PLOS ONE

RESEARCH ARTICLE

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

Claire Catherinet[1,2,3,4☯], **Diana Passaro**[1,2,3,4☯¤a], **Stéphanie Gachet**[1,2,3,4☯¤b], **Hind Medyouf**[1,2,3,4¤c], **Anne Reynaud**[1,2,3,4], **Charlène Lasgi**[5], **Jacques Ghysdael**[1,2,3,4,6*], **Christine Tran Quang**[1,2,3,4,6*]

1 Institut Curie, UMR3348, Orsay, France, **2** CNRS UMR3348, Centre Universitaire, Orsay, France, **3** University Paris-Saclay, Orsay, France, **4** PSL Research University, Paris, France, **5** Cytometry Platform, Institut Curie, Orsay, France, **6** INSERM U1278, Centre Universitaire, Orsay, France

☯ These authors contributed equally to this work.
¤a Current address: Université de Paris, Institut Cochin, INSERM, CNRS, Paris, France
¤b Current address: U944/UMR 7212 St-Louis Hospital, Paris, France
¤c Current address: Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, Germany
* Christine.tran-quang@curie.fr (CTQ); Jacques.Ghysdael@curie.fr (JG)

Citation: Catherinet C, Passaro D, Gachet S, Medyouf H, Reynaud A, Lasgi C, et al. (2021) NFAT transcription factors are essential and redundant actors for leukemia initiating potential in T-cell acute lymphoblastic leukemia. PLoS ONE 16(7): e0254184. https://doi.org/10.1371/journal.pone.0254184

Editor: Paula A. da Costa Martins, Maastricht University, NETHERLANDS

Received: February 2, 2021

Accepted: June 21, 2021

Published: July 7, 2021

Data Availability Statement: All transcriptomic files are available from https://doi.org/10.5061/dryad.xd2547dfk.

Funding: This work was supported by funds from Institut Curie, Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM), Ligue contre le Cancer and Fondation ARC pour la recherche contre le cancer. CC, DP and SG were supported by

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, NKX2.1/2.2, 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

pre-doctoral fellowships from the University Paris-Diderot and Ligue Nationale Contre le Cancer.

Competing interests: CTQ and JG have research agreements with Servier and Autolus Ltd. The remaining authors declare no competing financial interests. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

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 enlighted 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 Materiel 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; $Nfat1^{-/-}$; $Nfat2^{f/-}$; $Nfat4^{f/f}$ 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}; $Nfat1^{-/-}$; $Nfat2^{fl/fl}$; $Nfat4^{+/+}$) 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 *Nfat*4 (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 limit 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

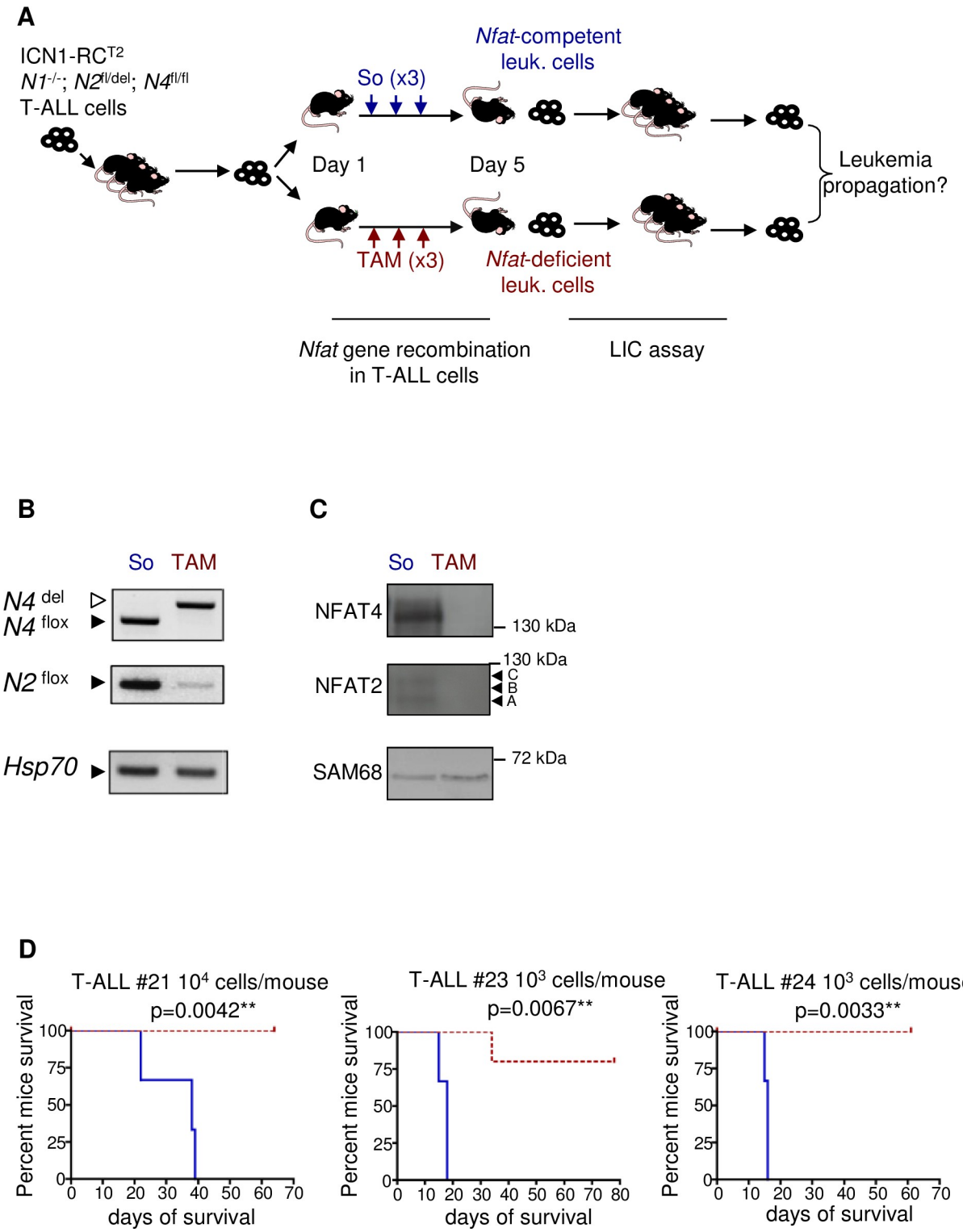

Fig 1. NFAT factors are required for leukemic initiating cell potential in T-ALL. (A) Schematic description of the experiment: Mice were i. v. injected with ICN1-induced leukemic cells obtained from 3 independent T-ALL (#21, #23, #24) of the indicated genotypes. When BM leukemia burden reached about 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

recipient mice under limit dilution conditions. *N1*: *Nfat1; N2*: *Nfat2; N4*: *Nfat4;* RC^{T2}: Rosa-Cre-ER^{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 $1x10^4$ (T-ALL #21) or $1x10^3$ (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).

https://doi.org/10.1371/journal.pone.0254184.g001

"recovered" panel compare red and blue tracings; Table 2). Leukemic cells recovered from terminally-leukemic recipients injected with RC^{T2}; *Nfat1*$^{-/-}$; *Nfat2*$^{+/+}$; *Nfat4* $^{del/del}$ 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[2+5+8] [18] (named NFAT1* 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*-NLS), further enhancing its nuclear accumulation and transcriptional activity [18]. ICN1; RC^{T2}; $CnB1^{fl/fl}$ T-ALL cells [8],

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.

