NFAT transcription factors are essential and redundant actors for leukemia initiating potential in T-cell acute lymphoblastic leukemia

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy with few available targeted therapies. We previously reported that the phosphatase calcineurin (Cn) is required for LIC (leukemia Initiating Capacity) potential of T-ALL pointing to Cn as an interesting therapeutic target. Calcineurin inhibitors have however unwanted side effect. NFAT transcription factors play crucial roles downstream of calcineurin during thymocyte development, T cell differentiation, activation and anergy. Here we elucidate NFAT functional relevance in T-ALL. Using murine T-ALL models in which Nfat genes can be inactivated either singly or in combination, we show that NFATs are required for T-ALL LIC potential and essential to survival, proliferation and migration of T-ALL cells. We also demonstrate that Nfat genes are functionally redundant in T-ALL and identified a node of genes commonly deregulated upon Cn or NFAT inactivation, which may serve as future candidate targets for T-ALL.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of T-cell progenitors that represents about 15% of pediatric and 25% of adult acute lymphoblastic leukemia cases. Genome-wide transcriptional profiling analysis enabled to classify T-ALL into different molecular subgroups characterized by abnormal expression of several transcription factors including TAL1/2, LMO1/2, TLX1/3, NXX2.1/2.2, HOXA, as the result of genetic rearrangements or other modes of deregulation (for review [1]). Another T-ALL subgroup encompasses cases characterized by a transcriptional signature resembling that of early T cell progenitors (ETP subgroup). Across these subgroups, a number of additional, recurrent alterations are found in
tumor suppressor genes/loci, including CDKN2A or PTEN and in oncogenes, most notably NOTCH1, which harbors functionally relevant activating mutations in the majority of T-ALL cases [2, 3].

Besides genetic and epigenetic oncogenic cues, T-ALL development also depends upon specific micro-environmental signals (for review [4]). Recent evidence has shown that bone marrow (BM)-stroma produced CXCL12 acting through its CXCR4 receptor on T-ALL cells is essential to leukemia development and initiating potential [5, 6]. We notably found that cell-surface expression of CXCR4 in T-ALL depends upon the activation of calcineurin (PPP3, named Cn thereafter) [6], a calcium-dependent phosphatase that we previously showed to be critical to T-ALL cell survival, proliferation, migratory activity and leukemia initiating potential using both pharmacological and genetic approaches[7, 8]. However, these studies also showed that restoring normal CXCR4 cell surface expression in Cn-deficient T-ALL failed to correct their impaired leukemia initiating potential [6], indicating the existence of other Cn effectors critical to T-ALL biology.

NFAT transcription factors are important effectors of calcium/calcineurin signaling in normal T cell development and in many aspects of mature T cell functions (for review, [9]). NFAT factors are composed of a DNA binding domain structurally related to that of the REL/NFkB family protein, a regulatory domain in which 12–14 serine residues located in specific regions (SRR1; SP1-SP3) are targeted for phosphorylation and N- and C-terminal activation domains [9]. In unstimulated cells, NFAT proteins are hyperphosphorylated in their SRR/SP motifs by the cooperative action of several protein kinases, including glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1) and dual specificity tyrosine phosphorylation-regulated kinases (DYRK) [9] and are sequestered as a supramolecular cytoplasmic complex [10]. Stimuli increasing calcium concentration and resulting in calcineurin activation lead to dephosphorylation of NFAT factors, resulting in their nuclear translocation and transcriptional activity [9].

Three members of the Nfat gene family, namely Nfat1 (Nfatc2), Nfat2 (Nfatc1) and Nfat4 (Nfatc3) are expressed in the T cell lineage and regulate many aspects of T cell functions. Although the picture is far from being complete, biochemical and gene inactivation studies have enlightened both specific, redundant or antagonistic functions of these genes in thymocyte development and mature T cell functions [11–17].

In this study, we show that Nfat1, Nfat2 and Nfat4 are critical effectors of calcineurin in T-ALL that act mostly in a redundant fashion in regulating leukemia initiating potential and the expression of several genes/pathways that could mediate the pro-oncogenic properties of Cn/NFAT activation.

Results

NFAT transcription factors are required for T-ALL leukemia initiating potential

We found that constitutive deletion of the Nfat1 gene did not affect T-ALL onset in both the transgenic TEL-JAK2 and in the BM transplantation model of ICN1-induced T-ALL (S1 Fig), suggesting either no function or a redundant of Nfat genes in T-ALL. We thus generated Nfat1-deficient ICN1-driven T-ALLs carrying in addition conditional, floxed alleles of Nfat2 and Nfat4 and the Rosa-Cre-ERT2 transgene (RC T2) (see Material and Methods) to compare the leukemia initiating potential (LIC activity) of NFAT-proficient and NFAT-deficient T-ALL cells. Three independent primary T-ALL (#21; #23; #24) of ICN1; RC2; Nfat1"; Nfat2 "; Nfat4" genotype were generated. To produce the Nfat-proficient and Nfat-deficient versions of each of these T-ALL, leukemic cells were injected into wild type secondary wild type recipient mice and sub-terminally leukemic mice were treated either with carrier solvent (So) or with
tamoxifen (Tam) to induce Cre-mediated deletion of Nfat2 and Nfat4 (see Fig 1A for a scheme of the experiment). Three successive, daily treatment with Tamoxifen were required to obtain efficient deletion of the Nfat4 floxed alleles (Fig 1B) associated with undetectable NFAT4 protein expression (Fig 1C) and a clear but incomplete deletion of Nfat2 accompanied by a strong decrease in NFAT2 protein isoforms expression (Fig 1B and 1C). In this experimental setting in which loss of NFAT expression was experienced by leukemic cells for about 2 days (see Methods), we observed no effect on tumor burden and leukemic cell survival (S2A and S2B Fig). To compare the leukemia inducing potential of the Nfat-deficient and Nfat-proficient versions of each of these T-ALL, these cells were transplanted under limit dilution conditions into new hosts and leukemia recurrence and mouse survival monitored over time (Fig 1A for a scheme). While 10^4 (T-ALL #21) or 10^3 (T-ALL #23; #24) Nfat-proficient cells were sufficient to re-initiate leukemia in all recipients, none (T-ALL #21, #24) or only 1/6 recipient mice (T-ALL #23) infused with the same number of Nfat-deficient T-ALL cells succumbed to leukemia (Fig 1D and Table 1). The differential phenotype between Nfat-proficient and Nfat-deficient leukemic cells did not result from a non-specific, toxic effect of tamoxifen treatment or Cre activation since the leukemia initiating potential of ICN1-driven T-ALL that are wild type for all 3 Nfat genes and that carry the RC^T2 transgene was unaffected by Tam treatment (S3 Fig; S1 Table). These results demonstrate that inactivation of NFAT function impairs the leukemia initiating potential of T-ALL cells.

