymus, spleen, liver, lymph node and bone marrow is similar to a conditional Scl transgenic that developed T cell malignancy [36]. In that report, ISP CD8 cells were derived from DP rather than DN cells. Intriguingly, both Fli-1 and Scl are basic helix-loop-helix transcription factors that are involved in early hematopoietic specification from the hemangioblast [37].

One of the main mechanisms proposed for Fli-1 erythroblast survival in erythroleukaemia and increased B cell survival in H2-Kb Fli-1 mice is upregulation of Bel-2 [7]. Increased Bel-2 has also been shown to play a major role in a zebrafish model of pre-T LBL [38]. However, we found no evidence of Bel-2, Bel-xL or Mcl-1 mRNA upregulation in Fli-1 pre-T LBL. On the contrary, expression of all pro-survival Bel-2 family members appeared downregulated; a phenotype more reminiscent of E2A deficient lymphomas [39].

This suggested the involvement of another, possibly more T cell specific signalling pathway. We decided to focus on Notch1 since activating mutations of Notch1 are common to both human T-ALL and mouse pre-T LBL [40]. Intracellular NOTCH1 protein was indeed increased in all Fli-1 pre-T LBL cells and was associated with 5' Notch1 deletions and PEST mutations. As expected, all Fli-1 pre-T LBL also expressed high levels of Notch1 mRNA, but the elevated Notch1 mRNA level in preleukaemic Fli-1 mice was unforeseen. As Fli-1 is expressed dynamically throughout the DN to DP transition [6], ectopic Fli-1 expression may alter the balance of a number of pre-TCR transcription factors leading to ectopic signalling of a number of genes including Notch1. SCL, for example, heterodimerises with E2A and HEB and downregulates their expression [41,42]. E2A and NOTCH1 directly regulate Notch1 transcription in pre-B-selected thymocytes and Notch1 activation is one of the early events in SCL-induced leukaemogenesis [36,43]. NOTCH1 also feeds back into this pathway by inducing SCL degradation [44]. A similar role has been proposed for ETS1 and E2A in T cell development and FLI-1 and E2A in B cell development [45,46]. Alternatively, although not mutually exclusive, Fli-1 overexpression may create a preleukaemic
environment where Notch1 mutations accrue much more readily than normal [34]. We have shown that Fli-1 cells accumulate at the DN3 stage and studies have shown that the Notch1 and pre-TCR signalling pathways cooperate at this stage during T cell development and transformation [43,47]. Leukaemic transformation might happen at a later stage as evidenced by the presence of 5’ Notch1 deletions in pre-T LBL, but not in preleukaemic cells. These specific Notch1 deletions are introduced by inappropriate Rag2 dependent recombination (Table S3) and Rag2 expression is highest in small resting DPs as seen in pre-T LBL [23,48]. Indeed, Fli-1 binding sites have been found in the Notch1 promoter in haemopoietic cells [49](Table S2).

The role of NOTCH1 and NOTCH1 gain-of-function mutations in human T-ALL, (including activating deletions) as well as its use as a target in the treatment of T-ALL has been well documented [50,51,52]. There is also ample evidence for abnormal FLI-1 expression in human haematological malignancies including T cell malignancies [Figure S4, [53,54]]. We have shown that Fli-1 appears to collaborate with Notch1 to induce pre-T LBL.

It therefore could be envisaged that the synergistic use of Fli-1 inhibitors and γ-secretase inhibitors would provide a potent therapeutic combination for human T-ALL. Given that FLI-1 inhibitors have already been identified using human Ewing’s sarcoma and erythroleukaemic cell lines, the Fli-1 mouse model developed here should be informative in elucidating pathways critical for inhibiting Fli-1-induced T cell proliferation [55,56].

Supporting Information

Figure S1 Total thymocyte numbers (GFP+ and GFP−) for mice transplanted with either MigR1 control or Fli-1 analysed at 10–12 weeks post-transplant (MigR1 GFP%: 84.2, 41.1 and 85; Fli-1 GFP%: 83.4, 86.2 and 76.8). (TIF)

Figure S2 Thymic phenotypes of Fli-1 T-LBL. Thymi from 9 Fli-1 mice were analysed by flow cytometry for expression of CD4 and C8. Mouse numbers are indicated at the top of each plot. Mouse #516 is an example of a TCR- Fli-1 tumour. The remainder of the mice have TCRβ on their surface. (TIF)

Figure S3 Fli-1 spleen and bone marrow have the same phenotype as Fli-1 thymus, liver and lymph node. Fli-1 spleen and bone marrow were analysed for CD4, CD8 and TCRβ expression by flow cytometry. Data representative of 6 MigR1 mice and 13 Fli-1 mice from at least 3 independent transplants. (TIF)

Figure S4 Human FLI-1 and NOTCH1 mRNA expression levels across a large number of healthy (light grey) and pathological (dark grey) human tissues retrieved from the IST database system developed by MediSapiens Ltd. The current version (4.3) contains 20,218 human tissue and cell line samples analysed by Affimetrix gene expression microarrays. (Kiplinen et al. Genome Biol. 2008;9 (9): R139. Antio et al. BMC Bioinformatics. 2009, Jan 30; 10 Suppl 1:S24).

(TIF)

Table S1 Insertion sites in Fli-1 tumours. A. Number of insertion sites in Fli-1 tumours identified by Southern Blot and LM-PCR and cloned for sequence analysis. B. Insertion site analysis of Fli-1 integrations: position of Fli-1 integration (≤: insertion downstream or >: upstream of indicated position) and genes identified in or near the integration site. (DOC)

Table S2 Notch1 PEST mutations. The PEST domain of murine Notch1 was amplified from genomic DNA isolated from total thymus or spleen and gel purified and sequenced. 1 position in GenBank sequence no. AL735411. RTIL: Radiation Induced Thymic Lymphoma. (DOC)

Table S3 5’ Notch1 deletion sequences. Rearrangements in Notch1 deduced from sequencing of PCR products shown in figure 6C. RAG dependent recombination occurred between the −8191 RSS and the +3575 RSS described in reference 24 or a newly identified −7090 RSS and the +3575 RSS. The −7090 RSS was determined using the Recombination Signal Sequence Site http://www.itb.cnr.it/rss/. (RIC score of −45.2 compared to −41.2 for the −8191 RSS and −66.3 for the +3575 RSS); RSS: cryptic RAG signal sequences and their location relative to the position of the ATG start codon in exon 1 of Notch1. GL: sequence of the germ line DNA flanking the breakpoints. 1 2: Indicate two different clones within the same tumour. (DOC)

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Author Contributions

Conceived and designed the experiments: DJI MS. Performed the experiments: DJI MS AC SD CB. Analyzed the data: DJI MS AC SD CB. Contributed reagents/materials/analysis tools: CB. Wrote the paper: DJI MS AC SD CB AW.

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