Leukemia id	Treatment of donor mice	Number of cells injected in recipients	Number of injected recipients	Number of leukemic recipients (time to death, days)	Statistics	% leukemic cells in the BM ± SEM of recipients
#21	So	4.10^6	3	3 (13;13;13)		63,9±6
	TAM	4.10^6	3	**3*** (17;17;17)	p = 0,025	64,9±2,8
	So	1.10^4	3	3 (22;39;38)		46,2±13,4
	TAM	1.10^4	6	0	p = 0,004	NA
	So	1.10^3	3	1 (21)		65,9
	TAM	1.10^3	6	0	ns	NA
#23	So	4.10^6	3	3 (13;14;13)		47,6±2,9
	TAM	4.10^6	5	**5*** (20;20;25;22;29)	p = 0.004	42,9±3,9
	So	1.10^4	3	3 (14;15;18)		38,1±11,1
	TAM	1.10^4	6	**6*** (22;25;25;25;29;52)	p = 0,001	37,6±13
	So	1.10^3	3	3 (18;15;18)		36,2±9,1
	TAM	1.10^3	6	**1*** (34)	p = 0,006	49,3
#24	So	4.10^6	3	3 (15;15;15)		58,8±5,7
	TAM	4.10^6	6	**6*** (17;17;18;18;18;23)	p = 0,004	42,4±5,5
	So	1.10^4	3	3 (15;15;15)		31,3±3
	TAM	1.10^4	6	**2*** (18;37)	p = 0,004	39,4±8,1
	So	1.10^3	3	3 (15;15;16)		9±1,6
	TAM	1.10^3	6	0	p = 0,003	NA

Asterisks indicate that leukemic cells recovered from these mice showed $NFAT2^{fl/del}$ and $NFAT4^{fl/fl}$ 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.

https://doi.org/10.1371/journal.pone.0254184.t001

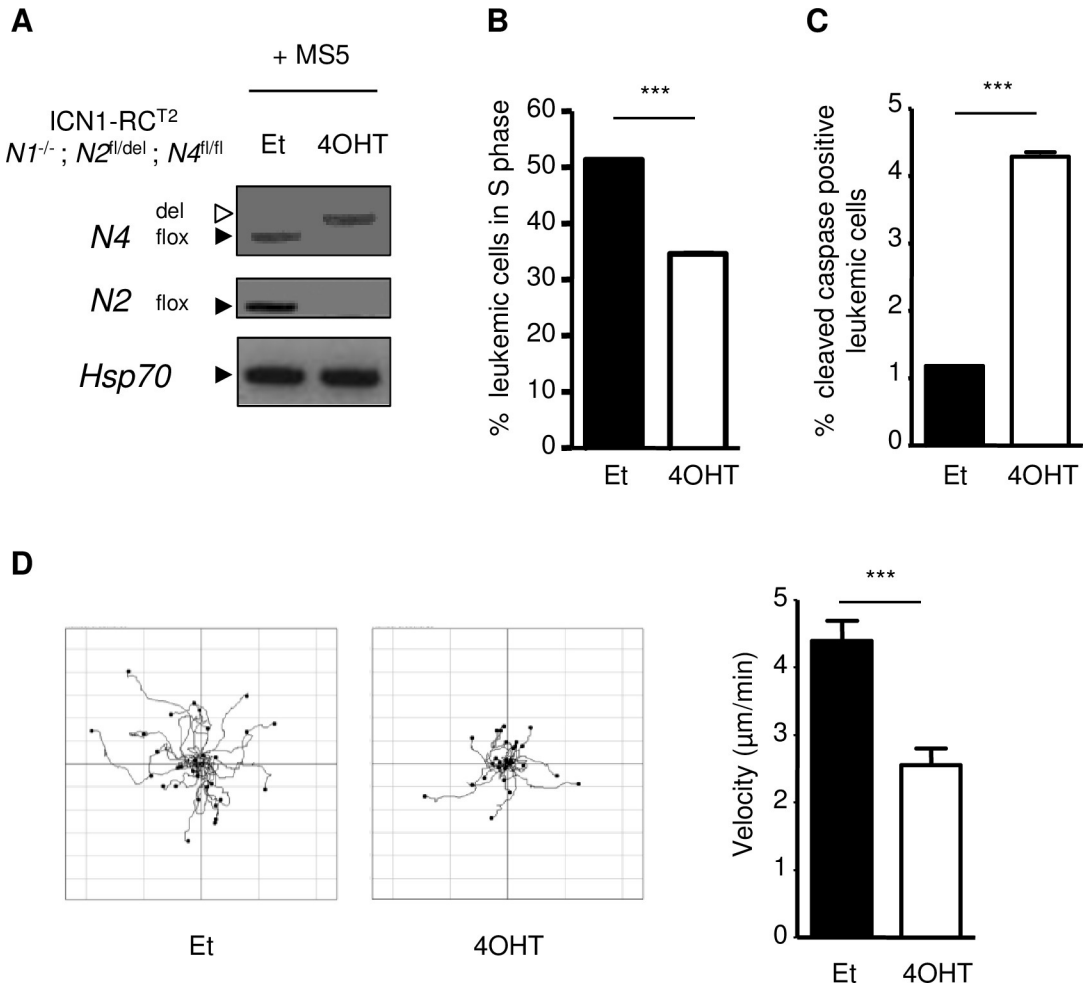

Fig 2. NFAT transcription factors regulate cell survival, proliferation and migration of ICN1-induced T-ALL *in vitro*. **(A)** T-ALL #21 leukemic cells were co-cultured on MS5 stromal cells and treated with solvent (ethanol, Et) or 4OHT to induce deletion of *Nfat* floxed alleles. Leukemic cells genotypes were analyzed 5 days later by PCR for the floxed (flox) and deleted (del) alleles of *Nfat4* 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).