We next investigated whether NFAT transcription factors were intrinsically involved in survival, proliferation and migration of leukemic cells. ICN1; RC^T2; Nfat1^-/-; Nfat2^fl/del; Nfat4^fl/fl T-ALL cells were co-cultivated in vitro with MS5 cells as MS5 stromal cells are required for survival and proliferation of ICN1 T-ALL cells ex vivo [8] and treated either with 4-hydroxytamoxifen (4OHT) to induce deletion of Nfat2 and Nfat4 or with the carrier solvent (Et), as control [6, 8]. Nfat inactivation (Fig 2A) was accompanied by impaired S phase progression as measured by BrdU pulse-labeling (Fig 2B), increased apoptosis as analyzed by caspase 3 activation (Fig 2C) and decreased cell migration as determined by time lapse videomicroscopy (Fig 2D). In contrast, ICN1; RC^T2; Nfat1^+/+; Nfat2^+/+; Nfat4^+/+ T-ALL cell survival and migration were not affected by 4OHT treatment (S4 Fig), showing the specificity of the observed phenotypes.

NFAT transcription factors have redundant functions in T-ALL

Given their redundant, agonistic or antagonistic roles during T cell development, we next investigated whether expression of individual members of the NFAT family would be sufficient to sustain leukemia initiating potential of T-ALL cells. To this end, we generated ICN1-driven tumors in which only Nfat4 (ICN1; RC^T2; Nfat1^-/-; Nfat2^fl/del; Nfat4^+/+) or only Nfat2 (ICN1; RC^T2; Nfat1^-/-; Nfat2^+/+; Nfat4^fl/fl) will remain expressed following genetic inactivation of the other family members following Tam treatment and analyzed the leukemia initiating potential of these cells (See Fig 3A for a scheme of the experiment). When injected under limiting dilution conditions, the leukemia initiating potential of T-ALL cells expressing only Nfat4 (Fig 3B, left, “input” panel) was comparable to that of cells expressing both Nfat2 and Nfat4 (Fig 3C, left panel, compare red and blue tracings; Table 2). Of note, T-ALL cells recovered from terminally leukemic recipient mice injected with RC^T2; Nfat1^-/-; Nfat2^del/del; Nfat4^+/+ T-ALL cells kept their original Nfat genotypes (Fig 3B, left, “recovered” panel), indicating that the mere expression of NFAT4 is sufficient to maintain the leukemia initiating potential of T-ALL. Likewise, recipient mice injected under limiting dilution conditions with leukemic cells expressing only Nfat2 (Fig 3B, right “input” panels) all succumbed to T-ALL, although with a slight delay as compared to mice infused with their respective control (Fig 3C, right panel, compare red and blue tracings; Table 2).
Fig 1. NFAT factors are required for leukemic initiating cell potential in T-ALL. (A) Schematic description of the experiment: Mice were i.v. injected with ICN1-induced leukemic cells obtained from 3 independent T-ALL (#21, #23, #24) of the indicated genotypes. When BM leukemia burden reached about 10–15% leukemic cells in these recipients, mice received 3 successive daily injection of either carrier solvent (So, n = 3) or tamoxifen (TAM, n = 6) to induce Nfat2 and Nfat4 floxed alleles deletion. Mice from both groups became terminally ill 2 days later and were sacrificed. Nfat-proficient (blue label, 1) and Nfat-deficient (red label, 2) leukemic cells were flow cytometry-sorted (tNGFR + cells), genotyped for Nfat floxed (flox) and deleted (del) alleles and compared for their ability to re-initiate leukemia in wild-type secondary
NFAT transcription factors are essentials in T-ALL

recipient mice under limit dilution conditions. N1: Nfat1; N2: Nfat2; N4: Nfat4; RC\textsuperscript{T2}: Rosa-Cre-ER\textsuperscript{T2} transgene; ICN1: intracellular NOTCH1; LIC: leukemia initiating cell. (B) PCR-based genotyping for Nfat2 and Nfat4 floxed and deleted alleles in tNGFR+ T-ALL cells obtained from mice treated with So (1) or Tam (2), as schematized in panel A. (C) Western blot analysis of NFAT2 and NFAT4 expression in leukemic cells of mice treated with So (1) or Tam (2), as schematized in panel A. SAM68 and STAT5 expression are used as loading controls. Arrowheads indicate the A, B and C NFAT2 isoforms. (D) Kaplan-Meier survival curve of mice infused with 1x10\textsuperscript{4} (T-ALL #21) or 1x10\textsuperscript{3} (T-ALL #23; 24) Nfat-proficient (blue tracing) or Nfat-deficient (red tracing) cells as described in (A). Mice were followed overtime for T-ALL recurrence and recipient mice survival (n = 3–6; log-rank test).

