NFATc1 and NFATc2 regulate glucocorticoid resistance in pediatric T-cell acute lymphoblastic leukemia through modulation of cholesterol biosynthesis and the WNT/β-catenin pathway

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SUPPLEMENTARY DATA

Table S1: Sequences of primers used for RQ-PCR

PRIMER SEQUENCES	Forward	Reverse
Human *NFATc1*	TGCAAGCCGAATTCTCTGG	GGGAAGGTAGGTGAAACGCTG
Human *NFATc2*	GATAGTGGGCAACACCAAAGTCC	TCTCGCCTTTCCCGCAGCTCAAT
Human *NFATc3*	ACCAGGTGAAGGAACAGGTG	GCAATCTCCTTGCCACTCTC
Human *NFATc4*	ACAGTGAACCGACTGCAGAG	TGCAGATCACAGGCAGAAAC
Human *ABCB1*	GAATGTTCAGTGGCTCCGAG	TGTATGTTGGCCTCCTTTGC
Human *TSC22D3*	GGAATAGGTGCCAAGGATCTGG	GCTTACATCTGGTCTCATGCTGG
Human *BCL2L11*	GGTCCTCCAGTGGGTATTTCTCTT	ACTGAGATAGTGGTTGAAGGCCTGG
Murine *Nfatc1*	CCCGTCACATTCTGGTCCAT	GCTGTAGCGTGAGAGGTTCA
Murine *Nfatc2*	GGCATCCATGAGAACAGCAG	CGATGGTGGCTCTCATGTTG
Human *GUSB*	GAAAATATGTGGTTGGAGAGTTCATT	CGGAGTGAAGATCCCCTTTTTA
Murine *Gapdh*	GCAAAGTGGAGATTGTTGCCAT	CCTTGACTGTGCCGTTGAATTT

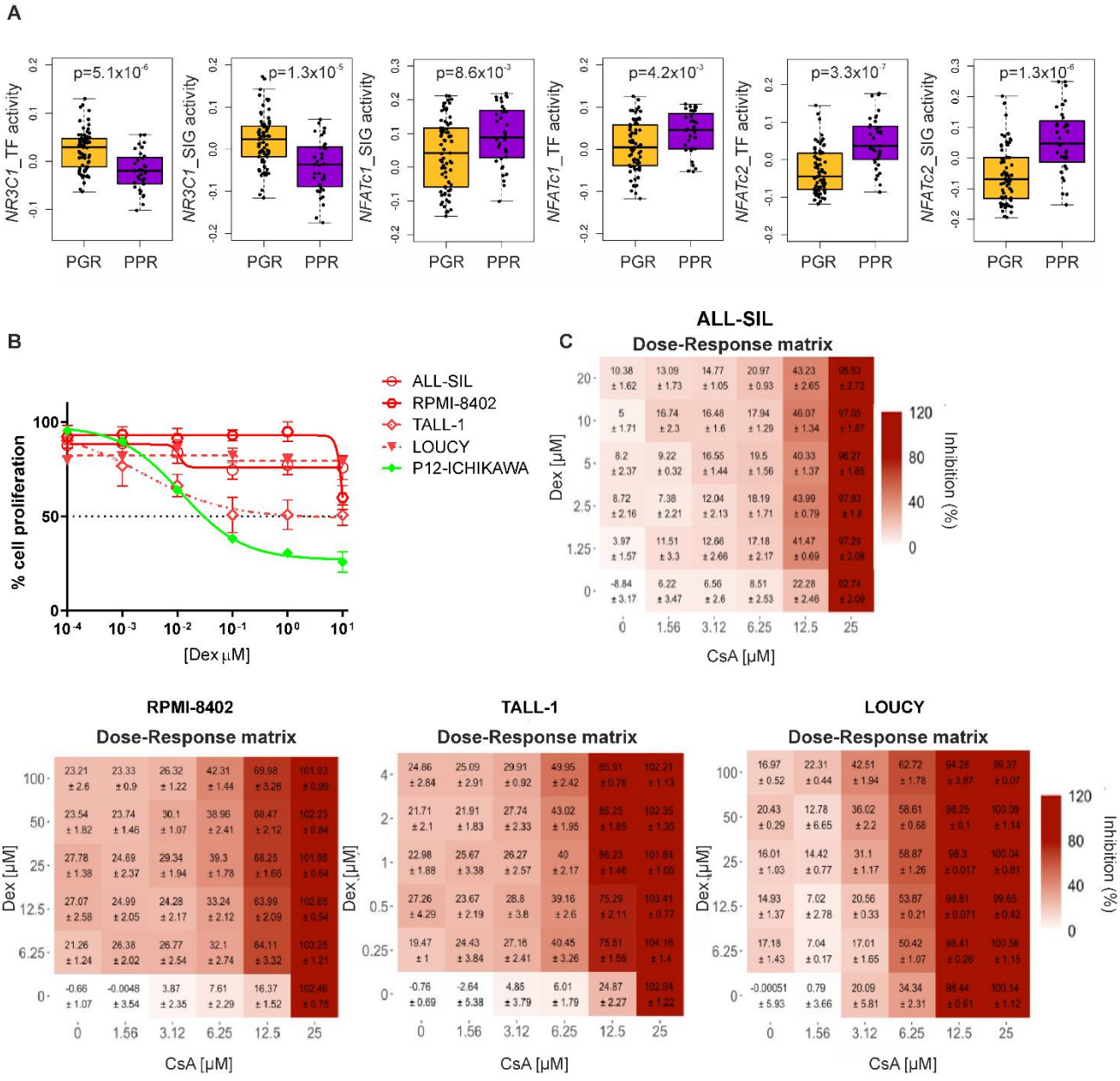

A
NR3C1_TF activity
p=5.1x10-6
NR3C1_SIG activity
p=1.3x10-5
NFATc1_SIG activity
p=8.6x10-3
NFATc1_TF activity
p=4.2x10-3
NFATc2_TF activity
p=3.3x10-7
NFATc2_SIG activity
p=1.3x10-6
PGR
PPR
B
% cell proliferation
[Dex µM]
ALL-SIL
RPMI-8402
TALL-1
LOUCY
P12-ICHIKAWA
C
ALL-SIL
Dose-Response matrix
Dex [µM]
CsA [µM]
Inhibition (%)
RPMI-8402
Dose-Response matrix
TALL-1
Dose-Response matrix
LOUCY
Dose-Response matrix
D
ALL-SIL
RPMI-8402
TALL-1
LOUCY
%AnnexV/PI-
DMSO
Cyclosporin A
Dex
Dex + Cyclosporin A

Figure S1: Diagnosed PPR T-ALL pediatric patients are characterized by high NFATc1 and NFATc2 transcriptional activity and signaling, whose inhibition by CsA reverts GC sensitivity. (A) Box plot showing the *NR3C1*, *NFATc1* and *NFATc2* transcriptional activity and signaling in PPR T-ALL pediatric patients compared to PGR one by NetBid2 inference (*NR3C1* TF activity p-value= $5.1*10^{-6}$; *NR3C1* SIG activity p-value=$1.3*10^{-5}$; *NFATc1* SIG activity p-value=$8.6*10^{-3}$; *NFATc1* TF activity p-value=$4.2*10^{-3}$; *NFATc2* TF activity p-value=$3.3*10^{-7}$; *NFATc2* SIG activity p-value=$1.3*10^{-6}$). (B) Percentage of cell proliferation measured by MTT assay in ALL-SIL, TALL-1, RPMI-8402 and LOUCY GC resistant cell lines treated with increasing concentrations of dex for 48 hours, $n \geq 3$ for all the experiments. (C) Dose-response matrix of cells' proliferation inhibition in ALL-SIL, RPMI-8402, TALL-1 and LOUCY GC resistant cells after 72 hours of treatment with CsA and dex. (D) Percentage of live cells (Annexin V/Propidium Iodide (PI) negative fraction) in ALL-SIL, RPMI-8402, TALL-1 and LOUCY cell lines treated with Growth Inhibition 50 (GI_{50}) value of CsA and dex for 72 hours. Results are presented as means + SEM (paired t test; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$), $n \geq 3$ for all the experiments.

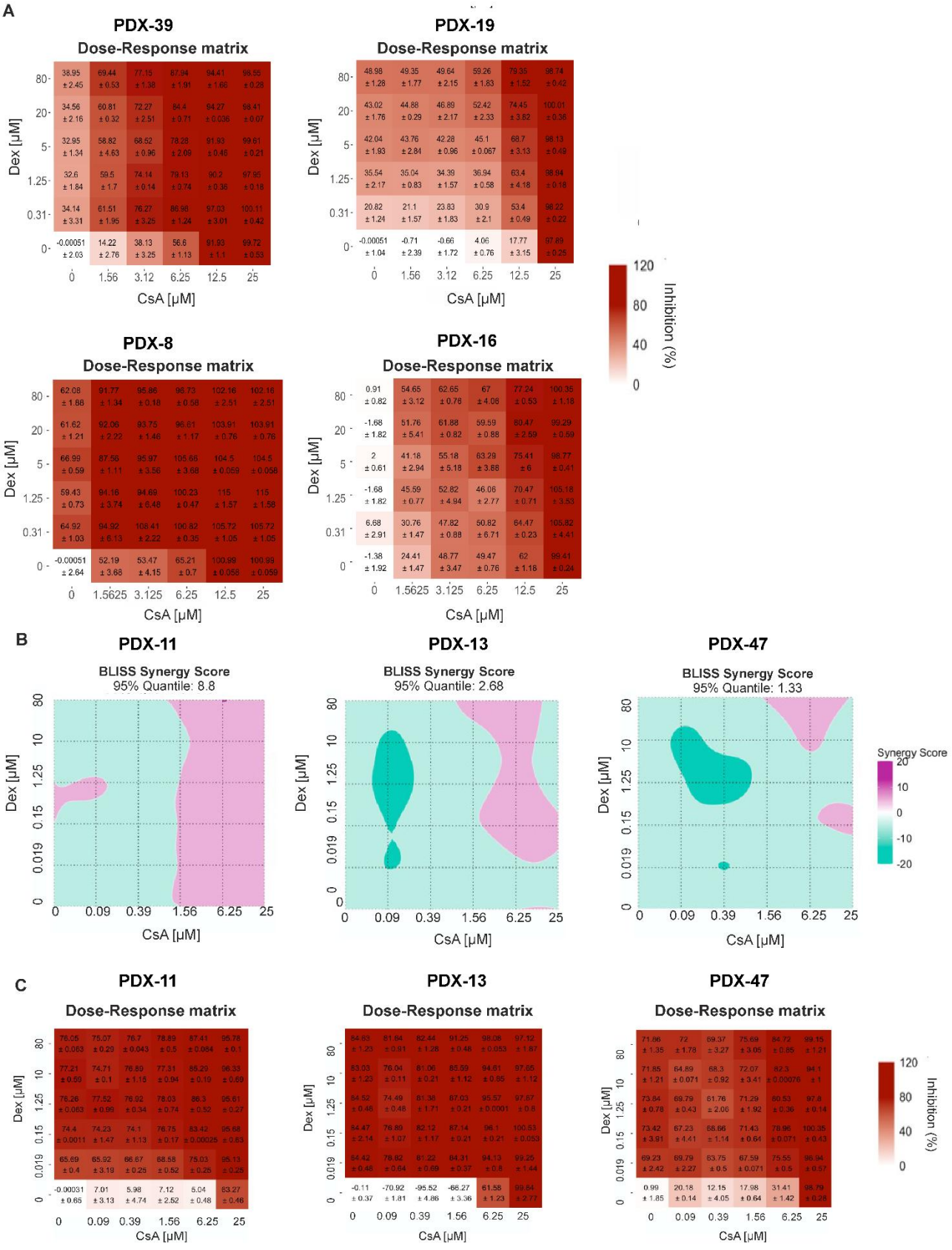

A
PDX-39
Dose-Response matrix
PDX-19
Dose-Response matrix
PDX-8
Dose-Response matrix
PDX-16
Dose-Response matrix
Dex [µM]
CsA [µM]
Inhibition (%)
B
PDX-11
PDX-13
PDX-47
BLISS Synergy Score
95% Quantile: 8.8
95% Quantile: 2.68
95% Quantile: 1.33
Synergy Score
C
PDX-11
PDX-13
PDX-47
Dose-Response matrix

Figure S2: In T-ALL GC sensitive cells, both CsA and dex synergize at high concentrations (A) Dose-response matrix of cells' proliferation inhibition in *ex vivo* primary cells from 4 GC resistant PDX mice after 72 hours of treatment with CsA and dex (B) 2D matrix representing Bliss synergy score of CsA and dex in reducing PDX GC sensitive cells' proliferation after 72 hours of treatment. (C) Dose-response matrix of cells' proliferation inhibition in *ex vivo* primary cells from 3 GC sensitive PDX mice after 72 hours of treatment with CsA and dex.