https://doi.org/10.1371/journal.pone.0254184.g002

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 *CnB1 (PPP3R1)* gene deletion and calcineurin inactivation [8] (Fig 4A for a schematic representation of the experiment). Leukemic cells were then co-cultured with MS5 stromal cells and followed over time. As shown in Fig 4B and as previously reported, 4-OHT treatment induced efficient *CnB1* 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

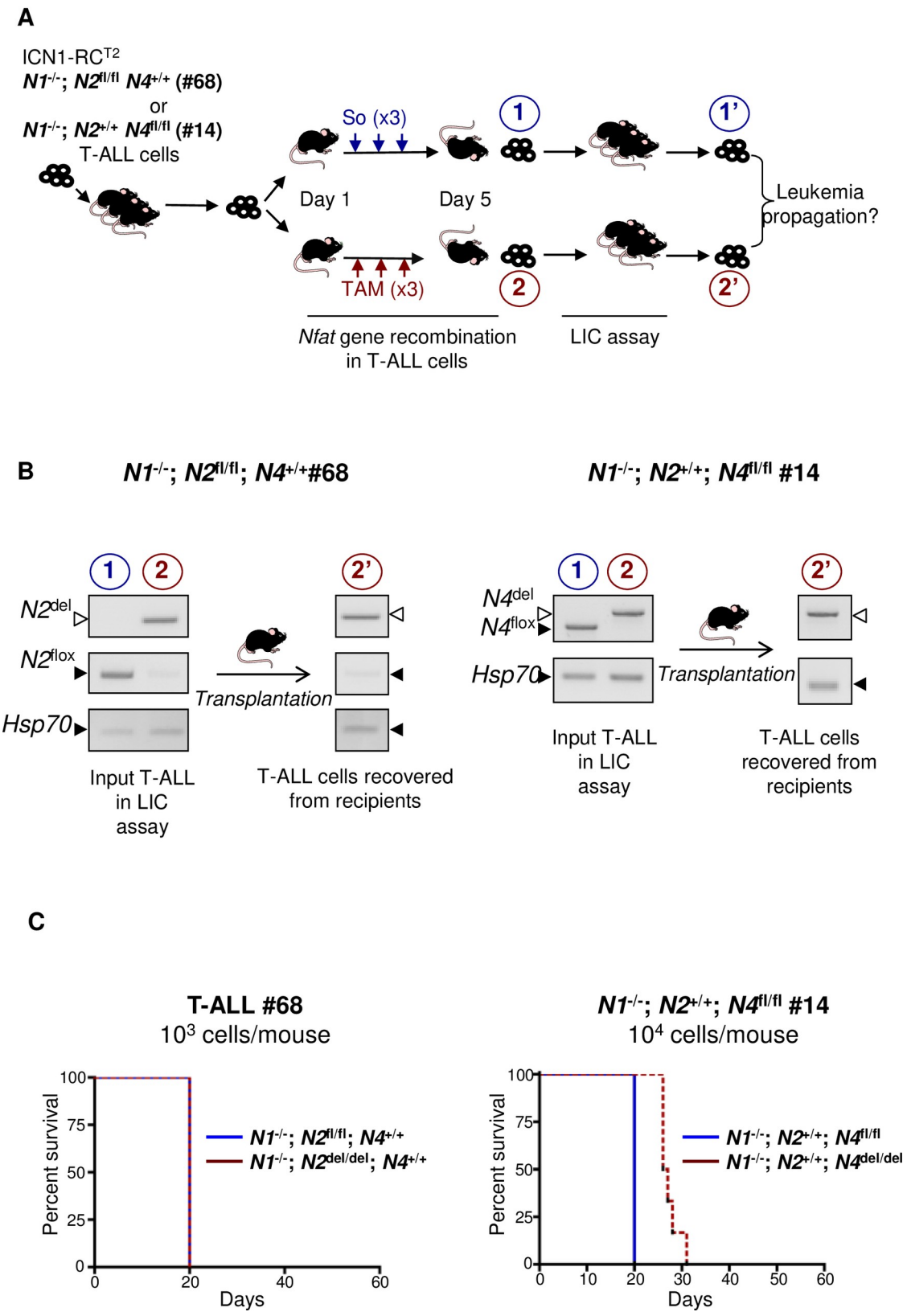

Fig 3. Functional redundancy of NFAT factors in T-ALL. (A) Schematic description of the experiment: mice were injected with leukemic cells obtained from either primary T-ALL #68 (ICN1; RCT2; *Nfat1*$^{-/-}$; *Nfat2*$^{fl/fl}$; *Nfat4*$^{+/+}$) or #14 (ICN1; RCT2; *Nfat1*$^{-/-}$;

Nfat2$^{+/+}$; *Nfat4*$^{fl/fl}$), that carry wild type alleles of *Nfat4* or *Nfat2*, respectively. When BM leukemia burden reached about 10–15% T-ALL 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* (T-ALL #68) or *Nfat4* (T-ALL #14) floxed alleles deletion thus resulting in T-ALLs relying upon only *Nfat4 (T-ALL #68) or only Nfat 2 (T-ALL #14)*. Mice from all groups became terminally leukemic 2 days later, sacrificed and *Nfat*-proficient (blue label, 1) or *Nfat*-defloxed (red label, 2) cells were flow cytometry-sorted and compared for their ability to re-initiate leukemia in secondary recipient mice under limit dilution conditions. Flow cytometry-sorted leukemic cells obtained from donor mice (1, 2) and retrieved from terminally ill recipients (1', 2') were genotyped for *Nfat* floxed and deleted alleles. **(B)** Left panels: PCR genotyping of *Nfat2* floxed and deleted alleles in input leukemic cells from T-ALL #68 (1 and 2; see schematic in A) and in leukemic cells recovered from one representative secondary recipient injected T-ALL cells (T-ALL #68, 2'). Right panels: PCR genotyping of Nfat4 floxed and deleted alleles in input leukemic cells from T-ALL #14 (1 and 2; see schematic in A) and in leukemic cells recovered from one representative secondary recipient injected with T-ALL #14 (2'). PCR for *Hsp70* is used as control. **(C)** Left panel: Kaplan-Meier survival curves of mice transplanted with T-ALL cells #68 expressing only *Nfat4* (red tracing) or co-expressing *Nfat2* and *Nfat4* (blue tracing). Right panel: Kaplan-Meier survival curves of mice transplanted with T-ALL cells #14 expressing only *Nfat2* (red tracing) or co-expressing *Nfat2* and *Nfat4* (blue tracing).

https://doi.org/10.1371/journal.pone.0254184.g003

exogenous CnB1 restored the ability of T-ALL cells to survive and proliferate (Fig 4C, left panel, blue tracing) concomitant with the rapid amplification of GFP+ cells (Fig 4C, right panel, blue tracing) that retain the parental CnB$^{del/del}$ genotype (Fig 4D). Interestingly, both NFAT1* and NFAT1*-NLS rescued the survival/proliferation defect of CnB1-deficient T-ALL cells (Fig 4C red and orange tracing, respectively) albeit less efficiently as compared to exogenous CnB1. Cn-deficient T-ALL cells transduced with either of the NFAT1* vectors (GFP+) maintained the original CnB1-deleted genotype unlike the remaining fraction of non-transduced cells (GFP-) found in these cultures (Fig 4D). We conclude from these experiments that constitutive NFAT activity can restore the survival/proliferative properties of Cn-deficient T-ALL cells, indicating that NFAT are one of the major downstream effectors of Cn in T-ALL.