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"recovered" panel compare red and blue tracings; Table 2). Leukemic cells recovered from terminally-leukemic recipients injected with RC\textsuperscript{T2}; Nfat1\textsuperscript{−/−}; Nfat2\textsuperscript{+/+}; Nfat4\textsuperscript{del/del} T-ALL kept their original Nfat genotypes (Fig 3B; right panels), showing that LIC activity was effectively driven by NFAT2. Taken together, these experiments show that NFAT factors play an essential and redundant role in the leukemia initiating potential of T-ALL cells.

NFAT factors act downstream of Cn

Since NFAT deficiency essentially phenocopies Cn deficiency in T-ALL [8], we hypothesized that NFAT may function downstream of Cn in T cell leukemogenesis. To investigate this hypothesis, we analyzed whether expression of constitutively active NFAT mutants could compensate the cellular phenotypes linked to Cn inactivation. We used the constitutive mutant NFAT1\textsuperscript{2+5+8} [18] (named NFAT1\textsuperscript{−} thereafter) in which most serine residues targeted by NFAT kinases in the SRR1, SP2 and SP3 motifs were mutated into alanine, thus mimicking calcineurin-induced dephosphorylation and the same mutant carrying in addition a SV40 nuclear localization signal (NLS) motif at its C-terminus (NFAT1\textsuperscript{−}-NLS), further enhancing its nuclear accumulation and transcriptional activity [18]. ICN1; RC\textsuperscript{T2}; CnB1\textsuperscript{fl/fl} T-ALL cells [8], recipient mice under limit dilution conditions.

Table 1. Comparison of the leukemia initiating potential of Nfat-proficient and Nfat-deficient versions of T-ALL #21, #23, and #24, generated as schematized in Fig 1A.

<table>
<thead>
<tr>
<th>Leukemia id</th>
<th>Treatment of donor mice</th>
<th>Number of cells injected in recipients</th>
<th>Number of injected recipients</th>
<th>Number of leukemic recipients (time to death, days)</th>
<th>Statistics</th>
<th>% leukemic cells in the BM ± SEM of recipients</th>
</tr>
</thead>
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<td>#21</td>
<td>So 4.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (13;13;13)</td>
<td>p = 0.025</td>
<td>63.9±6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAM 4.10\textsuperscript{6}</td>
<td>3</td>
<td>3* (17;17;17)</td>
<td></td>
<td>64.9±2.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>So 1.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (22;39;38)</td>
<td></td>
<td>46.2±13.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAM 1.10\textsuperscript{6}</td>
<td>6</td>
<td>0</td>
<td>p = 0.004</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>So 1.10\textsuperscript{6}</td>
<td>3</td>
<td>1 (21)</td>
<td></td>
<td>65.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAM 1.10\textsuperscript{6}</td>
<td>6</td>
<td>0</td>
<td>ns</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>#23</td>
<td>So 4.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (13;14;13)</td>
<td></td>
<td>47.6±2.9</td>
<td></td>
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<tr>
<td></td>
<td>TAM 4.10\textsuperscript{6}</td>
<td>5</td>
<td>5* (20;20;25;22;29)</td>
<td>p = 0.004</td>
<td>42.9±3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>So 1.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (14;15;18)</td>
<td></td>
<td>38.1±11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAM 1.10\textsuperscript{6}</td>
<td>6</td>
<td>6* (22,25,25,25,29;52)</td>
<td>p = 0.001</td>
<td>37.6±13</td>
<td></td>
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<tr>
<td></td>
<td>So 1.10\textsuperscript{6}</td>
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<td>3 (18;15;18)</td>
<td></td>
<td>36.2±9.1</td>
<td></td>
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<tr>
<td></td>
<td>TAM 1.10\textsuperscript{6}</td>
<td>6</td>
<td>1* (34)</td>
<td>p = 0.006</td>
<td>49.3</td>
<td></td>
</tr>
<tr>
<td>#24</td>
<td>So 4.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (15;15;15)</td>
<td></td>
<td>58.8±5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAM 4.10\textsuperscript{6}</td>
<td>6</td>
<td>6* (17;17;18;18;18;23)</td>
<td>p = 0.004</td>
<td>42.4±5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>So 1.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (15;15;15)</td>
<td></td>
<td>31.3±3</td>
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<tr>
<td></td>
<td>TAM 1.10\textsuperscript{6}</td>
<td>6</td>
<td>2* (18;37)</td>
<td>p = 0.004</td>
<td>39.4±8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>So 1.10\textsuperscript{6}</td>
<td>3</td>
<td>3 (15;15;16)</td>
<td></td>
<td>9±1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAM 1.10\textsuperscript{6}</td>
<td>6</td>
<td>0</td>
<td>p = 0.003</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Asterisks indicate that leukemic cells recovered from these mice showed NFAT2\textsuperscript{fl/del} and NFAT4\textsuperscript{fl/fl} genotypes indicating that mice succumbed because of the survival and expansion of a minor population of leukemic cells that escaped full NFAT2 and NFAT4 gene deletion that were undetectable in the input.

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were retrovirally transduced at the same m.o.i. either with the GFP control vector (MIG), or MIG vectors encoding HA-tagged version of either CnB1, or with the constitutive NFAT1 mutants and concomitantly treated with 4OHT to induce deletion of Nfat floxed alleles. Leukemic cells genotypes were analyzed 5 days later by PCR for the floxed (flox) and deleted (del) alleles of Nfat4 and the floxed allele of Nfat2. PCR for Hsp70 is used as control. As shown in Fig 4B and as previously reported, 4-OHT treatment induced efficient CnB1 (PPP3R1) gene deletion and resulted in a strong arrest in T-ALL expansion (Fig 4C), with the few cells found under these co-culture conditions resulting from the survival of a minor population of cells that escaped full CnB1 gene deletion, detectable by PCR (Fig 4D, MIG lanes). As expected, expression of