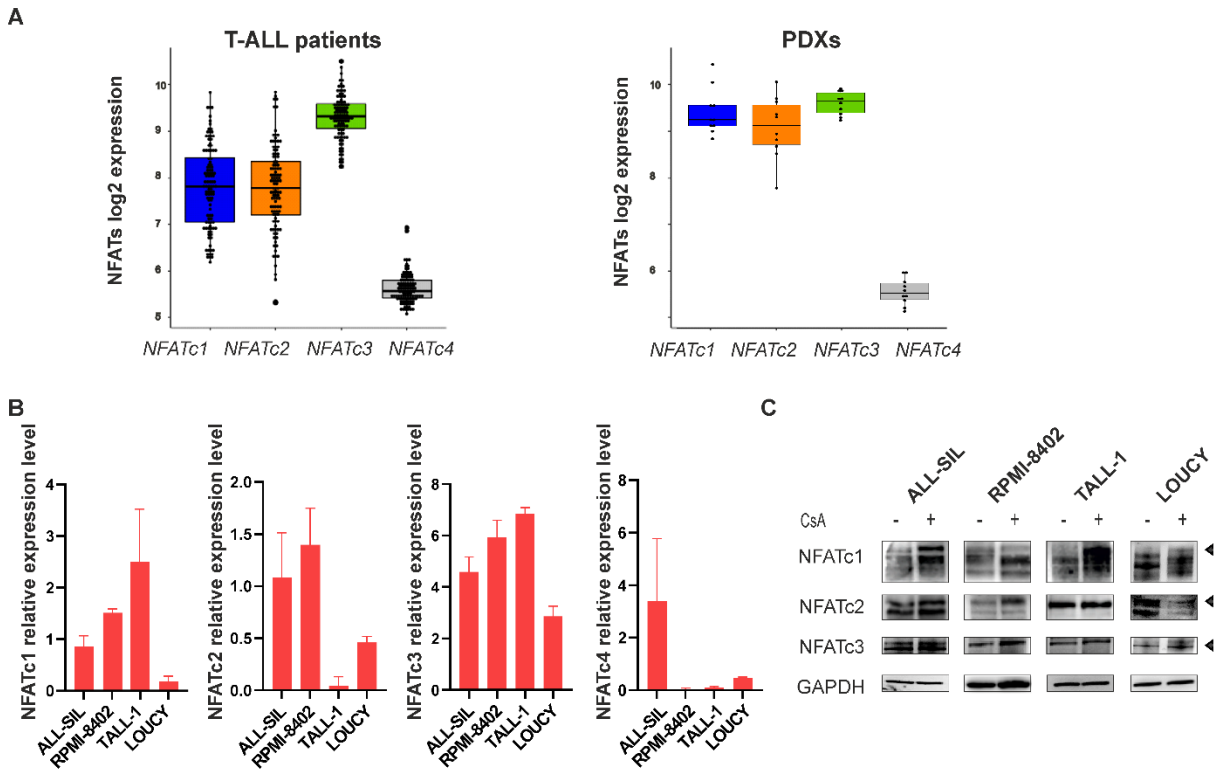


Figure S3: NFATs family expression levels. (A) *NFATc1*, *NFATc2*, *NFATc3* and *NFATc4 log2* expression levels obtained by transcriptome analysis in a cohort of 104 pediatric T-ALL patients at the diagnosis (on the left) and in 10 PDX T-ALL primary cells (on the right). (B) *NFATc1*, *NFATc2*, *NFATc3* and *NFATc4* expression normalized on *Glucuronidase Beta (GUS)* mRNA level in the four GC resistant T-ALL cell lines, measured by RQ-PCR; $n \geq 3$ for all the experiments. (C) Western Blot (WB) analysis of NFATc1, c2, c3 protein expression in ALL-SIL, RPMI-8402, TALL-1 and LOUCY cell lines treated with CsA GI_{50} for 1 hour.

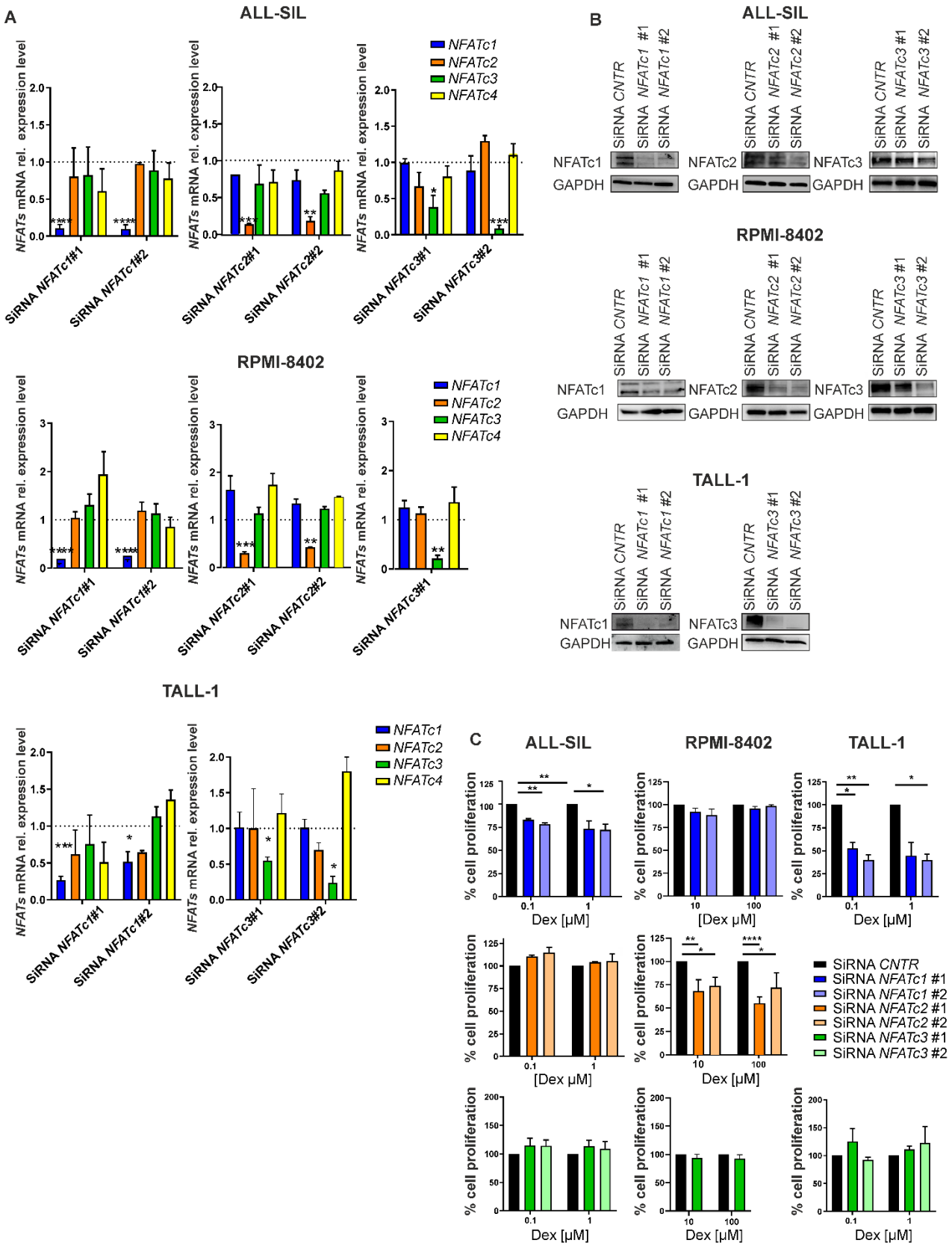

A
ALL-SIL
NFATs mRNA rel. expression level
NFATc1
NFATc2
NFATc3
NFATc4
SiRNA NFATc1#1
SiRNA NFATc1#2
SiRNA NFATc2#1
SiRNA NFATc2#2
SiRNA NFATc3#1
SiRNA NFATc3#2
RPMI-8402
TALL-1
B
ALL-SIL
SiRNA CNTR
SiRNA NFATc1 #1
SiRNA NFATc1 #2
SiRNA NFATc2 #1
SiRNA NFATc2 #2
SiRNA NFATc3 #1
SiRNA NFATc3 #2
NFATc1
NFATc2
NFATc3
GAPDH
RPMI-8402
TALL-1
C
ALL-SIL
RPMI-8402
TALL-1
% cell proliferation
Dex [μM]
SiRNA CNTR
SiRNA NFATc1 #1
SiRNA NFATc1 #2
SiRNA NFATc2 #1
SiRNA NFATc2 #2
SiRNA NFATc3 #1
SiRNA NFATc3 #2

Figure S4: Exclusively *NFATc1* or *NFATc2* specific gene silencing sensitizes T-ALL GC resistant cells to GC treatment. (A) *NFATs* mRNA expression levels measured by RQ-PCR after 24 hours of *NFATc1, c2 or c3* transient specific gene silencing with two different siRNAs in ALL-SIL, RPMI-8402 and TALL-1 cell lines. (B) WB analysis of NFATc1, c2, c3 and GAPDH protein after 48 hours from *NFATc1*, *c2* or *c3* transient gene silencing in ALL-SIL, RPMI-8402 and TALL-1 cells. (C) Percentage of cell proliferation in ALL-SIL, RPMI-8402 and TALL-1 cell lines transiently silenced for *NFATc1* (blue), *NFATc2* (orange) and *NFATc3* (green) gene expression after 48 hours of treatment with increasing concentration of dex. Results are presented as means + SEM (paired t-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$), $n \geq 3$ for all the experiments.