NFAT-dependent transcriptome in T-ALL

Although we expect NFAT1, 2 and 4 factors to regulate distinct sets of genes, their activity in T-ALL likely relies upon the regulation of shared pathways severely impinging upon leukemia

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.

Leukemia id	Treatment of donor mice	Number of cells injected in recipients	Number of injected recipients	Number of engrafted recipients (time to death, days)	Statistics	% leukemic cells in the BM ± SEM of recipients
N1$^{-/-}$;N2$^{f/f}$; N4$^{+/+}$						
#68	So	1.10^{6}	3	3 (10;10;10)		32,6±2,2
	TAM	1.10^{6}	6	6 (10;10;10;10;10;10)	ns	26,6±1
	So	1.10^{4}	3	3 (16;16;16)		35,1±1,4
	TAM	1.10^{4}	6	6 (16;16;16;17;17;17)	ns	30,9±3,2
	So	1.10^{3}	3	3 (20;20;20)		34,2±3,4
	TAM	1.10^{3}	5	5 (20;20;20;20;20)	ns	30,7±1,5
	So	1.10^{2}	3	2 (20;31)		38,8±5,1
	TAM	1.10^{2}	6	4 (20;20;29;46)	ns	28,3±2,1
N1$^{-/-}$;N2$^{+/+}$; N4$^{f/f}$						
#14	So	1.10^{6}	3	3 (17;17;17)		72,7±3,6
	TAM	1.10^{6}	6	6 (18;18;20;20;21;21)	p = 0,005	62,2±12,0
	So	1.10^{4}	3	3 (20;20;20)		61,2±6,4
	TAM	1.10^{4}	6	6 (26;26;26;27;28;31)	p = 0,005	61,4±10,4
	So	1.10^{3}	3	3 (21;21;21)		66,2±3,4
	TAM	1.10^{3}	6	5 (27;27;35;38;38)	p = 0,05	51,8±0,8
	So	1.10^{2}	3	1 (28)		70,7
	TAM	1.10^{2}	6	1 (60)	ns	8,2