Fig 2. NFAT transcription factors regulate cell survival, proliferation and migration of ICN1-induced T-ALL in vitro. (A) T-ALL #21 leukemic cells were co-cultured on MS5 stromal cells and treated with solvent (ethanol, Et) or 4OHT to induce deletion of Nfat floxed alleles. Leukemic cells genotypes were analyzed 5 days later by PCR for the floxed (flox) and deleted (del) alleles of Nfat1 and the floxed allele of Nfat2. PCR for Hsp70 is used as control. (B) BrdU pulse-labeling analysis Nfat-proficient and Nfat-deficient 5 days after ethanol or 4OHT treatment, respectively. Percentage BrdU-positive leukemic cells is presented (data are represented as ±SEM; n = 3; Student’s t-test; *** p < 0.001). (C) At the same time point, Nfat-proficient (black) and Nfat-deficient (white) leukemic cells were analyzed for percentage of cells positive for cleaved caspase 3 (data are represented as mean ± SEM; n = 3; Student’s t-test; *** p < 0.001). (D) At the same time point, Nfat-proficient and Nfat-deficient leukemic cells were seeded on MS5 stromal cells and migration of individual cells (n = 30) recorded for 15 minutes by time-lapse videomicroscopy. In the flower plot diagrams (left), the starting point of each track is placed at the axis origins. In the right panel, velocity (μm/min) of Nfat-proficient (Et) versus Nfat-deficient (4OHT) leukemic cell was compared (data are represented as mean ± SEM; n = 3; Student’s t-test *** p < 0.001).

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Fig 3. Functional redundancy of NFAT factors in T-ALL. (A) Schematic description of the experiment: mice were injected with leukemic cells obtained from either primary T-ALL #68 (ICN1; RCT2; Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\)) or #14 (ICN1; RCT2; Nfat1\(^{-/-}\); Nfat2\(^{+/+}\); Nfat4\(^{+/+}\)).

(B) Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\) #68

(C) Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\)

T-ALL #68

10\(^3\) cells/mouse

Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\)

Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\)

T-ALL #14

10\(^4\) cells/mouse

Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\)

Nfat1\(^{-/-}\); Nfat2\(^{fl/fl}\); Nfat4\(^{+/+}\)
NFAT transcription factors are essentials in T-ALL

NFAT transcrip tion factors are essentials in T-ALL

Table 2. Comparison of the leukemia initiating potential of Nfat-floxed and Nfat-defloxed versions of T-ALL #68 and T-ALL #14, generated as described in Fig 3A.

<table>
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<tr>
<th>Leukemia id</th>
<th>Treatment of donor mice</th>
<th>Number of cells injected in recipients</th>
<th>Number of injected recipients</th>
<th>Number of engrafted recipients (time to death, days)</th>
<th>Statistics</th>
<th>% leukemic cells in the BM ± SEM of recipients</th>
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</thead>
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<tr>
<td>N1+/−;N2+/−;N4+/+</td>
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<td>1.10^6</td>
<td>3</td>
<td>3 (10;10;10)</td>
<td>ns</td>
<td>72.7±3.6</td>
</tr>
<tr>
<td>TAM</td>
<td>1.10^6</td>
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<td>6 (18;18;20;20;21;21)</td>
<td>p = 0.005</td>
<td>62.2±12.0</td>
<td></td>
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<tr>
<td></td>
<td>So</td>
<td>1.10^6</td>
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<td>6 (26;26;26;27;28;31)</td>
<td>p = 0.005</td>
<td>61.4±10.4</td>
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<tr>
<td>N2+/−;N4+/−;N1+/−</td>
<td>So</td>
<td>1.10^6</td>
<td>3</td>
<td>3 (17;17;17)</td>
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<td>72.7±3.6</td>
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<td>6 (18;18;20;20;21;21)</td>
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<td>1.10^6</td>
<td>3</td>
<td>3 (20;20;20)</td>
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<td>TAM</td>
<td>1.10^6</td>
<td>6</td>
<td>6 (26;26;26;27;28;31)</td>
<td>p = 0.005</td>
<td>61.4±10.4</td>
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<td>N1−/−;N2+/−;N4+/+</td>
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initiating potential. To gain insight into the molecular basis of NFAT oncogenic properties, we compared the transcriptome of NFAT-proficient and NFAT-deficient leukemic cells, obtained as described in Fig 1A, using 3 independent ICN1; RC[T2]; NFAT1^+/−; NFAT2^+/−; NFAT4^+/− T-ALL. Hierarchical clustering analysis clearly distinguished NFAT-proficient from NFAT-deficient cells (Fig 5A) with 343 probe sets being significantly deregulated (FC>1.5 and p<0.05, S2 Table). IPA analysis of this signature did not highlight deregulation of a specific pathway. However, we noticed the up-regulation of a number of genes encoding proteins that are either physiological regulators in T cells, e.g. B- and T-cell attenuator (Btla), an inhibitory receptor of T cell signaling belonging to the CD28 superfamily; Gimap7, a member of the immunity associated
NFAT transcription factors are essentials in T-ALL

Figure A: Heatmap showing gene expression patterns for So and Tam conditions.

Figure B: GO Processes analysis with UP (81%), DOWN (20%), and ALL (67%) categories.

Figure C: Network diagram highlighting genes like Tafsd10, Nra4a1, Gadd45b, Ikr2, and Cdk1a.

Figure D: Western blot analysis for Cdkn1a, Nra4a1, and Actin in T-ALL #21, #23, and #24.

Figure E: Graph showing percentage of annexin/positive cells among GFP+ leukemic cells with statistical significance P<0.0001.

Figure F: Cumulative cell number over days for MIG and MIG-CDKN1A.

Figure G: Percentage of GFP positive cells among leukemic cells over days.
NFAT transcription factors are essentials in T-ALL.