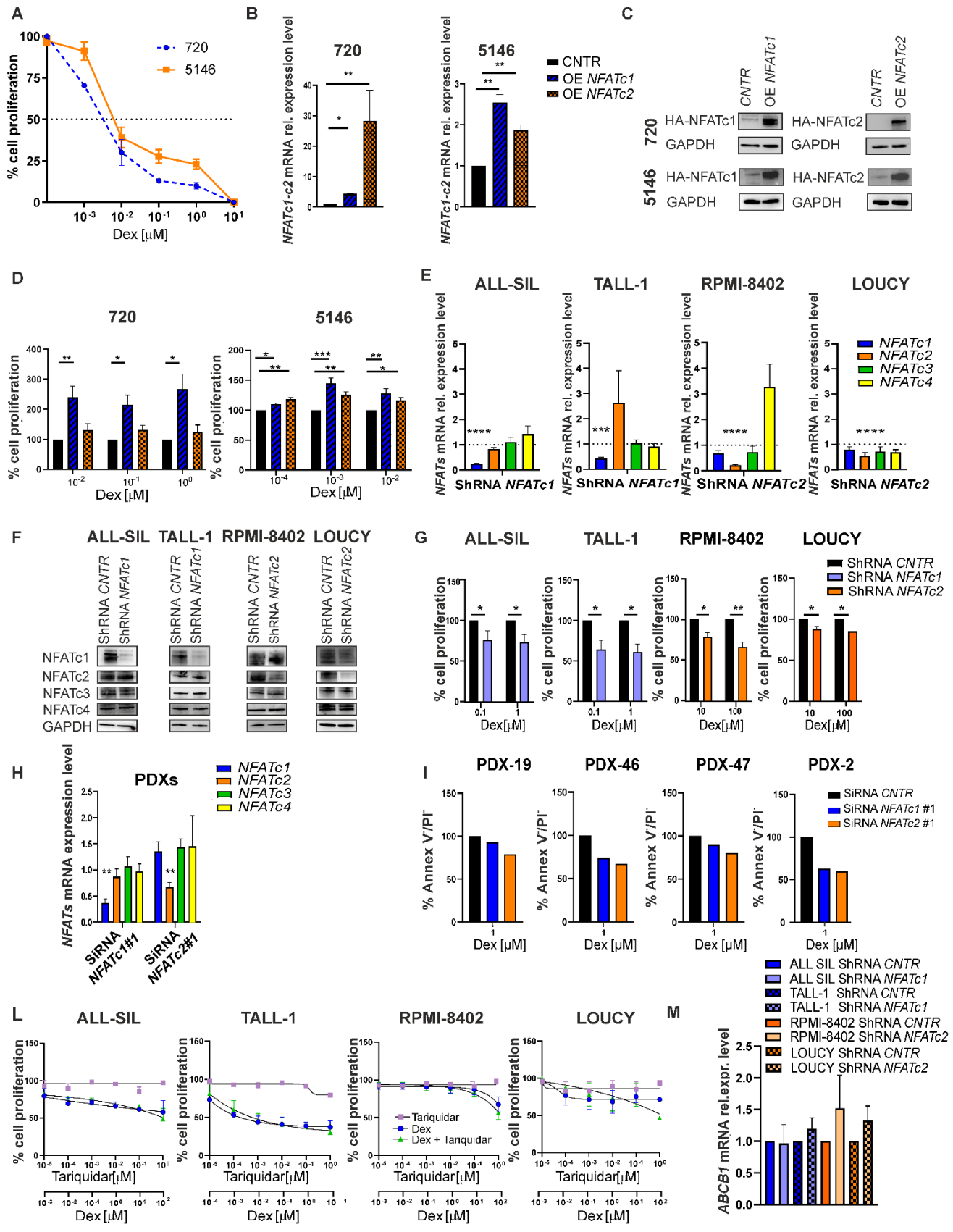

A
% cell proliferation
720
5146
Dex [μM]
B
720
5146
NFATc1-c2 mRNA rel. expression level
CNTR
OE NFATc1
OE NFATc2
C
CNTR
OE NFATc1
OE NFATc2
720
5146
HA-NFATc1
HA-NFATc2
GAPDH
D
720
5146
% cell proliferation
Dex [μM]
E
ALL-SIL
TALL-1
RPMI-8402
LOUCY
NFATs mRNA rel. expression level
ShRNA NFATc1
ShRNA NFATc2
NFATc1
NFATc2
NFATc3
NFATc4
F
ALL-SIL TALL-1 RPMI-8402 LOUCY
ShRNA CNTR
ShRNA NFATc1
ShRNA NFATc2
NFATc1
NFATc2
NFATc3
NFATc4
GAPDH
G
ALL-SIL
TALL-1
RPMI-8402
LOUCY
% cell proliferation
Dex[μM]
ShRNA CNTR
ShRNA NFATc1
ShRNA NFATc2
H
PDXs
NFATs mRNA expression level
NFATc1
NFATc2
NFATc3
NFATc4
SiRNA NFATc1#1
SiRNA NFATc2#1
I
PDX-19
PDX-46
PDX-47
PDX-2
% Annex V+/PI-
Dex [μM]
SiRNA CNTR
SiRNA NFATc1 #1
SiRNA NFATc2 #1
L
ALL-SIL
TALL-1
RPMI-8402
LOUCY
% cell proliferation
Tariquidar[μM]
Dex [μM]
Tariquidar
Dex
Dex + Tariquidar
M
ABCB1 mRNA rel.expr. level
ALL SIL ShRNA CNTR
ALL SIL ShRNA NFATc1
TALL-1 ShRNA CNTR
TALL-1 ShRNA NFATc1
RPMI-8402 ShRNA CNTR
RPMI-8402 ShRNA NFATc2
LOUCY ShRNA CNTR
LOUCY ShRNA NFATc2

Figure S5: NFATc1 and NFATc2 regulate T-ALL cells' response to GC without exploiting the ABC transporters. (A) Percentage of cell proliferation in 720 and 5146 cells treated with increasing concentration of dex for 48 hours. (B) *NFATc1-c2* mRNA expression level in 720 and 5146 CNTR and *NFATc1* or *c2* OE cells measured by RQ-PCR. (C) WB analysis of HA-*NFATc1* and HA-*NFATc2* and GAPDH proteins in 720 and 5146 CNTR and *NFATc1* or *c2* OE cells. (D) Percentage of cell proliferation in 720 and 5146 cells overexpressing (OE) *NFATc1* or *NFATc2* treated with increasing concentrations of dex for 48 hours. (E) *NFATs* mRNA expression level and (F) WB analysis of NFATc1, c2, c3 and GAPDH protein in stable ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down cells as well as in RPMI-8402 and LOUCY *NFATc2* gene silenced cells and relative controls. (G) Percentage of cell proliferation in ALL-SIL and TALL-1 *NFATc1* knock-down (in blue) and in RPMI-8402 and LOUCY *NFATc2* knock-down (in orange) cells and relative controls treated with increasing concentration of dex. (H) *NFATs* mRNA expression level measured by RQ-PCR in PDXs after 24 hours from transient *NFATc1* or *NFATc2* gene silencing. (I) Percentage of viable cells in primary cells from 4 different PDXs after transient *NFATc1* and *c2* gene silencing and 48 hours 1 μM dex treatment. Results are presented as means + SEM (paired t-test; * $p < 0.05$; ** $p < 0.01$), $n \geq 3$ for all the experiments. (L) Percentage of cell proliferation in ALL-SIL, TALL-1, RPMI-8402 and LOUCY after 72 hours of treatment with scalar concentration of tariquidar alone or in combination with dex, $n \geq 3$ for all the experiments. (M) *ABCB1* mRNA relative expression level in ALL-SIL and TALL-1 ShRNA *NFATc1* cells and in RPMI-8402 and LOUCY ShRNA *NFATc2* knock-down cells and the relative controls, $n \geq 3$ for all the experiments.

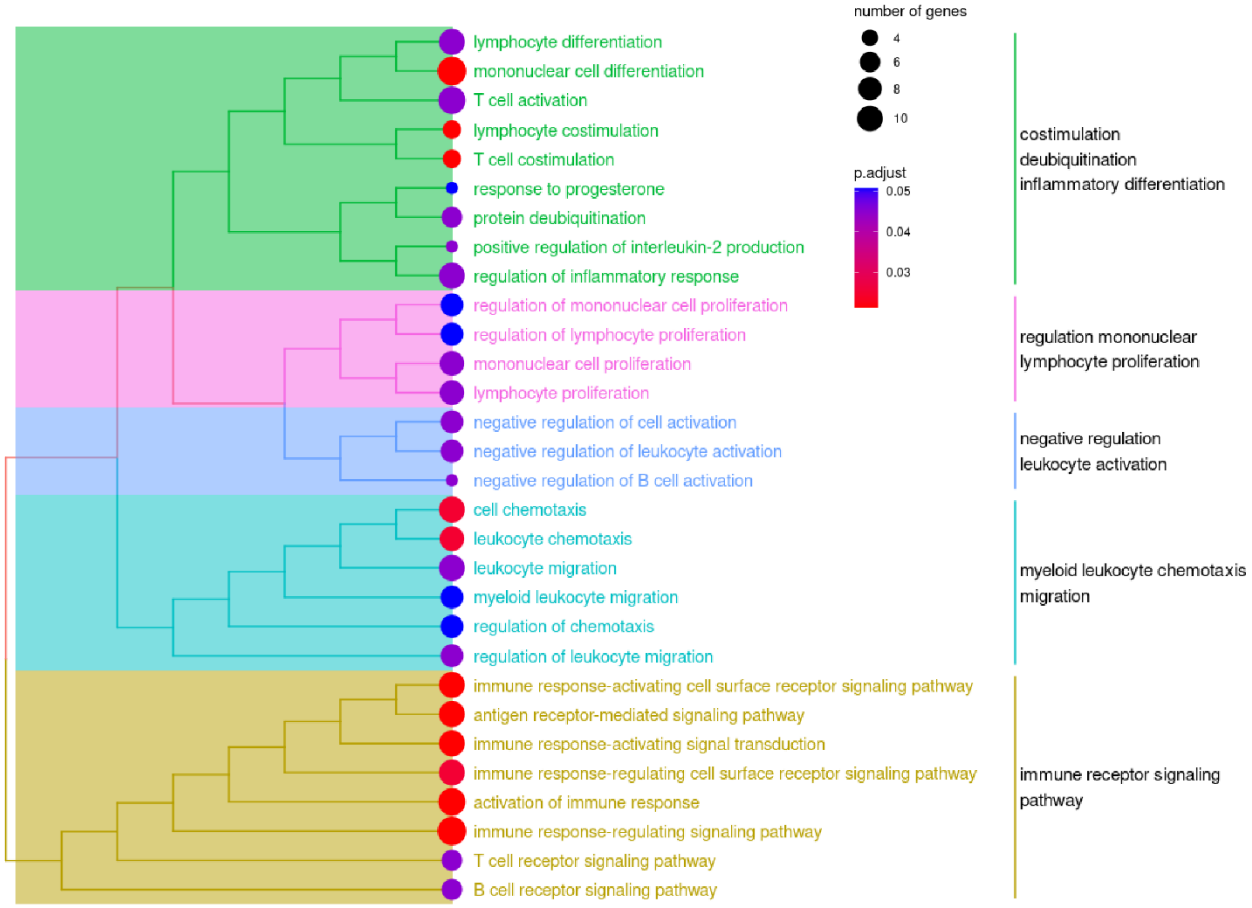
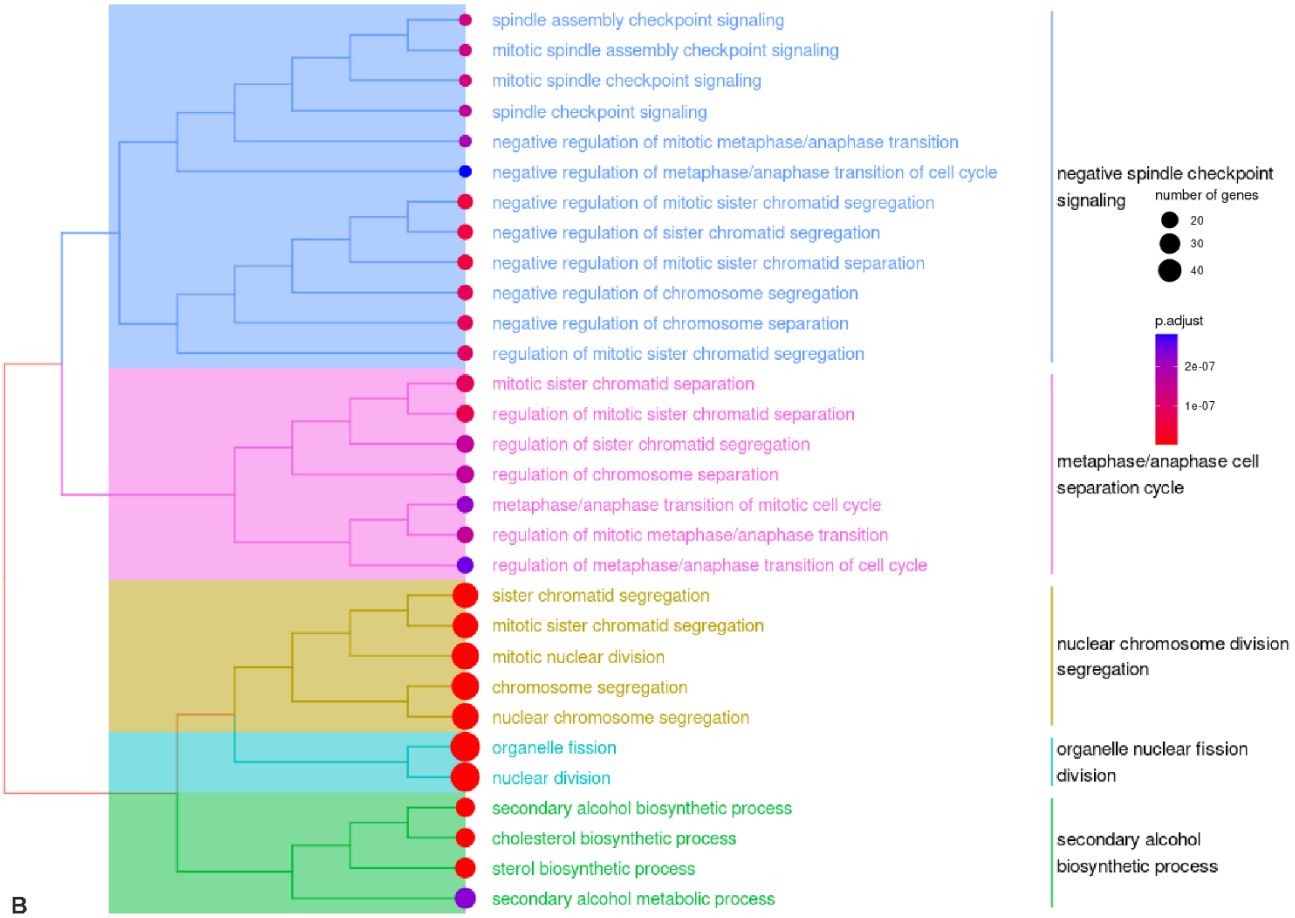


Figure S6: ***NFATc1* specific gene silencing upregulates genes involved in the negative T-cell activation.** Treeplots of over-represented GO-BP on upregulated in TALL-1 ShRNA *NFATc1* knock-down cells compared to controls. Dots size represents the gene count; circles color the adjusted p-value.