https://doi.org/10.1371/journal.pone.0254184.t002

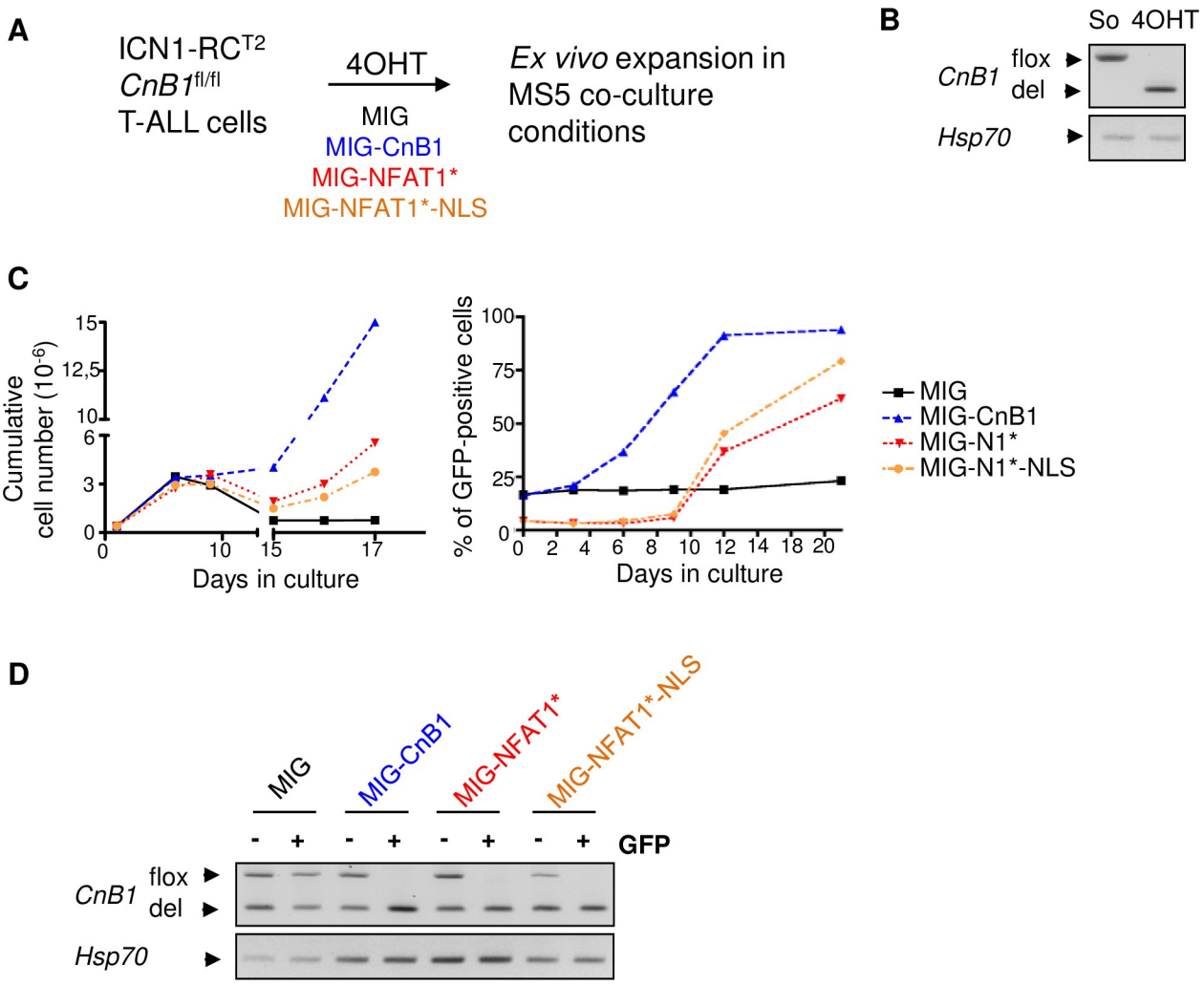

Fig 4. NFAT factors are major downstream effectors of calcineurin in T-ALL. (A) Schematic of the experiment: leukemic cells obtained from an ICN1-induced T-ALL carrying 2 floxed alleles of *CnB1* (T-ALL #3) were retrovirally transduced with MIG vectors encoding either HA-tagged CnB1, or HA-tagged NFAT1*, or HA-tagged NFAT1*-NLS, or the control vector without insert (MIG), co-cultured on MS5 stromal cells and immediately treated with 4OHT to delete *CnB1* floxed alleles. **(B)** PCR genotyping analysis for the floxed and deleted alleles of *CnB1* in cultured cells 2 days after 4OHT treatment. PCR for *Hsp70* is used as control. **(C)** Left panel: Expansion of CnB1-deleted leukemic cells transduced with MIG (black squares), MIG-CnB1 (blue triangle), MIG-NFAT1* (red triangles), MIG-NFAT1*-NLS (orange); expansion over time is reported as the cumulative cell numbers over 17 days in MS5 co-cultures. Right panel: in the same co-cultures, enrichment in GFP+, transduced cells, was followed over time. This experiment is representative of three independent experiments. **(D)** PCR genotyping analysis for the floxed and deleted alleles of *CnB1* in the GFP$^+$ (transduced) and GFP$^-$ (non-transduced) fractions of flow-cytometry sorted leukemic cells from the respective co-cultures at day 17. PCR for *Hsp70* is used as control.

https://doi.org/10.1371/journal.pone.0254184.g004

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*$^{fl/fl}$; *Nfat4*$^{fl/fl}$ 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

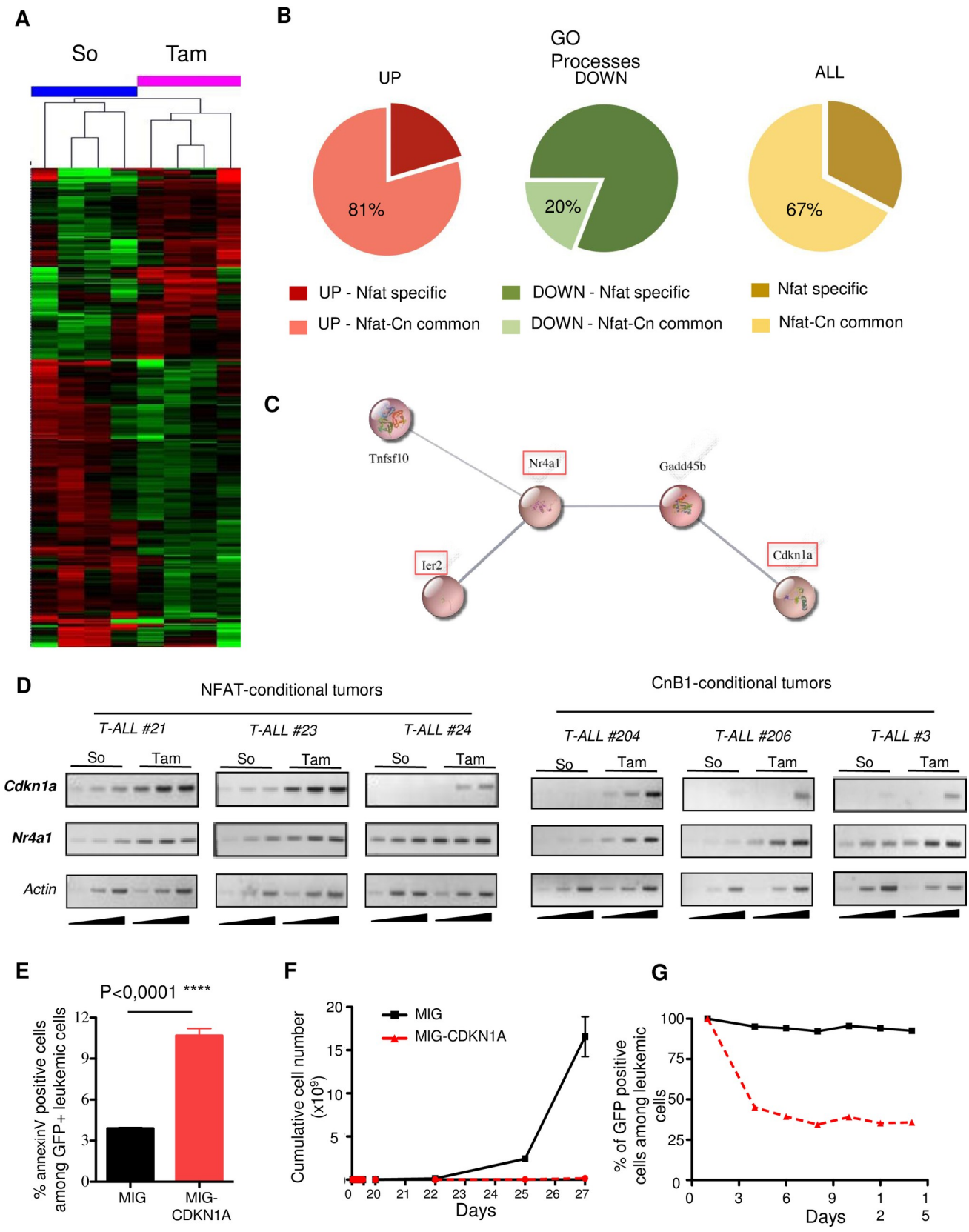
A
So
Tam
B
GO Processes
UP
DOWN
ALL
81%
20%
67%
UP - Nfat specific
UP - Nfat-Cn common
DOWN - Nfat specific
DOWN - Nfat-Cn common
Nfat specific
Nfat-Cn common
C
Tnfsf10
Nr4a1
Gadd45b
Ier2
Cdkn1a
D
NFAT-conditional tumors
CnB1-conditional tumors
T-ALL #21
T-ALL #23
T-ALL #24
T-ALL #204
T-ALL #206
T-ALL #3
So
Tam
Cdkn1a
Nr4a1
Actin
E
P<0,0001 ****
% annexinV positive cells among GFP+ leukemic cells
MIG
MIG-CDKN1A
F
Cumulative cell number (x10⁹)
MIG
MIG-CDKN1A
Days
G
% of GFP positive cells among leukemic cells
Days