GTases, regulators of lymphocyte survival and homeostasis; Nr4a1, a nuclear protein involved in thymocyte clonal deletion during negative selection; Irf4, a transcriptional regulator involved in multiple aspects of T cell development. Nfat inactivation also deregulated genes implicated in cell cycle regulation (e.g. up-regulation of Cdkn1A, Gadd45b; down-regulation of Evi5) (S2 Table). Of note, comparison of the NFAT-dependent transcriptome with that regulated by calcineurin in ICN1 T-ALL [8] identified 96 commonly deregulated probe sets (S3 Table). Gene enrichment analysis revealed a robust overlap between the calcineurin and NFAT-dependent transcriptome, particularly amongst the upregulated processes (Fig 5B; S3 Table). We identified a node of particular interest, accounting for proteins implicated in the regulation of cell proliferation and survival (Fig 5C). RT-PCR experiments performed in independent T-ALL confirmed that both Cdkn1a and Nr4a1 were up-regulated in response to either NFAT or CnB-1 inactivation (Fig 5D), validating at the molecular level the resemblance of CnB1 and NFAT loss of function phenotypes.

To gain insight into the functional relevance of this deregulated node, we next investigated the consequences of Cdkn1a overexpression on T-ALL expansion. For this, leukemic cells were retrovirally-transduced with the MIG control vector or MIG vector encoding p21\textsuperscript{CDKN1a}. Western blot analysis confirmed p21\textsuperscript{CDKN1a} overexpression as compared to MIG–transduced cells (S5 Fig). Enforced expression of p21\textsuperscript{CDKN1a} resulted in induction of apoptosis and impaired leukemic cell expansion in MS5 co-cultures (Fig 5E and 5F), with transduced cells (GFP+) rapidly being counter-selected (Fig 5G). Taken together, these results indicate an essential role of NFAT-regulated genes in orchestrating important leukemic phenotypes in T-ALL.

**Discussion**

We previously demonstrated through pharmacological and genetic approaches that calcineurin is important to T-ALL maintenance in vivo and ex vivo and critical to their leukemia propagating potential [7, 8]. We now demonstrate that the three NFAT factors activated in T-ALL, namely NFAT1, 2 and 4 are also essential to the survival/proliferation/migration properties and leukemia propagating potential of T-ALL. We found that only the concomitant inactivation of all three NFAT factors results in impaired LIC activity in T-ALL, as evidenced by the fact that expression of either NFAT2 alone or NFAT4 alone was sufficient to maintain this activity. This demonstrates clear functional redundancy for these factors in T-ALL. Since expression of a constitutive mutant of NFAT1 that mimics NFAT1 dephosphorylated, active state restores the survival and proliferation properties of calcineurin-deficient leukemic cells, our results highlight the central function of NFAT downstream of calcineurin activation in T-ALL.

The redundant function of NFAT factors in T-ALL contrasts with previous studies interrogating of NFAT involvement in other oncogenic settings. For example, while several reports
have shown that constitutively activated mutant of the isoform of NFAT2 (the so-called short NFAT2 isoform) transforms mouse 3T3L1 adipocytes and NIH3T3 fibroblasts in vitro [19–21], a constitutively activated phosphorylation mutant of NFAT1 (NFAT1* NLS in the present study) was unable to recapitulate those effects, and even inhibited cell transformation by constitutively active NFAT2 or the Ha-RAS oncprotein [21]. Likewise, NFAT1 ex vivo studies have suggested that NFAT1 has a non-redundant function in the invasive properties of breast carcinoma cell lines [22] while, in mice, the tumorigenic/metastatic potential of mammary tumor cells rather depends upon non redundant functions of NFAT1 and NFAT2 [23]. At the molecular level, NFAT2, but not NFAT1, is recruited to the c-MYC gene promoter leading to c-MYC over-expression, which is required for tumor maintenance together with the deregulation of other survival genes in aggressive B cell lymphomas [24]. The Nfat1 gene restrains the proliferation of naive T cells in vivo [25, 26] and exerts tumor suppressive functions in the mouse B cell lineage [27]. Yet, our data show that loss of NFAT1 function does not affect leukemia onset and outcome in T-ALL induced in mice by activated NOTCH1 (ICN1) or activated JAK2 (TEL/ETV6-JAK2), indicating lack of tumor suppressive function of Nfat1 in early T cell progenitors. Taken together, this indicates that the pro-oncogenic role of NFAT factors and their relative importance strongly depend upon the biological system studied.

Ca²⁺ signaling is essential in vivo for T-ALL as demonstrated by increased survival of mice injected with STIM1/2-deficient [28] or mice infused with CnB1-deficient T-ALL cells [8] as compared to their control counterparts. In the case of STIM1/2-deficient cells, the increased survival of mice was associated with the defective ability of T-ALL cells to activate cancer-induced inflammation. However, expression of neither pro-inflammatory cytokines (TNFa, IL-10, IL-16, IL32, CSF-1, CCL6, 9, CXCL9, 10) nor IFN-response genes (IRF1, 2, 7, 8, 9) identified as down-regulated in STIM1/2-deficient T-ALL [28] were found modulated in NFAT-deficient cells. In contrast, comparison of the NFAT-dependent transcriptome with that associated with calcineurin inactivation in ICN1-induced T-ALL [8] identifies a common signature, with 67% of NFAT regulated genes being also Cn-regulated. This Cn/NFAT signature in T-ALL differs from the NFAT-dependent signature characteristic of the TCR-dependent activation of peripheral T cells as no difference in expression in e.g. the genes encoding IL2, IL3, IL4, IL5, IL13, IFNg, GM-CSF was found upon CnB1 or Nfat deletion in T-ALL cells. Besides T cell differentiation and activation, NFAT is also involved in exhaustion of activated T cells to limit or constrain the immune response [16, 29]. Although a trend was observed for inhibition of the expression of Pdcd1, Lag-3 and Cta4 (genes encoding receptors involved in NFAT-mediated exhaustion) in Nfat-deficient T-ALL, their differential expression in our global transcriptomic analyses did not reach statistical significance.