A


spindle assembly checkpoint signaling
mitotic spindle assembly checkpoint signaling
mitotic spindle checkpoint signaling
spindle checkpoint signaling
negative regulation of mitotic metaphase/anaphase transition
negative regulation of metaphase/anaphase transition of cell cycle
negative regulation of mitotic sister chromatid segregation
negative regulation of sister chromatid segregation
negative regulation of mitotic sister chromatid separation
negative regulation of chromosome segregation
negative regulation of chromosome separation
regulation of mitotic sister chromatid segregation
mitotic sister chromatid separation
regulation of mitotic sister chromatid separation
regulation of sister chromatid segregation
regulation of chromosome separation
metaphase/anaphase transition of mitotic cell cycle
regulation of mitotic metaphase/anaphase transition
regulation of metaphase/anaphase transition of cell cycle
sister chromatid segregation
mitotic sister chromatid segregation
mitotic nuclear division
chromosome segregation
nuclear chromosome segregation
organelle fission
nuclear division
secondary alcohol biosynthetic process
cholesterol biosynthetic process
sterol biosynthetic process
secondary alcohol metabolic process
negative spindle checkpoint signaling
number of genes
20
30
40
p.adjust
2e-07
1e-07
metaphase/anaphase cell separation cycle
nuclear chromosome division segregation
organelle nuclear fission division
secondary alcohol biosynthetic process

B

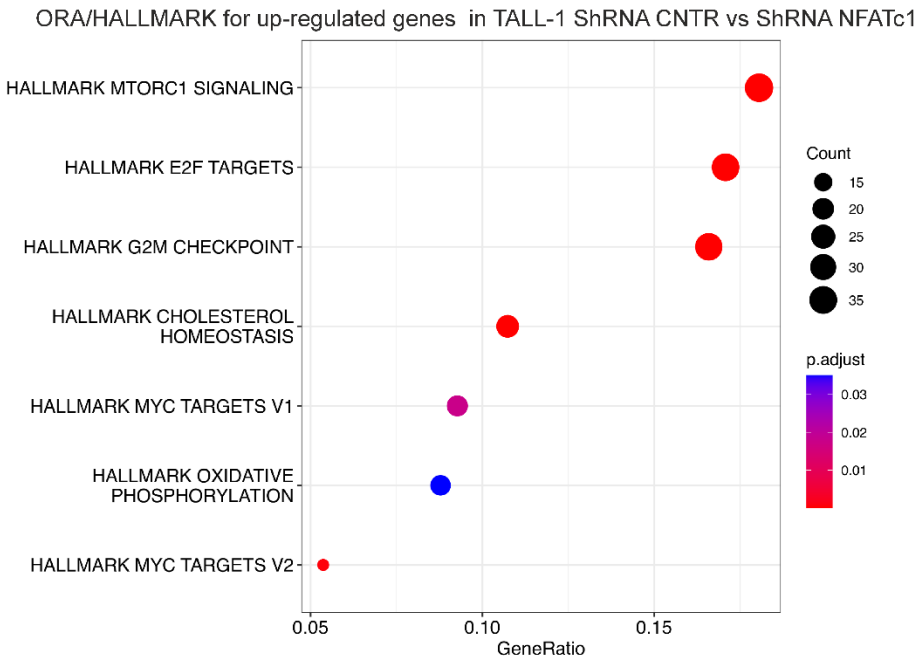

ORA/HALLMARK for up-regulated genes in TALL-1 ShRNA CNTR vs ShRNA NFATc1
HALLMARK MTORC1 SIGNALING
HALLMARK E2F TARGETS
HALLMARK G2M CHECKPOINT
HALLMARK CHOLESTEROL HOMEOSTASIS
HALLMARK MYC TARGETS V1
HALLMARK OXIDATIVE PHOSPHORYLATION
HALLMARK MYC TARGETS V2
0.05
0.10
0.15
GeneRatio
Count
15
20
25
30
35
p.adjust
0.03
0.02
0.01

Figure S7: ***NFATc1* specific gene silencing downregulates genes involved in cholesterol biosynthesis and MYC target.** (A) Treeplots of over-represented GO-BP on downregulated genes and (B) dot plot of downregulated hallmark MgSigDB gene sets in TALL-1 ShRNA *NFATc1* knock-down cells compared to controls. Dots size represents the gene count; circles color the adjusted p-value.

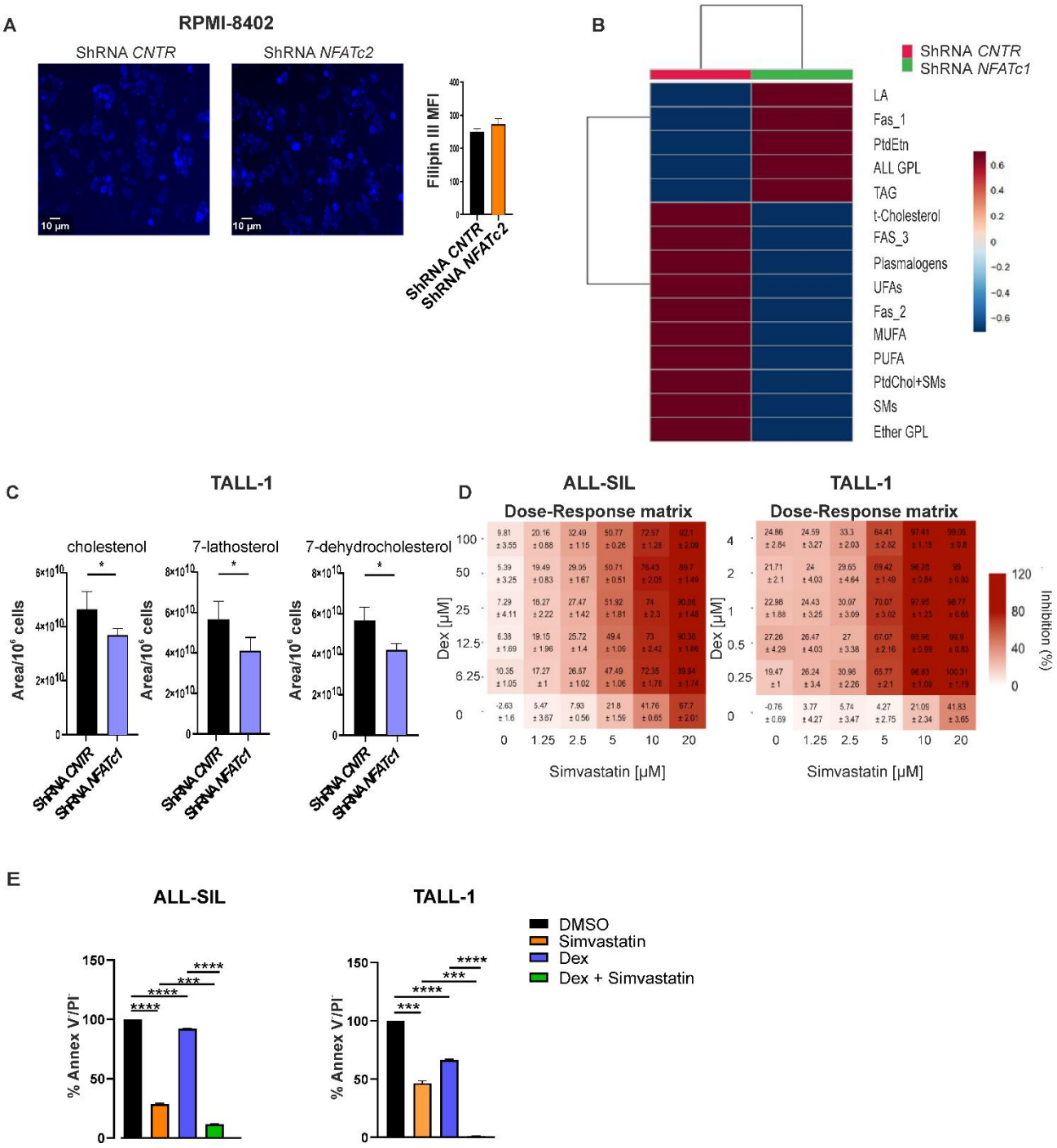


Figure S8: ***NFATc1* specific gene silencing downregulates intracellular cholesterol and simvastatin sensitizes T-ALL cells to dex treatment** (A) Intracellular unesterified cholesterol staining by Filipin III probe in RPMI-8402 ShRNA *NFATc2* knock-down cells and controls. On the right side Filipin III median MFI absolute quantification. (B) Heatmap of intracellular lipidome identified in organic fraction of TALL-1 ShRNA *CNTR* and TALL-1 ShRNA *NFATc1*. Abbreviations: Abbreviations: Fas, fatty acids: (Fas_1, Total Fatty Acyl chains at 1.56 ppm; Fas_2, methylene in FA at 1.29 ppm; Fas_3, ωCH3 methyl in FA at 0.89 ppm); GPL, glycerophospholipids; LA, Linoleic acid; MUFA, monounsaturated fatty acids; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PUFAs, polyunsaturated

fatty acids; SM, sphingomyelin; TAG, triacylglycerides; total cholesterol (t-cholesterol) UFAs, unsaturated fatty acids. (C) Bar plots showing the level of intracellular cholestenol, 7-lathosterol and 7-dehydrocholesterol in TALL-1 ShRNA NFATc1 compared to control cells detected by NMR technology (paired t test; * $p < 0.05$). (D) Dose-response matrix of proliferation reduction in ALL-SIL and TALL-1 GC resistant cells after 72 hours of treatment with simvastatin and dex. (E) Percentage of cell viability of ALL-SIL and TALL-1 cell lines (Annexin V/Propidium Iodide (PI) negative fraction) treated with 10 μM and 20 μM simvastatin and 25 μM or 1 μM dex respectively for 72 hours. Results are presented as means + SEM (paired t test; *** $p < 0.001$, **** $p < 0.000$), $n \geq 3$ for all the experiments.