Fig 5. NFAT-dependent transcriptome analysis in ICN1-induced T-ALL. (A) Global gene expression analyses of *Nfat*-proficient (So) and *Nfat*-deficient (Tam) leukemic cells obtained from T-ALL #21, #24, #23 (2 independent experiments) and sorted by flow cytometry from solvent (So)- or Tam-treated mice generated as described in Fig 1A. Hierarchical clustering of the different leukemias (top legends) in their *Nfat*-competent (So) and *Nfat*-deficient (Tam) versions was performed using a fold change ≥1,5 with a p value <0,05. The heatmap representation highlights up-regulated genes in red and down-regulated genes in green. **(B)** Pie charts showing the percentages of commonly deregulated GO processes in the Cn-dependent and *Nfat*-dependent transcriptome. **(C)** Predicted protein interaction map retrieved from the analysis of significantly upregulated genes in the T-ALL *Nfat*-dependent transcriptome. Orange boxes point to genes commonly deregulated in both NFAT- and Cn-dependent transcriptomes **(D)** *Nfat*-proficient, *Nfat*-deficient, *CnB1*-proficient and *CnB1*-deficient versions of the indicated T-ALL were sorted by flow cytometry and analyzed by semi-quantitative RT-PCR for the expression of *Cdkn1a* and *Nr4a1*. RT-PCR for expression of *β-actin* is used as control **(E)** Leukemic cells from ICN1-T-ALL were transduced with MIG vectors encoding CDKN1A or the control MIG vector without insert. Leukemic cells survival was analyzed by Annexin V staining at day 4 in co-cultures of the indicated leukemic cells with MS5 stromal cell (data are represented as ± SEM; n = 3; Student's t-test). **(F)** Expansion over time in MS5 co-cultures of leukemic cells transduced with the MIG and MIG-CDKN1A vectors described in E. **(G)** Percentage of transduced (GFP+) leukemic cells in co-cultures described in (F) was followed by flow cytometry.

https://doi.org/10.1371/journal.pone.0254184.g005

GTPases, 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 $p21^{CDKN1a}$. Western blot analysis confirmed $p21^{CDKN1a}$ overexpression as compared to MIG–transduced cells (S5 Fig). Enforced expression of $p21^{CDKN1a}$ 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 oncoprotein [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, 34, 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 Ctla4* (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 N*r4a1*, 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 therapeutical 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*$^{-/-}$; *Nfat2*$^{flox/del}$; *Nfat4*$^{flox/flox}$, or RC^{T2}; *Nfat1*$^{-/-}$; *Nfat2*$^{flox/flox}$; *Nfat4*$^{+/+}$, or RC^{T2}-*Nfat1*$^{-/-}$; *Nfat2*$^{+/+}$; *Nfat4*$^{flox/flox}$. 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 10^6 to 10^3 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 RC^{T2} 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 ($1x10^6$ 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:

NFAT2 $^{flox/+}$	5′-CCA TCT CTC TGA CCA ACA GAA GCC AGC-3′ 5′-CCT ATT TAA ACA CCT GGC TCC CTG CGT-3′
NFAT2 ^{del}	5′-CTA GGC CTG AGG CGT TCC ACC-3′ 5′-CCT GCC TCT CTC AGC CTT TGA-3′
NFAT4 $^{flox/del}$	5′-GCA AGA ACA GCA AGT GTA C-3′ 5′-TTG ACC TCA ACA TTC TGG AG-3′ 5′-CTG GTG ATG GTA GTG TAC-3′
HSP70	5′-GCT GAG AAG CAC CAG GAT TC-3′ 5′-CGG GGT CTC CTT TTC TGT CT-3′

https://doi.org/10.1371/journal.pone.0254184.t003

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:

CDKN1A	5′-AGT GTC CCG TTG TCT CTT CG-3′ 5′-ACA CCA GAG TGC AAG ACA AGC-3′
NR4A1	5′-GGA AGC TCA TCT TCT GCT CAG-3′ 5′-CCT TCA GAC AGC TAG CAA TGC G-3′
Actin	5′-GTG GGC CGC CCT AGG CAC CA-3′ 5′-TCT TTG ATG TCA CGC ACG ATT TC-3′

https://doi.org/10.1371/journal.pone.0254184.t004

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 ≥ 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 FACSCalibur™ 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).
(PPTX)

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).
(PPTX)

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; RC^{T2}; 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 (10^6 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 $1x10^6$ 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; RC^{T2}; $NFAT1^{+/+}$; $NFAT2^{+/+}$; $NFAT4^{+/+}$ control T-ALL (left panel) and ICN1; RCT2; $Nfat1^{-/-}$; $Nfat2^{fl/del}$; $Nfat4^{fl/fl}$ 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; $Nfat1^{-/-}$; $Nfat2^{fl/del}$; $Nfat4^{fl/fl}$ 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)

Acknowledgments

The authors thank E Belloir, C Alberti and C Roulle (Institut Curie animal facility) for expert technical assistance.

Author Contributions

Conceptualization: Jacques Ghysdael, Christine Tran Quang.

Formal analysis: Claire Catherinet, Diana Passaro, Stéphanie Gachet, Hind Medyouf, Charlène Lasgi, Christine Tran Quang.

Funding acquisition: Jacques Ghysdael.

Investigation: Claire Catherinet, Diana Passaro, Stéphanie Gachet, Hind Medyouf, Anne Reynaud, Jacques Ghysdael, Christine Tran Quang.

Methodology: Claire Catherinet, Diana Passaro, Stéphanie Gachet, Hind Medyouf, Anne Reynaud, Charlène Lasgi, Christine Tran Quang.

Supervision: Diana Passaro, Hind Medyouf, Jacques Ghysdael, Christine Tran Quang.

Validation: Claire Catherinet, Charlène Lasgi, Jacques Ghysdael, Christine Tran Quang.

Writing – original draft: Jacques Ghysdael, Christine Tran Quang.

Writing – review & editing: Diana Passaro, Stéphanie Gachet, Hind Medyouf, Jacques Ghysdael, Christine Tran Quang.