Instead, we found NFAT to impact T-ALL maintenance through deregulation of genes with demonstrated inhibitory properties on survival, cell cycle progression of normal T cell progenitors. Cdkn1a, a gene recurrently altered in T-ALL diagnostic samples through promoter methylation [30] and Nr4a1, a gene involved in clonal deletion of self-reactive T cells during thymic negative selection and in activated T cell exhaustion [31, 32] are up-regulated upon either CnB1 or Nfat inactivation and contribute to ICN1 T-ALL defective LIC properties. Available evidence indicates that these genes can be regulated by NFAT factors independently of their binding to specific DNA sequences but rather through protein–protein interactions with other transcription factors[33, 34]. We also found Cn/NFAT-dependent expression of Tox in ICN1-induced T-ALL. Tox encodes a protein that facilitates genomic instability in T-ALL and essential for T-ALL cell lines survival/ proliferation and for in vivo maintenance [35]. Because constitutive expression of CDKN1a (this study), or TOX knockdown [35] are sufficient to partially mimic the deleterious phenotypes induced upon NFAT or calcineurin
deletion, this suggests that the Cn and NFAT commonly deregulated genes are central mediators of the pro-oncogenic properties of Cn/NFAT pathway.

Besides these transcriptionally regulated candidates, we also identified CXCR4 cell surface expression being commonly modulated by Cn and NFAT. Since NFAT-deficient cells also present reduced CXCR4 cell surface expression level (S6 Fig), this defect could explain the migration default and impaired leukemia inducing potential as demonstrated for Cn-deficient leukemic cells [6], reinforcing a major role for Cn/NFAT axis in T-ALL.

Not surprisingly, we found the number of genes regulated by calcineurin [8] to be broader than that dependent upon NFAT (this study), indicating that in addition to NFAT, calcineurin likely acts through other effectors to enforce leukemia inducing potential in T-ALL. In line with this hypothesis, several new Cn interacting proteins were recently identified, the inactivation of which could synergize with Cn inhibition to impair T-ALL expansion [36, 37]. We also noticed a number of genes specifically deregulated upon Nfat deletion, but not upon Cnb1 deletion, indicating that NFAT activity can be critically regulated by upstream signaling pathways in addition to their Cn-mediated nuclear translocation. In normal early T cell progenitors, the IL7/IL7R/JAK3 signaling pathway directly regulates NFAT2 through phosphorylation on a tyrosine residue in its regulatory domain [11]. Moreover, NFAT2 activity was also identified as a target of PIM kinases independent of calcineurin activation [38]. High PIM1 expression is a biomarker in T-ALL cases with JAK/STAT activation and response of leukemic cells to endogenous IL7 [39, 40], with PIM targeting cooperating with chemotherapy to promote leukemic mice survival in T-ALL PDXs [40]. Nevertheless, we did not find NFAT activity to be modulated by IL7/IL2 in T-ALL cells (S7 Fig), leaving space to speculate on new NFAT regulators in this context.

While T-ALL is a highly heterogeneous disease, patient treatment relies mostly on general chemotherapeutic regimens with 20% and 50% of pediatric and adult cases relapsing respectively. The demonstration that Cn/NFAT signaling directly contributes to T-ALL LIC activity has important clinical implications, as new therapeutic developments against leukemia mostly rely on molecular targets of LIC cells, which are important to treatment resistance. Targeting calcineurin using CsA or FK506 has been investigated as therapeutic option in T-ALL mouse models [7]. Yet these drugs are immunosuppressive and might therefore affect the T-cell mediated, poorly documented anti-T-ALL response. Linked to its immunosuppressive properties, CsA and FK506 are associated in transplanted human patients with induction of secondary cancer [41] and ill-characterized off-target effects. Inhibitors of NFAT have been developed (INCA1, 2 and 6, JapA, MA242, compound 10), that act by preventing Cn/NFAT interaction [42], promoting NFAT degradation [43] or interfering with NFAT binding partners [44], thus being more selective compared with blocking calcineurin activity directly. Specificity of these inhibitors remains to be established in vivo. It would be interesting to analyze in the future whether these or other compounds represent valid therapeutic alternatives to overcome treatment limitations in T-ALL.

Materials and methods

Analysis of T-all mouse models

Mice carrying null (-) alleles of Nfat1 [25], the floxed (flox) and deleted (del) alleles of Nfat2 (a generous gift of Dr. A. Rao) and Nfat4 [12] (a generous gift of Dr. GR Crabtree) and the Rosa-Cre-ER T2 (RC T2) transgene [45] were maintained on a C57BL/6 genetic background. Mice were crossed to generate the following compound mice: RC T2; Nfat1-/-; Nfat2flox/del; Nfat4flox/flox, or RC T2; Nfat1-/-; Nfat2flox/flox; Nfat4+/+, or RC T2; Nfat1-/-; Nfat2+/+; Nfat4flox/flox. Primary ICN1 (intracellular NOTCH1 domain)-induced T-ALL were obtained following
retroviral-mediated gene transfer of bone marrow (BM) cells from the respective mice using either MIN-ICN1 or MIG-ICN1, which also encode either a truncated human NGFR from an IRES-tNGFR cassette or eGFP, respectively, as described [8]. T-ALL of the different genotypes emerged within 4–6 weeks and leukemic cells (tNGFR⁺ or GFP⁺) were collected from BM for further studies. To study Nfat function in T-ALL, leukemic cells of the indicated genotypes were intravenously (i.v) infused into secondary wild-type mice, conditions that result in synchronous leukemia engraftment in recipients. Engraftment was followed by sacrificing mice at regular time intervals and measuring % of tNGFR⁺ (GFP⁺) leukemic cells in BM. Cre activation was induced in leukemic cells by Tamoxifen (Tam) administration (Sigma-Aldrich; 1mg/mouse, three times at 24 hours intervals). Carrier solvent (corn oil, So) was used as control. Unless otherwise stated, Tam administration was started when leukemic burden reached 10–15% T-ALL cells in BM (usually 10–14 days after leukemic cells infusion). In these conditions, Cre-mediated loss of NFATs activity is experienced by leukemic cells 2 days later [8]. Mice were sacrificed when becoming moribund (5 days after the start of treatment). To study NFAT factors function in T-ALL leukemia initiating potential, NFAT-proficient and NFAT-deficient leukemic cells obtained from carrier solvent or Tam-treated mice were injected i.v at different doses (from 10⁶ to 10³ leukemic cells/mouse) into wild-type, syngeneic recipient mice. Time to death and leukemia burden, as analyzed by flow cytometry as % FSC large/tNGFR⁺ (or GFP⁺) leukemic blasts in BM, spleen and liver, were recorded. Mice were maintained under specific pathogen-free conditions in the animal facility of the Institut Curie. Experiments were carried out in accordance with the European Union and French National Committee recommendations, under agreement APAFIS #7393-2016102810475144-v1, which specifically approved this study.