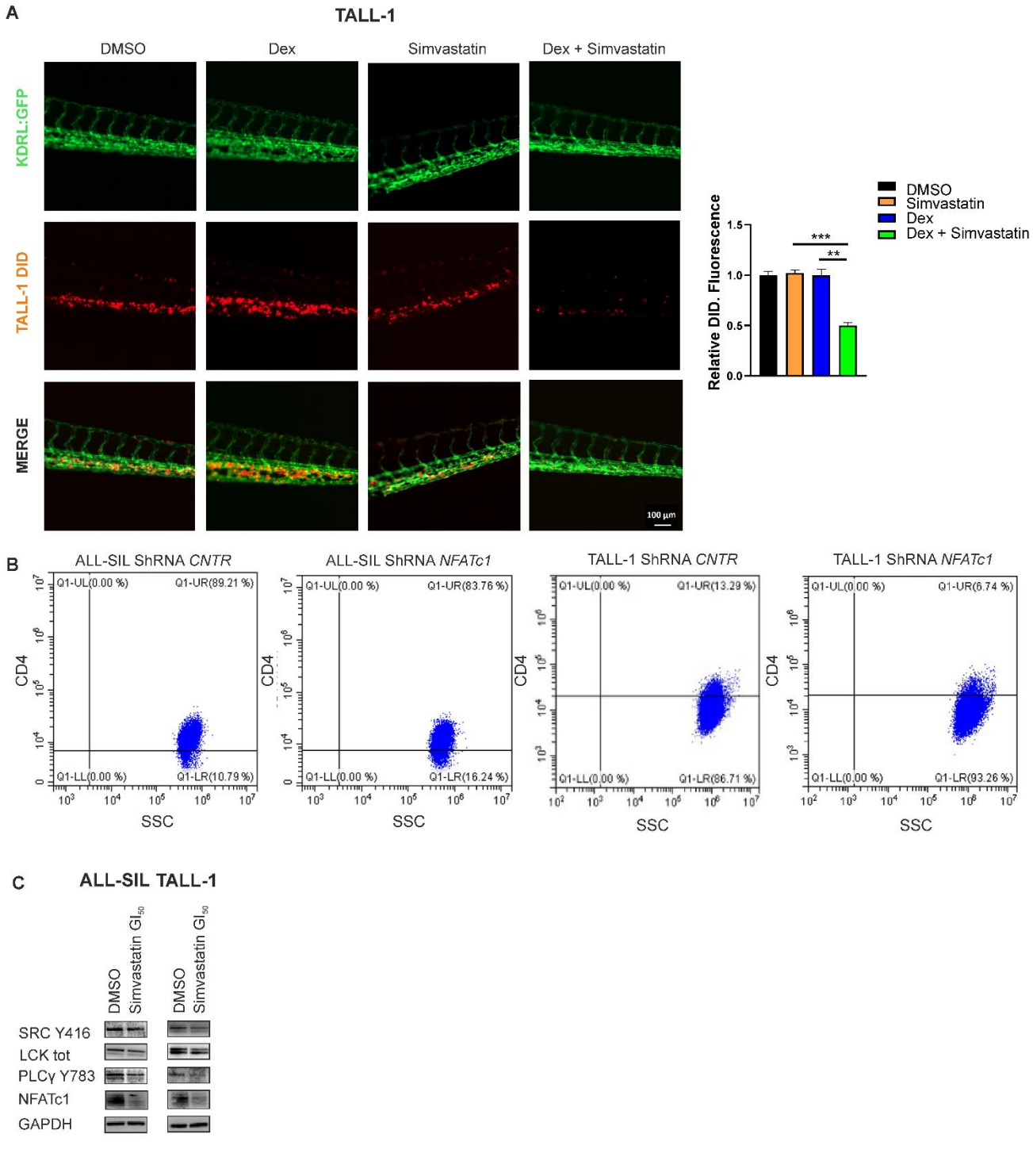

A
TALL-1
DMSO
Dex
Simvastatin
Dex + Simvastatin
KDRL:GFP
TALL-1 DID
MERGE
100 µm
Relative DID. Fluorescence
DMSO
Simvastatin
Dex
Dex + Simvastatin
***
**
B
ALL-SIL ShRNA CNTR
ALL-SIL ShRNA NFATc1
TALL-1 ShRNA CNTR
TALL-1 ShRNA NFATc1
CD4
SSC
Q1-UL(0.00 %)
Q1-UR(89.21 %)
Q1-LL(0.00 %)
Q1-LR(10.79 %)
Q1-UR(83.76 %)
Q1-LR(16.24 %)
Q1-UR(13.29 %)
Q1-LR(86.71 %)
Q1-UR(6.74 %)
Q1-LR(93.26 %)
C
ALL-SIL TALL-1
DMSO
Simvastatin GI50
SRC Y416
LCK tot
PLCγ Y783
NFATc1
GAPDH

Figure S9: **Simvastatin sensitizes T-ALL cells to dex treatment in the *in vivo* zebrafish model and negatively affects the TCR/LCK signaling** (A) Lateral view of the trunk region of Tg(Fli1:GFP) embryos injected with approximately 200 pre-labelled DiD^+ TALL-1 cells pre-treated *in vitro* with 20 μM Simvastatin or vehicle (DMSO) and then treated by adding dex directly into the fish water at a final 1 μM concentration or DMSO for 24 hours. On the right, quantification of the red intensity signal associated with transplanted cells. Scale bar, 100μm. Results are presented as means + SEM (unpaired t-test;** $p < 0.01$; *** $p < 0.001$), $n \geq 3$ for all the experiments. (B) Representative dot plots showing the number of $CD4^+$ cells in ALL-SIL and TALL-1 ShRNA *NFATc1* knock down cells and control cells. (C) WB analysis of NFATc1, SRC Y416, total LCK and PLCγ Y783 protein expression in ALL-SIL and TALL-1 treated with the respective GI_{50} value of simvastatin for 48 hours.

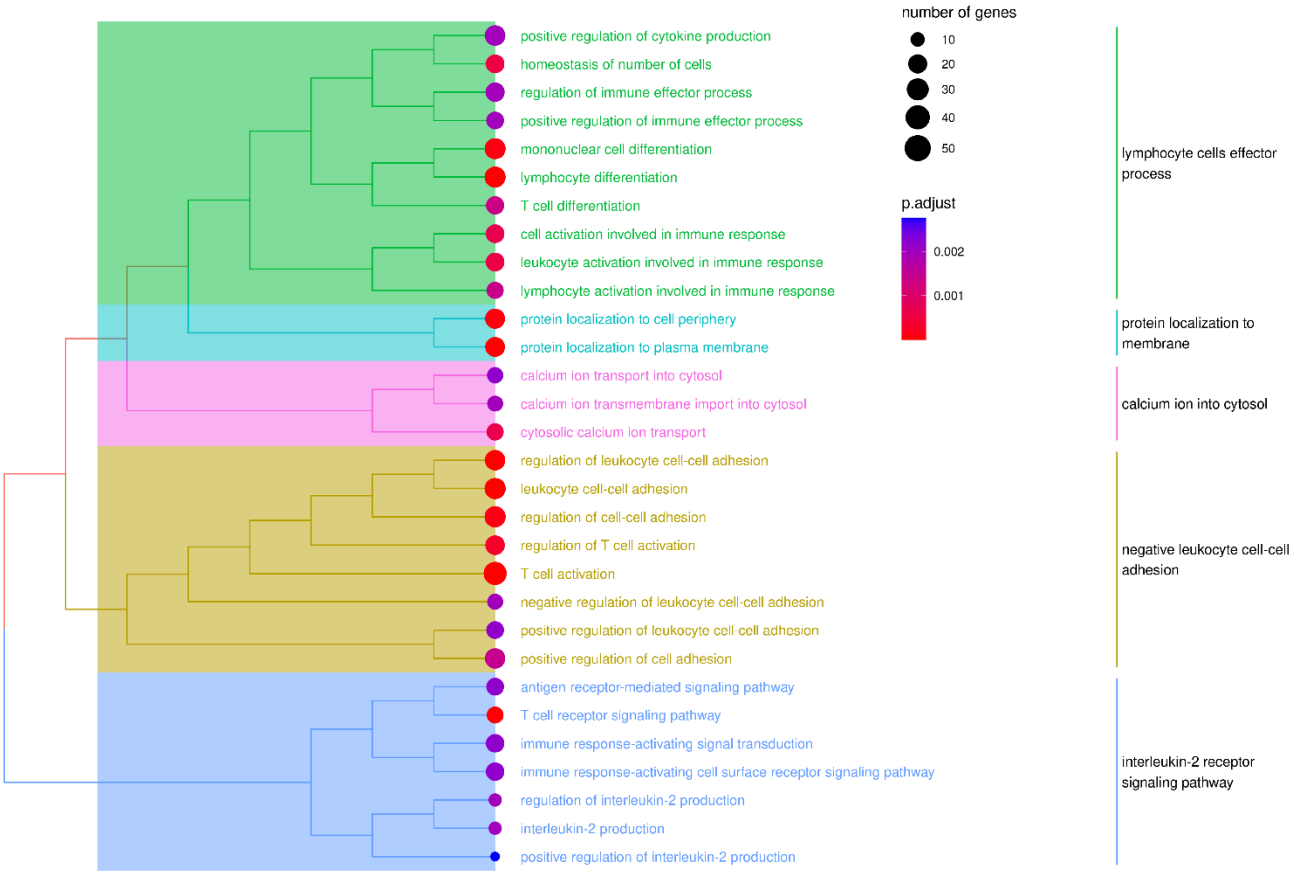


Figure S10: ***NFATc2* gene silencing induces T-cell differentiation.** (A) Treeplot showing upregulated GO-BP obtained by ORA in RPMI-8402 ShRNA *NFATc2* knock-down cells compared to controls. Filled circles size represents the number of genes; circles color the adjusted p-value.

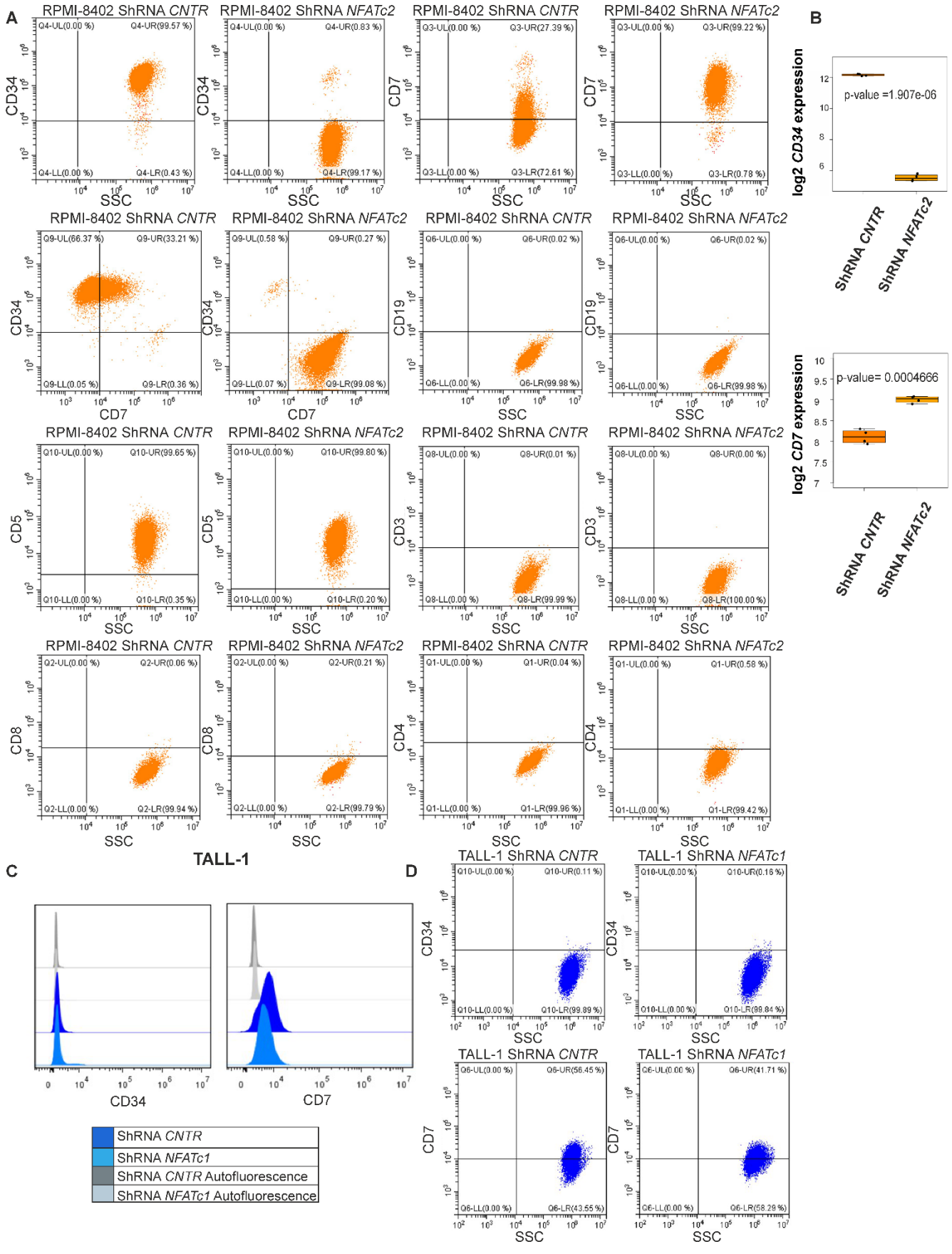

A
RPMI-8402 ShRNA CNTR
RPMI-8402 ShRNA NFATc2
CD34
CD7
CD19
CD5
CD3
CD8
CD4
SSC
B
log2 CD34 expression
p-value =1.907e-06
log2 CD7 expression
p-value= 0.0004666
ShRNA CNTR
ShRNA NFATc2
TALL-1
C
CD34
CD7
ShRNA CNTR
ShRNA NFATc1
ShRNA CNTR Autofluorescence
ShRNA NFATc1 Autofluorescence
D
TALL-1 ShRNA CNTR
TALL-1 ShRNA NFATc1

Figure S11: **Exclusively *NFATc2* gene silencing decreases the percentage of CD34$^+$ T-ALL cells and increases the CD7$^+$ one.** (A) Representative dot plots of CD34$^+$, CD7$^+$, CD5$^+$, CD3$^+$, CD8$^+$, CD4$^+$, CD19$^+$ RPMI-8402 ShRNA *NFATc2* knock down cells and controls and CD34$^+$ signal in relation to CD7$^+$ one. (B) *CD34* and *CD7* mRNA $\log_2$ value expression obtained from GEP analysis in RPMI-8402 ShRNA *NFATc2* knock down cells compared to controls (*CD34* p-value= 1.907 *10^6; *CD7* p-value= 0.0004666, Welch t-test). (C) Representative histogram showing the intensity expression of CD34$^+$ and CD7$^+$ cells in TALL-1 ShRNA *NFATc1* knock-down and control cells. (D) Representative dot plots of CD34$^+$ and CD7$^+$ expression in TALL-1 ShRNA *NFATc1* knock down cells and controls.