References

1. Girardi T, Vicente C, Cools J, De Keersmaecker K. The genetics and molecular biology of T-ALL. Blood. 2017; 129: 1113–1123. https://doi.org/10.1182/blood-2016-10-706465 PMID: 28115373
2. Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C, et al. Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. Science. 2004; 306: 269–271. https://doi.org/10.1126/science.1102160 PMID: 15472075
3. 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: 3181–3194. https://doi.org/10.1172/JCI35090 PMID: 18677410
4. Passaro D, Quang CT, Ghysdael J. Microenvironmental cues for T-cell acute lymphoblastic leukemia development. Immunol Rev. 2016; 271: 156–172. https://doi.org/10.1111/imr.12402 PMID: 27088913
5. Pitt LA, Tikhonova AN, Hu H, Trimarchi T, King B, Gong Y, et al. CXCL12-Producing Vascular Endothelial Niches Control Acute T Cell Leukemia Maintenance. Cancer Cell. 2015; 27: 755–768. https://doi.org/10.1016/j.ccell.2015.05.002 PMID: 26058075
6. Passaro D, Irigoyen M, Catherinet C, Gachet S, Da Costa De Jesus C, Lasgi C, et al. CXCR4 Is Required for Leukemia-Initiating Cell Activity in T Cell Acute Lymphoblastic Leukemia. Cancer Cell. 2015; 27: 769–779. https://doi.org/10.1016/j.ccell.2015.05.003 PMID: 26058076
7. Medyouf H, Alcalde H, Berthier C, Guillemin MC, dos Santos NR, Janin A, et al. Targeting calcineurin activation as a therapeutic strategy for T-cell acute lymphoblastic leukemia. Nat Med. 2007; 13: 736–741. https://doi.org/10.1038/nm1588 PMID: 17515895
8. Gachet S, Genescà E, Passaro D, Irigoyen M, Alcalde H, Clémenson C, et al. Leukemia-initiating cell activity requires calcineurin in T-cell acute lymphoblastic leukemia. Leukemia. 2013; 27: 2289–2300. https://doi.org/10.1038/leu.2013.156 PMID: 23689515
9. Müller MR, Rao A. NFAT, immunity and cancer: a transcription factor comes of age. Nat Rev Immunol. 2010; 10: 645–656. https://doi.org/10.1038/nri2818 PMID: 20725108
10. Sharma S, Findlay GM, Bandukwala HS, Oberdoerffer S, Baust B, Li Z, et al. Dephosphorylation of the nuclear factor of activated T cells (NFAT) transcription factor is regulated by an RNA-protein scaffold complex. Proc Natl Acad Sci. 2011; 108: 11381–11386. https://doi.org/10.1073/pnas.1019711108 PMID: 21709260
11. Patra AK, Avots A, Zahedi RP, Schüler T, Sickmann A, Bommhardt U, et al. An alternative NFAT-activation pathway mediated by IL-7 is critical for early thymocyte development. Nat Immunol. 2013; 14: 127–135. https://doi.org/10.1038/ni.2507 PMID: 23263556
12. Canté-Barrett K, Winslow MM, Crabtree GR. Selective Role of NFATc3 in Positive Selection of Thymocytes. J Immunol. 2007; 179: 103–110. https://doi.org/10.4049/jimmunol.179.1.103 PMID: 17579027
13. Oukka M, Ho I-C, de la Brousse FC, Hoey T, Grusby MJ, Glimcher LH. The Transcription Factor NFAT4 Is Involved in the Generation and Survival of T Cells. Immunity. 1998; 9: 295–304. https://doi.org/10.1016/s1074-7613(00)80612-3 PMID: 9768749

14. Peng SL, Gerth AJ, Ranger AM, Glimcher LH. NFATc1 and NFATc2 Together Control Both T and B Cell Activation and Differentiation. Immunity. 2001; 14: 13–20. https://doi.org/10.1016/s1074-7613(01)00085-1 PMID: 11163226

15. Ranger AM, Oukka M, Rengarajan J, Glimcher LH. Inhibitory Function of Two NFAT Family Members in Lymphoid Homeostasis and Th2 Development. Immunity. 1998; 9: 627–635. https://doi.org/10.1016/s1074-7613(00)80660-3 PMID: 9846484

16. Martinez GJ, Pereira RM, Äijö T, Kim EY, Marangoni F, Pipkin ME, et al. The Transcription Factor NFAT Promotes Exhaustion of Activated CD8+ T Cells. Immunity. 2015; 42: 265–278. https://doi.org/10.1016/j.immuni.2015.01.006 PMID: 25680272

17. Yissachar N, Sharar Fischler T, Cohen AA, Reich-Zeliger S, Russ D, Shifrut E, et al. Dynamic Response Diversity of NFAT Isoforms in Individual Living Cells. Mol Cell. 2013; 49: 322–330. https://doi.org/10.1016/j.molcel.2012.11.003 PMID: 23219532

18. Okamura H, Aramburu J, García-Rodríguez C, Viola JPB, Raghavan A, Tahiliani M, et al. Concerted Dephosphorylation of the Transcription Factor NFAT1 Induces a Conformational Switch that Regulates Transcriptional Activity. Mol Cell. 2000; 6: 539–550. https://doi.org/10.1016/s1097-2765(00)00053-8 PMID: 11030334

19. Mognol GP, Carneiro FRG, Robbs BK, Faget DV, Viola JPB. Cell cycle and apoptosis regulation by NFAT transcription factors: new roles for an old player. Cell Death Dis. 2016; 7: e2199–e2199. https://doi.org/10.1038/cddis.2016.97 PMID: 27100893

20. Neal JW, Clipstone NA. A Constitutively Active NFATc1 Mutant Induces a Transformed Phenotype in 3T3-L1 Fibroblasts. J Biol Chem. 2003; 278: 17246–17254. https://doi.org/10.1074/jbc.M300528200 PMID: 12598522

21. Robbs BK, Cruz ALS, Werneck MBF, Mognol GP, Viola JPB. Dual Roles for NFAT Transcription Factor Genes as Oncogenes and Tumor Suppressors. Mol Cell Biol. 2008; 28: 7168–7181. https://doi.org/10.1128/MCB.00256-08 PMID: 18809576

22. Jauliac S, López-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nat Cell Biol. 2002; 4: 540–544. https://doi.org/10.1038/ncb816 PMID: 12080349

23. Tran Quang C, Leboucher S, Passaro D, Fuhrmann L, Nourieh M, Vincent-Salomon A, et al. The calcineurin/NFAT pathway is activated in diagnostic breast cancer cases and is essential to survival and metastasis of mammary cancer cells. Cell Death Dis. 2015; 6: e1658–e1658. https://doi.org/10.1038/cddis.2015.14 PMID: 25719243

24. Pham LV, Tamayo AT, Li C, Bueso-Ramos C, Ford RJ. An epigenetic chromatin remodeling role for NFATc1 in transcriptional regulation of growth and survival genes in diffuse large B-cell lymphomas. Blood. 2010; 116: 3899–3906. https://doi.org/10.1182/blood-2009-12-257378 PMID: 20664054

25. Xanthoudakis S, Viola JPB, Shaw KTY, Luo C, Wallace JD, Bozza PT, et al. An Enhanced Immune Response in Mice Lacking the Transcription Factor NFAT1. Science. 1996; 272: 892–895. https://doi.org/10.1126/science.272.5263.892 PMID: 8629027

26. Hodge MR, Ranger AM, de la Brousse FC, Hoey T, Grusby MJ, Glimcher LH. Hyperproliferation and Dysregulation ofIL-4 Expression in NF-ATp-Deficient Mice. Immunity. 1996; 4: 397–405. https://doi.org/10.1016/s1074-7613(00)80253-8 PMID: 8612134