**Cell culture**

MS5 (mouse) bone marrow-derived stromal cells [46] were maintained in αMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen). NIH-3T3 (mouse) cells were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated donor bovine serum (Invitrogen). 293T and PlatE cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen). Leukemic cells were co-cultivated in RPMI (Invitrogen) supplemented with 15% FBS, IL2 and IL7 (10ng/ml; Peprotech). In vitro activation of RC₉₂ was performed by a 24h pulse treatment with 4-hydroxytamoxifen (4OHT, 1µM, Sigma Aldrich).

**Retroviral-mediated gene transfer**

The cDNAs encoding CnB1 (PPP3R1) [8], and the constitutively active mutant NFAT1 [2+5+8] and NFAT1 [2+5+8]-NLS (a generous gift of Dr A Rao) [18], were subcloned into MigR1, allowing their co-expression with GFP. Retroviral stocks were obtained by transfection of the PlatE packaging cell line with the desired retroviral vector using Lipofectamine, following manufacturer’s instructions (Lipofectamine 2000, Invitrogen). After 48 hours, the retroviral supernatant was collected and titrated on NIH3T3 cells. tNGFR⁺ ICN1 leukemic cells were spin-infected (1800g for 2 h at 30˚C) at the same multiplicity of infection in the presence of 4µg/ml polybrene (Sigma-Aldrich). Following infection, leukemic cells were co-cultured on MS5 for 48–72 hours before flow cytometry sorting or immediately infused into syngeneic mice (1x10⁶ cells/mouse) and expanded in vivo for further studies.

**PCR genotyping, RNA extraction and RT-PCR analyses**

Detection of the different alleles of Nfat1, Nfat2 and Nfat4 in genomic DNA of compound mice and leukemic cells was by PCR, using the following primers:
Total RNA was extracted using the RNeasy kit (QIAGEN) and reverse transcribed using random primers and the kit ImProm II Reverse Transcription System (Promega) according to the manufacturer’s instructions. The following primers were used for RT-PCR:

- **NFAT2**
  - Flox/+:
    - Forward: 5’-CCA TCT CTC TGA CCA ACA GAA GCC AGC-3’
    - Reverse: 5’-CCT ATT TAA ACA CCT GGC TCC CTG CGT-3’
  - Del:
    - Forward: 5’-CTA GCC CTG AGG CGT TCC ACC-3’
    - Reverse: 5’-CCT GCC TCT CTC AGC CTT TGA-3’
- **NFAT4**
  - Flox/del:
    - Forward: 5’-GCA AGA ACA GCA AGT GTA C-3’
    - Reverse: 5’-CTG GTG ATG GTA GTG TAC-3’
  - HSP70:
    - Forward: 5’-GCT GAG AAG CAC CAG GAT TC-3’
    - Reverse: 5’-CGG GGT CTC CTT TTC TGT CT-3’

Western blot

Western blot analyses were performed as described [6], using antibodies to NFAT2 (sc-7294), NFAT1 (sc-7296), NFAT4 (sc-8321), STAT5 (sc-835) and SAM-68 (sc-333), all from Santa Cruz Biotechnology.

Microarray analysis

Total RNA was isolated from flow-cytometry-sorted leukemic cells (tNGFR+) 5 days following treatment with either tamoxifen or carrier solvent, using the Rneasy kit (QIAGEN). cRNA synthesis and hybridization of Mouse GeneChip 430 2.0 arrays (Affymetrix) were according to the manufacturer’s instructions, as described (http://www-microarrays.u-strasbg.fr). A paired Student’s t-test was performed to compare gene intensities in the different biological replicates. Genes were considered significantly regulated when fold-change was $\geq 1.9$ and $p$ value $\leq 0.05$. Significantly deregulated genes from our dataset (NFAT) and from (Cn) [8] were used to perform pathways and processes enrichment analysis via the online STRING platform. The percentage of shared significantly upregulated, down regulated and overall deregulated processes are shown. Using a spring model via the STRING platform, we generated a network of predicted associated upregulated proteins.

Flow cytometry, apoptosis, proliferation and migration analyses

Surface staining of tNGFR leukemic cells was performed with PE-conjugated anti-human CD271 antibody (BD Biosciences). Apoptosis assays were carried out as described [8], using PE-conjugated-anti-active Caspase-3 antibody (BD Biosciences). BrdU incorporation assays were performed as described [6], using APC-conjugated anti-BrdU antibody (BD Biosciences). Flow cytometry acquisitions were carried out on a FACS Calibur™ analyzer (BD Biosciences) equipped to detect 4 fluorescent parameters with the assistance of BD CellQuest Software (BD...
Biosciences) and data were analyzed with FlowJo Software (Tree Star). ICN1 leukemic cells were sorted on a FACSAria™ III (BD Biosciences) cell sorter on the basis of tNGFR and/or GFP expression with the assistance of BD FACSDiva Software (BD Biosciences). Migration analyses were performed by videomicroscopy, as described [6].