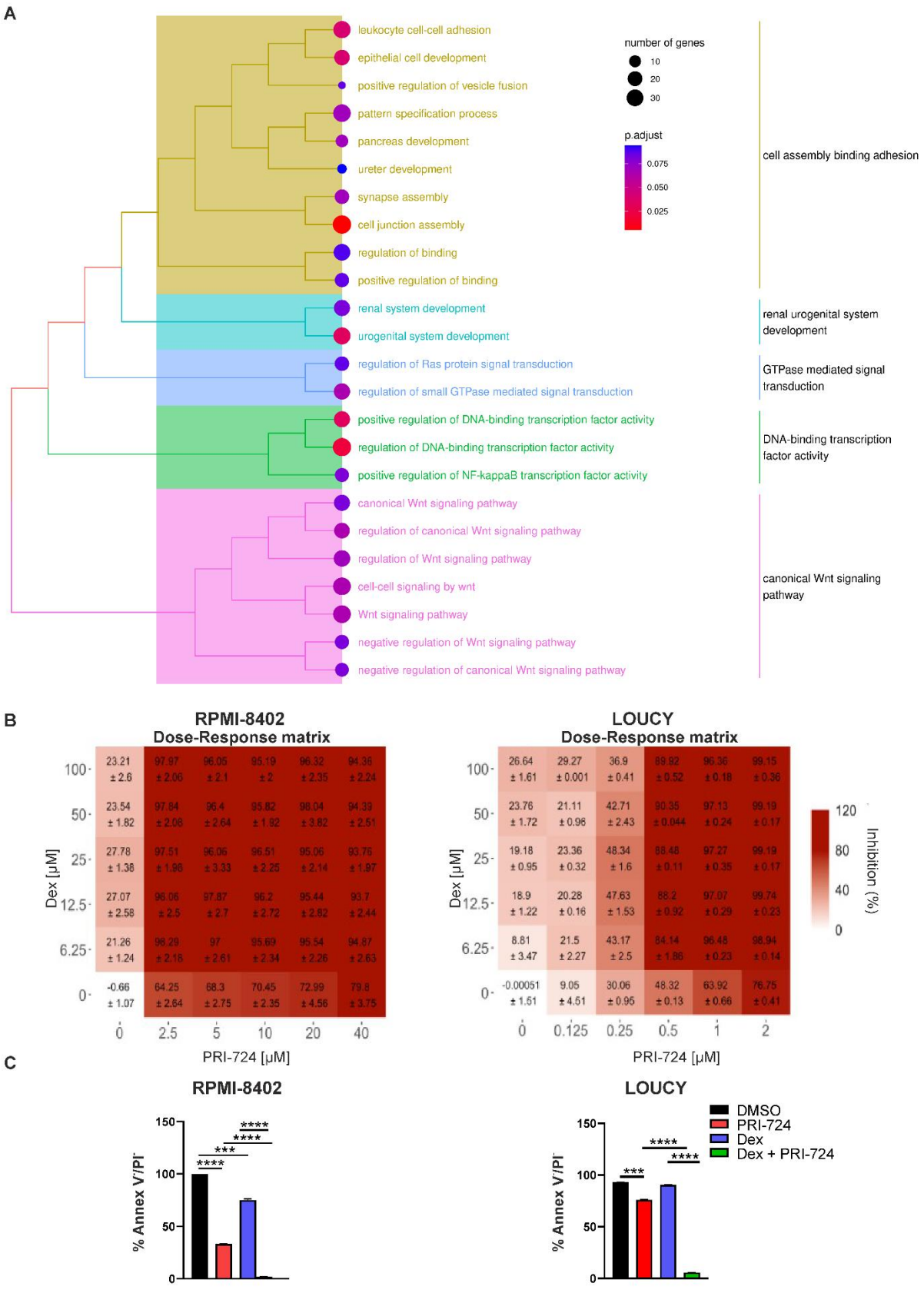

A
leukocyte cell-cell adhesion
epithelial cell development
positive regulation of vesicle fusion
pattern specification process
pancreas development
ureter development
synapse assembly
cell junction assembly
regulation of binding
positive regulation of binding
renal system development
urogenital system development
regulation of Ras protein signal transduction
regulation of small GTPase mediated signal transduction
positive regulation of DNA-binding transcription factor activity
regulation of DNA-binding transcription factor activity
positive regulation of NF-kappaB transcription factor activity
canonical Wnt signaling pathway
regulation of canonical Wnt signaling pathway
regulation of Wnt signaling pathway
cell-cell signaling by wnt
Wnt signaling pathway
negative regulation of Wnt signaling pathway
negative regulation of canonical Wnt signaling pathway
number of genes
10
20
30
p.adjust
0.075
0.050
0.025
cell assembly binding adhesion
renal urogenital system development
GTPase mediated signal transduction
DNA-binding transcription factor activity
canonical Wnt signaling pathway
B
RPMI-8402
Dose-Response matrix
LOUCY
Dose-Response matrix
Dex [μM]
PRI-724 [μM]
Inhibition (%)
C
RPMI-8402
LOUCY
% Annex V+/PI-
DMSO
PRI-724
Dex
Dex + PRI-724

Figure S12: *NFATc2* gene silencing negatively affects WNT/β-catenin pathway whose inhibition restores GC response in T-ALL cells. (A) Treeplot showing downregulated GO-BP obtained by ORA in RPMI-8402 ShRNA *NFATc2* knock-down cells compared to controls. Filled circles size represents the number of genes in over-represented terms, whereas circles color the adjusted p-value. (B) Dose-response matrix of proliferation reduction in RPMI-8402 and LOUCY GC resistant cells after 72 hours of treatment with PRI-724 and dex. (C) Percentage of cell viability in RPMI-8402 and LOUCY cells treated with PRI-724 and dex alone or in combination for 72 hours. Results are presented as means + SEM (paired t test; *** $p < 0.001$; **** $p < 0.0001$), $n \geq 3$ for all the experiments.

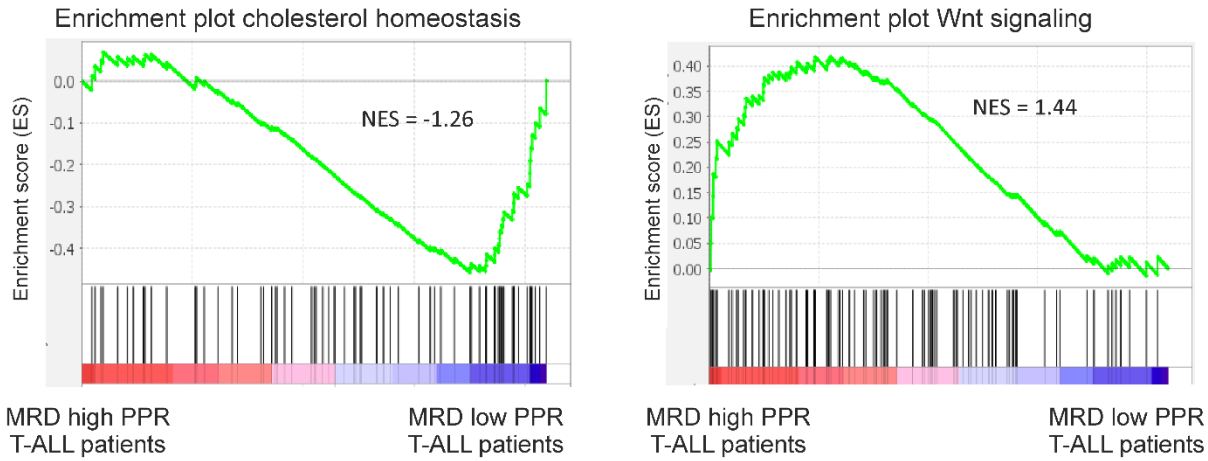


Figure S13: MRD positive and negative PPR T-ALL patients are characterized by an enrichment of genes belonging to Wnt signaling and cholesterol homeostasis respectively. GSEA for cholesterol homeostasis and Wnt signaling in MRD (+78) high and low PPR T-ALL pediatric patients at diagnosis (normalized enrichment score (NES) = -1.26 for cholesterol homeostasis, NES =1.44 for Wnt signaling, p.value<0.25.

EXTENDED METHODS

Cell culture

ALL-SIL (DSMZ, Braunschweig, Germany Cat# ACC-511), TALL-1 (DSMZ Cat# ACC-521), RPMI-8402 (DSMZ Cat# ACC-290, RRID:CVCL_1667), LOUCY (DSMZ Cat# ACC-394) P12-ICHIKAWA (DSMZ Cat# AC-34), 720 and 5146 cell lines were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) with 10-20% Fetal Bovine Serum (FBS; Thermo Fisher Scientific), glutamine (2 mM/L; Thermo Fisher Scientific), penicillin (100 U/mL; Thermo Fisher Scientific) and streptomycin (100 mg/mL; Thermo Fisher Scientific) and maintained at 37°C in a humidified atmosphere with 5% CO_2. Each cell line was periodically tested for mycoplasma infection. All the human cell lines were purchased from DSMZ whereas the 720 and 5146 mouse cell lines were kindly gifted by Prof. Ntziachristos and Prof. Kelliher. Primary cells from patients derived xenograft (PDX) were cultured in minimum essential medium α (Thermo Fisher Scientific) with 10% FBS, 10% human serum (Thermo Fisher Scientific), penicillin (100 U/mL), human IL-7 (10 ng/mL; Peprotech, Cranbury, NJ, USA), human stem cell factor (50 ng/mL; Peprotech, Rocky Hill, NJ), human FLT3-ligand (20 ng/mL; Peprotech), and insulin (20 nM; Sigma-Aldrich, St. Louis, MO).

In vitro treatments

To assess GC sensitivity, ALL-SIL, TALL-1, RPMI-8402, LOUCY and P12-ICHIKAWA cell lines were treated with increasing concentration of dex dissolved in DMSO (D1756, Sigma-Aldrich,). Of note, the percentage of cell proliferation above or below 50% at 1 µM of dex was used as cut-off to select T-ALL

GC resistant and sensitive cell lines respectively [1]. ALL-SIL, TALL-1, RPMI-8402 SiRNA NFATc1, NFATc2 or NFATc3 and controls were treated with dex for 48 hours (0.1-1 μM for ALL-SIL and TALL-1, 10-100 μM for RPMI-8402). Similarly, ALL-SIL and TALL-1 ShRNA NFATc1, as well as RPMI-8402 and LOUCY ShRNA NFATc2 stable knock-down cells were treated with increasing concentration of dex (0.1-100 μM) for 48 hours. The 720 and 5146 mouse T-ALL cell lines overexpressing NFATc1 or NFATc2 and controls were treated with increasing concentration of dex (0.0001-1 μM). ALL-SIL, TALL-1, RPMI-8402 and LOUCY cell lines were treated with increasing concentration of tariquidar and dex alone or in combination for 72 hours. To assess the influence of cholesterol in GCs response, ALL-SIL and TALL-1 NFATc1 knock-down cells as well as the control cells were cultivated or not in medium plus cholesterol 1X (S5442, Sigma-Aldrich) for 24 hours, followed by 48 hours of dex (1-10 nM) treatment in normal medium or in medium plus cholesterol 1X. Similarly, RPMI-8402 and LOUCY cell lines were pre-treated with exogenous WNT3a ligand 30 ng/mL for 24 hours and followed by dex (0.1-10 μM) for 48 hours. Cell proliferation after all treatments was assessed by the MTT test.
To assess the impact of Calcineurin/NFAT pathway inhibition by CsA in combination with GC on T-ALL cells' viability , ALL-SIL, TALL-1, RPMI-8402 and LOUCY cell lines were treated with the CsA GI_{50} value (6,2 μM ALL-SIL, 2 μM TALL-1, 15 μM RPMI-8402 and 8 μM LOUCY) and a fixed concentration of dex (10 μM ALL-SIL, 1 μM TALL-1, 50 μM RPMI -8402 and 8 μM LOUCY) for 72 hours. Primary T-ALL cells derived from PDXs transiently silenced for NFATc1 or NFATc2 were treated with 1 μM dex, after 24 hours of siRNA transfection. Similarly, to evaluate the effect of cholesterol biosynthesis and WNT/β-catenin signaling inhibition in combination with dexamethasone on T-ALL GC resistant cells' viability, ALL-SIL and TALL-1 were treated with 10 μM simvastatin for 24 hours and then treated with dex (25 μM ALL-SIL and 1 μM TALL-1), RPMI-8402 and LOUCY cell lines were pre-treated with 2.5 μM and 1 μM PRI-724 respectively for 24 hours and then treated with 6.25 μM dex for 48 hours. Apoptosis of cell lines treated with CsA, simvastatin, PRI-724 alone or in combination with dexamethasone, as well as in NFATc1 or NFATc2 silenced cells was assessed by the AnnexinV–FLUOS staining kit.