27. May SL, Zhou Q, Lewellen M, Carter CM, Coffey D, Highfill SL, et al. Nfatc2 and Tob1 Have Non-Overlapping Function in T Cell Negative Regulation and Tumorigenesis. PLOS ONE. 2014; 9: e100629. https://doi.org/10.1371/journal.pone.0100629 PMID: 24945807

28. Saint Fleur-Lominy S, Maus M, Vaeth M, Lange I, Zee I, Suh D, et al. STIM1 and STIM2 Mediate Cancer-Induced Inflammation in T Cell Acute Lymphoblastic Leukemia. Cell Rep. 2018; 24: 3045–3060.e5. https://doi.org/10.1016/j.celrep.2018.08.030 PMID: 30208327

29. Chen J, López-Moyado IF, Seo H, Lio C-WJ, Hempleman LJ, Sekiya T, et al. NR4A transcription factors limit CAR T cell function in solid tumours. Nature. 2019; 567: 530–534. https://doi.org/10.1038/s41586-019-0985-x PMID: 30814732

30. Davies C, Hogarth LA, Dietrich PA, Bachmann PS, Mackenzie KL, Hall AG, et al. p53-independent Epigenetic Repression of the p21WAF1 Gene in T-cell Acute Lymphoblastic Leukemia. J Biol Chem. 2011; 286: 37639–37650. https://doi.org/10.1074/jbc.M111.272336 PMID: 21903579

31. Cainan BJ, Szychowski S, Ka-Ming Chan F, Cado D, Winoto A. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. Immunity. 1995; 3: 273–282. https://doi.org/10.1016/1074-7613(95)90113-2 PMID: 7552993

32. Seo H, Chen J, González-Avalos E, Samaniego-Castruita D, Das A, Wang YH, et al. TOX and TOX2 transcription factors cooperate with NR4A transcription factors to impose CD8+ T cell exhaustion. Proc Natl Acad Sci. 2019; 116: 12410–12415. https://doi.org/10.1073/pnas.1905675116 PMID: 31152140

33. Santini MP, Talora C, Seki T, Bolgan L, Dotto GP. Cross talk among calcineurin, Sp1/Sp3, and NFAT in control of p21WAF1/CIP1 expression in keratinocyte differentiation. Proc Natl Acad Sci. 2001; 98: 9575–9580. https://doi.org/10.1073/pnas.161299698 PMID: 11493684

34. Youn H-D, Chatila TA, Liu JO. Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. EMBO J. 2000; 19: 4323–4331. https://doi.org/10.1093/emboj/19.16.4323 PMID: 10944115

35. Lobbardi R, Pinder J, Martinez-Pastor B, Theodorou M, Blackburn JS, Abraham BJ, et al. TOX Regulates Growth, DNA Repair, and Genomic Instability in T-cell Acute Lymphoblastic Leukemia. Cancer Discov. 2017; 7: 1336–1353. https://doi.org/10.1158/2159-8290.CD-17-0267 PMID: 28974511

36. Tosello V, Saccomani V, Yu J, Bordin F, Amadori A, Piovan E. Calcineurin complex isolated from T-cell acute lymphoblastic leukemia (T-ALL) cells identifies new signaling pathways including mTOR/AKT/S6K whose inhibition synergize with calcineurin inhibition to promote T-ALL cell death. Oncotarget. 2016; 7: 45715–45729. https://doi.org/10.18632/oncotarget.9933 PMID: 27304189

37. Wigington CP, Roy J, Damle NP, Yadav VK, Blikstad C, Resch E, et al. Systematic Discovery of Short Linear Motifs Decodes Calcineurin Phosphatase Signaling. Mol Cell. 2020; 79: 342–358.e12. https://doi.org/10.1016/j.molcel.2020.06.029 PMID: 32645368

38. Eerola SK, Santio NM, Rinne S, Kouvonen P, Corthals GL, Scaravilli M, et al. Phosphorylation of NFATC1 at PIM1 target sites is essential for its ability to promote prostate cancer cell migration and invasion. Cell Commun Signal. 2019; 17: 148. https://doi.org/10.1186/s12964-019-0463-y PMID: 31730483

39. La Starza R, Messina M, Gianfelici V, Pierini V, Matteucci C, Pierini T, et al. High PIM1 expression is a biomarker of T-cell acute lymphoblastic leukemia with JAK/STAT activation or t(6;7)(p21;q34)/ TRB@-PIM1 rearrangement. Leukemia. 2018; 32: 1807–1810. https://doi.org/10.1038/s41375-018-0031-2 PMID: 29479063

40. De Smedt R, Morscio J, Reunes L, Roels J, Bardelli V, Lintermans B, et al. Targeting cytokine- and therapy-induced PIM1 activation in preclinical models of T-cell acute lymphoblastic leukemia and lymphoma. Blood. 2020; 135: 1685–1695. https://doi.org/10.1182/blood.2019003880 PMID: 32315407

41. Wimmer CD, Angele MK, Schwarz B, Pratschke S, Rentsch M, Khandoga A, et al. Impact of cyclosporine versus tacrolimus on the incidence of de novo malignancy following liver transplantation: a single center experience with 609 patients. Transpl Int. 2013; 26: 999–1006. https://doi.org/10.1111/tri.12165 PMID: 23952102

42. Roehrl MHA, Kang S, Aramburu J, Wagner G, Rao A, Hogan PG. Selective inhibition of calcineurin-NFAT signaling by blocking protein–protein interaction with small organic molecules. Proc Natl Acad Sci. 2004; 101: 7554–7559. https://doi.org/10.1073/pnas.0401835101 PMID: 15131267

43. Wang W, Cheng J-W, Qin J-J, Hu B, Li X, Nijampatnam B, et al. MDM2-NFAT1 dual inhibitor, MA242: Effective against hepatocellular carcinoma, independent of p53. Cancer Lett. 2019; 459: 156–167. https://doi.org/10.1016/j.canlet.2019.114429 PMID: 31181320

44. Mognol GP, González-Avalos E, Ghosh S, Spreafico R, Gudlur A, Rao A, et al. Targeting the NFAT:AP-1 transcriptional complex on DNA with a small-molecule inhibitor. Proc Natl Acad Sci. 2019; 116: 9959–9968. https://doi.org/10.1073/pnas.1820604116 PMID: 31019078

45. Seibler J, Zevnik B, Küter-Luks B, Andreas S, Kern H, Hennek T, et al. Rapid generation of inducible mouse mutants. Nucleic Acids Res. 2003; 31: e12–e12. https://doi.org/10.1093/nar/gng012 PMID: 12582257

46. Suzuki J, Fujita J, Taniguchi S, Sugimoto K, Mori KJ. Characterization of murine hemopoietic-supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines. Leukemia. 1992; 6: 452–458. PMID: 1375698