Statistics
Statistical analyses were performed with GraphPad Prism (version 6.0; GraphPad Software, Inc.). The data are expressed as mean ± standard deviation (s.d) of n = 3 or more determinations. Unpaired two-tailed Student’s t tests were used to analyze experimental data between two groups. For three or more groups, a one-way ANOVA was performed using Tukey’s test. Overall survival of mice infused with ICN1-induced T-ALL was calculated according to the Kaplan-Meier method. Log-rank test was used to analyze survival curves comparisons. Differences were considered statistically significant at p < .05 (*), p < .01 (**) or p < .001 (***)

Supporting information
S1 Fig. Nfat1 gene inactivation does not affect leukemia development in TEL-JAK2 and ICN1-induced T-ALL. (A) TEL-JAK2 transgenic mice were crossed and backcrossed with mice inactivated for Nfat1 to generate cohorts of TEL-JAK2+/0; Nfat1+/− (n = 17) and TEL-JAK2+/0; Nfat1−/− (n = 18) littermates. As reported previously, expression of the TEL-JAK2 fusion oncogene in mouse lymphoid lineage induced T-ALL with high penetrance. These cohorts were followed over time for T-ALL onset and mouse survival (log-rank test; ns: non-significant). (B) Mice carrying ICN1-induced T-ALL, a well characterized T-ALL model induced by activated NOTCH1, with the indicated genotypes (Nfat1+/+, n = 35; Nfat1−/−, n = 28) were followed over time for T-ALL onset and recipient mice survival (log-rank test; ns: non-significant).

S2 Fig. Analysis of the consequences of short-term Nfat inactivation in ICN1-induced T-ALL. The Nfat-proficient and Nfat-deficient versions of T-ALL #21 were generated as described in Fig 1A. Mice were sacrificed when terminally ill, 2 days after the end of So and Tam treatments (1 and 2 in Fig 1A). (A) Leukemic burden (% tNGFR+ cells) in BM was analyzed by flow cytometry at the time mice were killed (data are represented as ± SEM; n = 3; Student’s t-test; ns: non-significant). (B) Apoptosis in leukemic cells described in (A) was analyzed by measuring caspase 3 activation by flow cytometry (data are represented as ± SEM; n = 3; Student’s t-test; ns: non-significant).

S3 Fig. Rosa-Cre activation does not affect leukemia propagation after transplantation. (A) Schematic representation of the experiment: Mice were injected with leukemic cells obtained from T-ALL ICN1; RC1°; NFAT1−/−; NFAT2−/−; NFAT4−/−. When BM leukemia burden reached about 10–15% leukemic cells in recipients, mice received 3 successive daily injection of either carrier solvent (So, n = 3) or tamoxifen (TAM, n = 6). Terminally ill mice from both groups were sacrificed 2 days later and leukemic cells from So-treated (blue label, 1) or Tam-treated (red label, 2) cells (10⁶ cells/mouse) were transplanted in wild-type secondary recipients that were followed for leukemia recurrence. (B) Kaplan-Meier survival curve of recipient mice infused with 1x10⁶ T-ALL #RC2 cells. Mice were followed overtime for tumor recurrence and recipient mice survival. (C) Leukemic burden analysis of recipient mice (n = 3 for each group) infused with T-ALL #RC2 cells expressing NFAT factors Nfat-proficient (data
are represented as ± SEM; n = 3; Student’s t test; ns: non-significant).

(PPTX)

S4 Fig. T-ALL RC#2 leukemic cells were co-cultured on MS5 stromal cells and treated with solvent (ethanol, Et) or 4OHT. (A) 5 days after ethanol or 4OHT treatment leukemic cells were analyzed for percentage of cells positive for cleaved caspase 3 (data are represented as mean ± SEM; n = 3; Student’s t-test; ns = not significant). (B) At the same time point EtOH and 4OHT-treated leukemic cells were re-seeded on fresh MS5 stromal cells and migration of individual cells (n = 30) recorded for 15 minutes by time-lapse video-microscopy. In the flower plot diagrams (left), the starting point of each track is placed at the axis origins. (C) In the right panel, velocity (μm/min) of leukemic cell was compared (data are represented as mean ± SEM; n = 3; Student’s t-test; ns = not significant).

(PPTX)

S5 Fig. ICN1-driven T-ALL cells were transduced with MIG vectors encoding Cdkn1a or the control MIG vector without insert. Leukemic cells co-cultured on MS5 stromal cells for 2 days were analyzed by western blot for P21 expression. SAM68 is used as loading control.

(PPTX)

S6 Fig. ICN1; RC2; NFAT1+/−; NFAT2+/−; NFAT4+/− control T-ALL (left panel) and ICN1; RCT2; Nfat1−/−; Nfat2fl/fl; Nfat4fl/fl leukemic cells (right panel) obtained from solvent or Tamoxifen-treated mice as in Fig 1 were analyzed for CXCR4 surface expression by flow cytometry as previously described [6]. MFI quantification obtained for independent mice of each group. Data are represented as mean ± SEM (n = 3 for control and n = 9 for ICN1; RCT2; Nfat1−/−; Nfat2fl/fl; Nfat4fl/fl leukemic cells. ns, not significant; Student’s t test).

(PPTX)

S7 Fig. ICN1-driven T-ALL cells were stably transduced with an NFAT-Luc reporter gene and luciferase activity was measured 24h after IL2/IL7 (100ng/mL) stimulation. Note the lack of induction of luciferase activity as compared to untreated cells.

(PPTX)

S1 Table. Comparison of the leukemia initiating potential of ICN1; RC2; Nfat +/+ T-ALL.

(DOCX)

S2 Table. NFAT-dependent transcriptome in T-ALL.

(DOCX)

S3 Table. Overlap between NFAT- and Calcineurin-dependent transcriptomes.

(DOCX)

S1 Raw images.

(PDF)

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