Transcriptome analysis

Transcriptome data from 104 T-ALL pediatric patients at diagnosis, belonging to the AIEOP-BFM ALL2000/R2006 therapeutic protocol, and 10 T-ALL patient derived xenograft (PDX) were available and previously collected [2,3]. GEP data were obtained by GeneChip Human Genome U133 Plus 2.0 assays, normalized using RMA algorithm and processed with sva R package for batch effect removal. NetBID2 (v2.0.3, https://jyyulab.github.io/NetBID/) [4], a data-driven network-based inference pipeline was applied to identify driver transcription factors (TFs) and signaling factors (SIGs) in T-ALL patients. SJARACNe algorithm was applied with default parameters (IQR threshold 0.5 and IQR.loose_thre 0.1) to reconstruct the interactome, based on transcriptomic data. Then, NetBID2 allowed us to calculate TFs and SIGs expressions and activities, and to identify driver TFs and SIGs in PPR and PGR patients. In detail, Bayesian method was used for differential gene expression and driver activity analyses, with gaussian error distribution and pooling set to "full". Volcano plot was built using a customized function based on draw.volcanoPlot, setting logFC threshold to 0.03 and p-value threshold to 0.05. Pearson correlation was computed and represented using Hmisc (v5.1-0) and corrplot (v0.92) R packages. Hierarchical clustering analysis on patients was computed using Euclidean distance and Ward.d distance. Gene Set Enrichment Analysis (GSEA) between PPR HR vs non-HR patients according to MRD values at day +78, was

performed using “cholesterol homeostasis” and “WNT signaling” gene set in MSigDB database collection.

Total RNA was extracted from TALL-1 ShRNA *NFATc1* and from RPMI-8402 ShRNA *NFATc2* knock-down cells and controls after 12 hours of 1 μM and 10 μM dex treatment respectively using the RNeasy Mini Kit (Qiagen). Similarly, total RNA was extracted from P12-ICHIKAWA and TALL-1 cells treated or not with 1 μM dex for 12 hours. Next, the RNA was quantified using the Qubit RNA High Sensitivity Assay (Thermo Fisher Scientific) and the RNA quality was verified by Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, DE). Transcriptomic data were obtained using Clariom S Human arrays (Affymetrix, Santa Clara, CA, USA). Specifically, CEL files were normalized using the Robust Multichip Average (RMA) algorithm of the oligo-R package (v1.58.0). Differentially expressed genes (DEGs) in TALL-1 ShRNA *NFATc1* knock-down (470 genes downregulated and 114 upregulated) and RPMI-8402 ShRNA *NFATc2* knock-down cells DEGs (755 upregulated and 826 downregulated) were computed using Significant Analysis of Microarray R package (samr package, v3.0), with a False Discovery Rate (FDR) cut-off of 0.05. DEGs in P12-ICHIKAWA (142 upregulated and 95 downregulated) and TALL-1 cells (12 upregulated and 1 downregulated) treated or not with dex were computed using Linear Model for Microarray Data R package (limma R package, v3.50.3), adjusted p-value < 0.05. GSEA was performed in TALL-1 ShRNA *NFATc1* knock-down cells treated or not with dex, as well as in RPMI-8402 ShRNA *NFATc2* knock-down cells, in presence or absence of dex, using GSEA v4.2.2 with genes ranked by signal-to noise ratio and statistically significant gene sets determined by 1000 permutations and gene set permutations. Probe sets were collapsed to genes using max probe mode. A p-value cut-off of 0.05 was used in both the comparisons. MSigDB c5: GO:BP:Response to corticosteroid was used as tested gene set. Moreover, Over Representation Analyses (ORA) were applied to determine DEGs functional relationships of each comparison (i.e TALL-1 ShRNA *NFATc1* knock-down cells and the relative control, as well as for RPMI-8402 ShRNA *NFATc2* gene silenced cells compared to control ones). ORA analyses were performed with clusterProfiler R package (v4.2.2) using Gene Ontology (GO-BP) (www.geneontology.org) and MSigDB (www.gsea-msigdb.org) databases (c2: Chemical Genetic Perturbation (CGP) and Hallmarks) with an adjusted p-value cut-off of 0.1. The treeplot function (enrichplot v1.14.2 R package) was applied to generate ward.D hierarchical clustering after pairwise similarities of enriched terms calculated using JACCARD’S similarity coefficient (enrichplot v1.14.2 R package) and clusterProfiler. Data was deposited in the GEO repository at accession number: GSE254001.

Xenotransplantation model and *in vivo* drug treatment

The Tg(fli1: EGFP) zebrafish embryos were raised, staged, and maintained, as previously described [5,6]. Two-day-old Tg(fli1:EGFP) zebrafish embryos were anesthetized (0.003% tricaine) and positioned on 3% agarose. TALL-1 ShRNA *NFATc1*, RPMI-8402 ShRNA *NFATc2*, and control cells were labeled with Vybrant® DIL (Invitrogen) following the manufacturer’s protocol. Approximately 200 cells were resuspended in PBS (pH 7.4) and implanted using a pneumatic picopump equipped with borosilicate glass capillary needles (OD/ID: 1.00/0.75 mm, WPI, USA) within the duct of Cuvier of each embryo. After the injection procedure, the embryos were raised at 34°C and animals showing less than 100 cells after 2 hours from the transplant were discarded from the analysis. The TALL-1 ShRNA *NFATc1* and ShRNA *CNTR* injected embryos were incubated with 1 μM dex dissolved in fish water (0.3 g/L “Instant Ocean” Sea Salts and 0.08 g/L $CaSO_4*2H_2O$), whereas the RPMI-8402 ShRNA *NFATc2* cells embryos

and relative controls were incubated with 10 μM dex and each animal was photographed live with a Zeiss LSM800 confocal microscope after 24 hours treatment. Similarly, non-fluorescent TALL-1 cells were pre-treated in vitro with 20 μM simvastatin, and labeled with the Vybrant® DiD cell-labeling solution (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Approximately 200 TALL-1 cells pretreated or not with simvastatin were resuspended in PBS (pH 7.4) and implanted in zebrafish embryos as previously described. Following, a treatment with 1 μM dex dissolved in fish water was performed. At least 30 embryos per group were analyzed from three independent experiments. Embryos incubated with DMSO only were used as control.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The effect of drug treatments or NFATs gene silencing on cell lines and primary T-ALL cells' proliferation was measured using MTT assay. Specifically, an equal number of cells were plated in triplicate in a 96-well plate and incubated with 10 μl MTT (Sigma-Aldrich) for 4 hours. The Growth Inhibition 50 (GI_{50}, compound concentration required to inhibit cell proliferation by 50%) was calculated by plotting the data as a logarithmic function of (x) when viability was 50%. Control cells viability was set to 100%.

High-Throughput drug synergism screening (HTS)

The HTS was performed as previously described [7]. Briefly, 13000-20000 c/well of primary T-ALL cells derived from PDXs were seeded in 384-well plates in 18 μl of RPMI growth medium with 20% FBS, 10 ng/mL human IL-7, 50 ng/mL human stem cell factor, 20 ng/mL human FLT3-ligand and 20 nM insulin and were immediately pre-treated with 2 μl of 10X CsA. After 24 hours, cells were then treated with 2 μl of 10X dex as single treatments or in combination in a 6x6 matrix design. 7000c/well of GC resistant T-ALL cell line models were seeded in 384-well plates in 24 μl of complete RPMI growth medium. T-ALL cells were immediately pre-treated with 3 μl of 10X DRUG (Simvastatin for ALL-SIL and TALL-1 and PRI-724 for RPMI-8402 and LOUCY. After 24 hours, cells were treated with 3 μl of 10X dex as single treatments or in combination in a 6x6 matrix design. Each drug was tested in a 6-point 2-fold dose-response curve, and each dose was tested in duplicate within each plate, in two independent experiments. A gas-permeable sealing membrane (Breathe-Easy, Merck, Readington Township, NJ) was used to avoid evaporation during treatment incubation. After 48 hours of treatment, 3 μl of resazurin (10X) was added to each well to reach a final concentration of 44 μM, incubated for 3 hours at 37°C, and then the fluorescence signal was measured at 590 nm using a multi-well plate reader (Spark, Tecan, Männedorf, Switzerland). To ensure treatment reproducibility, all procedures, including cell seeding, drug dilutions, cell treatments and application of resazurin solution, were carried out through a 96-channel robotic liquid handler (Microlab STAR 96-CORE, Hamilton, Bonaduz, Switzerland). The Z-prime (Z') quality control metrics were calculated for each plate to measure the separation between the positive control (1 μM bortezomib) and negative control (0.5% DMSO). For this screening, only plates with acceptable quality metrics (Z' > 0.4) were further analyzed. Raw data were normalized according to the following equation: cell viability (%) = $x-POSNEG-POS*100$, where x is the relative fluorescence units (RFU) collected from each single well, NEG is the mean of intraplate negative controls (0.5% DMSO) and POS is the mean of intraplate positive controls (1 μM bortezomib). SynergyFinder R package was applied to compute the Bliss synergy score.

Annexin V/Propidium Iodide (PI) staining

Annexin V–FLUOS staining kit (Roche, Basel, Switzerland) was used to detect the percentage of cell viability, apoptosis and necrosis of cell lines and primary cells treated with CsA, simvastatin or PRI-724 as well as in *NFATc1* or *NFATc2* silenced cells in combination with dexamethasone. Samples were

analyzed by flow cytometric analysis (Cytomics FC500, Beckman Coulter, CA). DMSO-treated cells' vitality was set to 100%.

NFATs transient and stable gene silencing and overexpression

For transient gene silencing, two different Small-interfering (si)RNA (Thermo Fisher Scientific, WLM, USA) sequences targeting the coding region of each NFAT member were used at 50 pM to selectively silence human *NFATc1, c2 or c3* gene. A negative control siRNA (stealth RNAiTM siRNA Negative Control, Thermo Fisher Scientific) was used as control in each experiment. Cell transfection was performed using the Nucleofector systems (Amaxa Biosystems, Lonza Sales Ltd., Basel, Switzerland) according to the manufacturer's instructions as previously described [8]. *NFATs* specific gene silencing was verified by RQ-PCR after 24 hours and by WB analysis after 48 hours from siRNA transfection. Specifically, *NFATc1*, *NFATc2* and *NFATc3* transient gene silencing was performed in ALL-SIL, TALL-1 and RPMI-8402, except for *NFATc2* gene silencing that was conducted only in ALL-SIL and RPMI-8402, since by RQ-PCR data *NFATc2* is not expressed in TALL-1. *NFATc1* or *NFATc2* stable gene knock-down was obtained employing lentiviral plasmids containing *NFATc1* or *NFATc2* short hairpin RNA (ShRNA) expression cassette and as reference a control ShRNA sequence (ShRNA *CNTR*) from a lentiviral construct carrying a scramble RNA (Merck, Darmstadt, DE). In detail, the viral particles were produced by transfecting 293T cells (ATCC, Manassas, VA) with the 2nd-generation packaging plasmids. Specifically, lentiviral vector-containing supernatants were collected after 48 hours of transfection, centrifuged to eliminate 293T cells and filtered with a 0.45 μM filter to discard any cellular debris. Viral particles were titrated by serial dilution on NIH-3T3 cells (DSMZ Cat# ACC-59, RRID: CVCL_0594). One microgram of p24 equivalent of lentiviral vector-containing supernatant was used to transduce $3*10^6$ cells, centrifuging at 32°C for 2.5 hours at 2500 Revolutions Per Minute (RPM). After infection, transduced cells were seeded and selected by culturing in medium with 1 μg/μL puromycin. *NFATc1* or *NFATc2* specific gene silencing was verified by RQ-PCR and by WB experiments. 720 and 5146 mouse T-ALL cells ($0.2*10^6$) were transduced, at the multiply of infection (MOI) 100, with the lentiviral vector PGK.GFP LV containing a GFP sequence and the *NFATc1* or *c2* mouse gene sequence conjugated with the human influenza hemagglutinin (HA) or with the control lentiviral vector containing the GFP tag sequence [9]. Transduction was performed in medium serum free for 12 hours. 720 and 5146 transduced cells have been left to grow at least for one week and then fluorescence-activated cell sorting (FACS) analysis was used to select GFP^+ cells and exclude the pseudo transduction products. *NFATc1* or *NFATc2* mRNA overexpression was verified by RQ-PCR and the increase in NFATc1 or c2 protein level was determined by WB using an anti-HA antibody.

Immunoblotting

Whole cell protein lysates were prepared in RIPA buffer (Sigma-Aldrich) plus protease and phosphatase inhibitors (Sigma-Aldrich) as previously described [10] and following the manufacturer's instructions. Protein concentration in each sample was assessed using the BCA protein assay (Thermo Fisher Scientific). Protein lysates were analyzed by SDS–polyacrylamide gel electrophoresis as previously described. The following primary antibodies were used: NFATc1 (Santa Cruz Biotechnology, CA, USA, Cat# sc-7294, RRID:AB_2152503) NFATc2 (Santa Cruz Biotechnology, Cat# sc-7296, RRID:AB_628012), NFATc3 (Abcam, CB, UK, Cat# ab245501), NFATc4 (Abcam, Cat# ab3447, RRID:AB_303809), SRC Y416 (Cell Signaling Technology, MA, USA, Cat # CS2101,), LCK (Cell Signaling Technology, Cat # CS2657 and CS2752), GAPDH (Genetex, Irvine, CA, USA, Cat# GTX 8627408), Phospholipase C Gamma (PLCγ Y783) (GeneTex Cat# GTX133465, RRID:AB_2886998), anti-HA (Abcam, Cat # ab1818,), β-catenin (Abcam, Cat # ab2365), LRP6 (Cell Signaling Technology,

Cat # CS2560), TCF-4 (Cell Signaling Technology, Cat # CS2569), HHEX (Abcam, Cat # ab79392), GR (Cell Signaling Technology, Cat# CSD4X9).

RNA extraction, cDNA synthesis and quantitative Real Time PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, DE) following the manufacturer's instruction. SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) was used to retro transcribe 1 µg of RNA following the manufacturer's instruction. Gene expression was assessed by RQ-PCR using the QPCR Platinum Sybr Mix (Thermo Fisher Scientific). Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method, normalizing to the expression of human *Glucuronidase Beta* (*GUSB*) or the murine *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and to the average ratio of control cells which was arbitrarily defined as 1. Primer sequences for each gene are reported in Table S1.

GR-luciferase assays

The GR luciferase reporter plasmids GR-GRE-luciferase reporter was kindly donated by Prof. De Bosscher (Ghent University). TALL-1 and ALL-SIL cells expressing control or ShRNA *NFATc1*, as well as RPMI-8402 and LOUCY cells expressing ShRNA *NAFTc2* or control, were transiently transfected via electroporation with the GR-GRE-luciferase reporter plasmid and the pMAXGFP control vector. Briefly, cells were washed with PBS, and 3.0×10^6 cells per transfection were resuspended in Ingenio® Electroporation Solution (Mirus, WI, USA) with a total of 5 µg plasmid DNA (4 µg GR-GRE-luciferase and 1 µg pMAXGFP). Electroporation was performed using the Amaxa Nucleofector system according to the manufacturer's protocol, as previously described[10]. Following electroporation, cells were seeded at 1×10^6 cells/mL in complete medium and, after 24 hours, treated with 10 µM dexamethasone or DMSO for 6 hours. Luciferase and GFP signals were measured using a Spark Tecan plate reader. The GFP signal was used to control for transfection efficiency and to normalize luciferase activity across samples. GR transcriptional activity was expressed as the ratio of normalized luciferase signal in dexamethasone-treated cells versus DMSO-treated controls.

Filipin III staining

Intracellular cholesterol amount in ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down as well as in RPMI-8402 ShRNA *NFATc2* knock-down and in the relative control cells was assessed by Cholesterol Cell-Based Detection Assay Kit (Cayman Chemical, MI, USA) according to manufacturer's instructions. Briefly, $1.5*10^4$ ALL-SIL and TALL-1 ShRNA *CNTR* and ShRNA *NFATc1* as well as RPMI-8402 ShRNA *CNTR* and ShRNA *NFATc2* cells were seeded in a 96-well plate pre-coated for 30 minutes with 1 mg/mL of fibronectin to let the cells attach to the plate. Cells were cultivated overnight and fixed with the Cell-Based Assay fixative solution for 10 minutes. Subsequently, cells were incubated with Filipin III probe. Signal was assessed using an excitation of 340-380 nm and an emission to 385-470 nm. Filipin III Median Fluorescence Intensity (MFI) for each image of ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down and controls as well as RPMI-8402 ShRNA *CNTR* and ShRNA *NFATc2* were calculated using the ImageJ software.

Nuclear Magnetic Resonance (NMR) spectroscopy

For the extraction of lipid metabolites, TALL-1 ShRNA *CNTR* and ShRNA *NFATc1* cell pellets were processed as previously described [11]. Briefly, the dried organic samples were resuspended in 750 µL of solution CDCl3:CD3OD (2:1; v/v), containing 0.02% tetramethylsilane (TMS) for chemical shift for NMR analyses. 1H-NMR analyses were performed at 25°C at 600 MHz (14.1 T Bruker AVANCE Neo spectrometer; Karlsruhe, Germany, Europe) on organic cell extracts using standard 1D Bruker library 1H NMR spectra. A total of 512 scans were collected into 32,768 data points using a spectral width of

12500 Hz, an acquisition time of 1.31 s and a relaxation delay (d1) of 5 seconds. Prior to Fourier transformation, each FID (free induction decay) was zero-filled to 65,536 points and multiplied by a 0.3 Hz exponential line-broadening function. Subsequently, spectra were manually phased, baseline corrected, and chemical shifts referenced internally to TMS at d = 0.00 ppm by using Topspin software 4.1. Relative quantification (area) of lipid signals in organic fractions was normalized to the number of cells. Heatmap and multivariate analyses were performed by MetaboAnalyst5.0 tool. An unpaired two-tailed Student t-test was applied for the comparison between NMR data groups.

ChIP and droplet digital PCR (ddPCR) analysis

ChIP experiments were performed as described previously [12]. Briefly, $25*10^6$ of TALL-1 or RPMI-8402 cells were crosslinked with 1% of formaldehyde for 10 minutes and lysed in lysis buffer plus protease inhibitors (50 mM Hepes-KOH, 7.5pH, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100) for 10 minutes at 4°C. Next, the nuclei were lysed in nucleus lysis buffer (10 mM TRIS HCl, 1 mM EDTA, 0.1% SDS), followed by sonication using Covaris M220 Focused-ultrasonicator instrument to obtain DNA fragments. A preclearing step of chromatin was performed, incubating the total amount of sonicated chromatin with 75 µl of protein G Dynabeads (Thermo Scientific) in reaction buffer. Contemporary, protein G Dynabeads were incubated with 2.5 ug of NFATc1 or 7.5 ug of NFATc2 and an equal amount of IgG as negative control for 8 hours and 4°C then washed with a buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl 8.1pH, 167 mM NaCl). Next, beads-antibody complexes and sonicated chromatin were incubated overnight at 4°C with rotation. Complexes bound to the beads were washed using buffers with increasing salt concentration: 2X with Low Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl 8.1pH, 150 mM NaCl), 1X with High Salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl 8.1pH, 500 mM NaCl) and 1X with TE buffer (100 mM Tris-HCl 8.1pH, 1 mM EDTA). After the last washing step, the ChIP DNA was eluted by resuspending the chromatin-antibody-beads complexes in elution buffer (100 mM NaHCO3, 1% SDS) at 65°C for 15 minutes, followed by treatment with RNase and proteinase K. The crosslinks were then reversed, and the DNA was precipitated using ethanol and glycogen.

To investigate the molecular binding of NFATc1 and NFATc2 to specific DNA sequences we analyzed NFATc1 or NFATc2 ChIP samples by ddPCR technique using EvaGreen Digital PCR Supermix (Bio-Rad) and the following couples of primers self-designed based on JASPAR (https://jaspar.uio.no) predicted NFATc1 or c2 DNA binding sequence:

GENE

PRIMER SEQUENCE
NFATc1/c2 DNA BINDING SEQUENCE

Hmgcs1

F: TGTCTGCCCAGGTGATGAAAATTTCCA

R: CCTTTCACTTCAGCCTCCCT

Dhcr7

F: TGCTCAAACAGAAACCAAACCATGGAAA

R: GGACCTACCCTCTCAGTGTC

Ebp

F: TTTGATCAGGGCAGGGACAGTGGAAA

R: TCCCACTCAGAGCAACCAAT

Lrp6

F: TTGCAGTGTTTCCCCATTCGTTTTCCT

R: TCTTTGCTGACTCCTCCCAT

The promoter region of *MyoD* gene served as negative control [13]:

MyoD F: CTCTGCTCCTTTGCCACAAC; *MyoD* R: GAGTGCTCTTCGGGTTTCAG.

Specifically, each sample was emulsified to obtain droplets that were processed in a standard thermal cycler according to manufacturer's instructions. Next, the QX200 Droplet Digital PCR (ddPCR™) System (Bio-Rad) was used to analyze the amplification signals for each sample (NFATc1 or NFATc2 ChIP samples and IgGs). The quantity of DNA was measured as DNA copies/µl and normalized on the relative IgG sample, obtaining the enrichment over IgG ratio plotted on bar graphs.

Lipid Rafts (LRs) staining

To detect plasma membrane LRs in ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down cells and controls, the Vybrant™ Alexa Fluor™ 488 Lipid Raft Labeling Kit (Thermo Fisher Scientific) was employed according to the manufacturer's instructions. Images were captured by the Zeiss LSM800 Airyscan microscope and the number of LRs for each cell was analyzed by ImageJ software.

LCK staining

ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down and control cells were stained for LCK expression by immunofluorescence (IF) assay. In detail, $1*10^6$ of cells were fixed using formaldehyde 4% in PBS1X for 15 minutes at 4°C. Cells were washed and permeabilized with Triton 0.1X in PBS1X for 10 minutes at room temperature. Subsequently, cells were incubated with LCK antibody (CS2657, Cell Signaling Technology) diluted 1:100 in PBS1X overnight at 4°C. Cells were washed and incubated with an Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Cat # A11032, RRID: AB_2534091) diluted 1:200 in PBS1X for 30 minutes at room temperature. Cells were analyzed through the Zeiss LSM800 Airyscan microscope. The LCK median fluorescence intensity for each image of ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down and controls was calculated using the ImageJ software.

Flow Cytometry analysis

CD4 staining was performed in ALL-SIL and TALL-1 ShRNA *NFATc1* knock-down cells and in the control, incubating $5*10^5$ the cells with 10 µL of the CD4 (SK3) FITC (BD345768, Beckton Dickinson, Franklin Lakes, NJ, USA) for 20 minutes at room temperature in the dark. $5*10^5$ RPMI-8402 ShRNA *NFATc2* knock down cells and controls were stained with a 10 µL of antibody mix (CD34 APC-A Becton Dickinson (BD), NJ, USA, Cat# 345804; CD7 PC5-5A Beckman Coulter (BC) CA, USA, Cat# IM3613; CD45 APC-A750-A, BD Cat# 348815; CD3 K0525-A, BC Cat# B00068; CD19 PB450-A, BC Cat# B49213; CD5 PC7-A, BD Cat# 348810; CD4 FITC-A, BD Cat# 345768; CD8 PE-A, BD Cat# 345773) for 30 minutes at room temperature in the dark. $5*10^5$ TALL-1 ShRNA *NFATc1* knock-down cells and controls were stained with a 10 µL of antibody mix (CD4 FITC-A, BD Cat# 340422; CD8 PE-A, BD Cat# 555367; CD5 PeCy5-A, BD Cat# 555354; CD34 PeCy7-A, BD Cat# 34879; CD45 APC-A750-A, BD Cat# 348795; CD19 APC-A, BD Cat# 340722; CD3 K0525-A, BC Cat# B00068) for 30 minutes at room temperature in the dark. After washing, cells were analyzed through Cytoflex Beckman Coulter. Intensity Fluorescence analyses were performed by FlowJo v7.6.5.

Reference:

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