TET2 as a tumor suppressor and therapeutic target in T-cell acute lymphoblastic leukemia

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Pediatric T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy resulting from overproduction of immature T-cells in the thymus and is typified by widespread alterations in DNA methylation. As survival rates for relapsed T-ALL remain dismal (10 to 25%), development of targeted therapies to prevent relapse is key to improving prognosis. Whereas mutations in the DNA demethylating enzyme TET2 are frequent in adult T-cell malignancies, TET2 mutations in T-ALL are rare. Here, we analyzed RNA-sequencing data of 321 primary T-ALLs, 20 T-ALL cell lines, and 25 normal human tissues, revealing that TET2 is transcriptionally repressed or silenced in 71% and 17% of T-ALL, respectively. Furthermore, we show that TET2 silencing is often associated with hypermethylation of the TET2 promoter in primary T-ALL. Importantly, treatment with the DNA demethylating agent, 5-azaacytidine (5-aza), was significantly more toxic to TET2-silenced T-ALL cells and resulted in stable re-expression of the TET2 gene. Additionally, 5-aza led to up-regulation of methylated genes and human endogenous retrogene (HERVs), which was further enhanced by the addition of physiological levels of vitamin C, a potent enhancer of TET activity. Together, our results clearly identify 5-aza as a potential targeted therapy for TET2-silenced T-ALL.

Significance

Pediatric T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy in need of novel targeted therapies to prevent relapse and lessen treatment toxicity. We reveal frequent (~88%) transcriptional silencing or repression of the tumor suppressor TET2 in T-ALL. We show that loss of TET2 in T-ALL is correlated with hypermethylation of the TET2 promoter and that TET2 expression can be rescued by treatment with the DNA demethylating agent, 5-azaacytidine (5-aza). We further reveal that the TET2 cofactor vitamin C exerts a strong synergistic effect on global transcriptional changes when added to 5-aza treatment. Importantly, 5-aza treatment results in increased cell death, specifically in T-ALL cells lacking TET2. Thus, we clearly identify 5-aza as a potentially targeted therapy for TET2-silenced T-ALL.

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barriers to transformation as clearly facilitated by TET2 loss in adult T-cell malignancies. Increasing intensity of chemotherapy has improved prognosis of T-ALL from 50% to 75% between 1970 and 2000 (24). However, the last 20 y have seen only marginal improvements in outcome, with survival rates for relapsed T-ALL remaining just 10% to 25% (25). In addition, the long and intensive course of chemotherapy employed in T-ALL frequently results in serious, often fatal, side effects (26). Thus, a key clinical challenge to increasing survival in T-ALL is development of targeted therapies to prevent relapse and decrease toxicity of treatment (25, 26).

Here, we investigated the expression of the TET2 gene in healthy human progenitor T cells, pediatric T-ALL patients, and T-ALL cell lines, revealing that TET2 was frequently silenced in T-ALL and that silencing was associated with aberrant hypermethylation of the TET2 promoter. Furthermore, we found that treatment with the DNA demethylating agent, 5-azaacytidine (5-aza), was significantly more toxic to TET2-silenced T-ALL cells and resulted in stable re-expression of the TET2 gene. The 5-aza treatment was associated with up-regulation of both methylated genes and HERVs. Moreover, 5-aza-mediated transcriptional reprogramming was associated with TET2 expression levels and was further enhanced by addition of physiological levels of vitamin C, a potent enhancer of TET2 activity. Together, our results reveal TET2 as a potential tumor suppressor in T-ALL and a marker for response to 5-aza in this poorly understood malignancy.

Results

The Tumor Suppressor TET2 Is Frequently Silenced in T-ALL. Whereas mutations in TET2 have been sporadically reported in T-ALL, no systematic analysis of TET2 mutation frequency in T-ALL has been performed to date. Meta-analysis of targeted exome sequencing, whole exome sequencing, and whole genome sequencing of leukemic blasts from 854 T-ALL patients (Dataset S1) confirmed that TET2 mutations were infrequent in T-ALL (0.7%) (Fig. 1A and SI Appendix, Fig. S1A). In addition, the paucity of IDH1/2 mutations (1.9%) suggested that metabolic inhibition of TET2 by aberrant 2-HPG production was also uncommon in T-ALL (Fig. 1A). Mutations in TET2 or IDHs were similarly rare in 24 analyzed T-ALL cell lines (SI Appendix, Fig. S1B).

Since TET2 has recently been reported to be frequently mis-expressed (30), we next investigated if TET2 expression was similarly altered in T-ALL. To understand the potential consequence of any TET2 expression change observed in T-ALL, we first needed to establish normal TET2 expression patterns across human tissues. The TET2 gene has two isoforms, a full-length transcript, TET2a, and a shorter transcript, TET2b, which lacks the C-terminal catalytic domain (Fig. 1B). By analyzing RNA-sequencing data from 27 healthy tissues from the Human Protein Atlas (27), no expression of TET2b was detected in any tissue except bone marrow, in which expression of TET2b was considerably lower than TET2a (Fig. 1C). Additionally, qPCR with isoform-specific probes in an independent panel of 25 normal human tissues confirmed absence of TET2b expression in all tissues, including bone marrow (Fig. 1D). Our results cast doubt on the validity of TET2b as a genuine transcript in humans, the annotation of which was based on a single sequence observed in a single fetal kidney complementary DNA (cDNA) library (28).

Indeed, TET2b-specific exonic sequences showed no conservation across vertebrates and a lack of mapped reads in seven fetal RNA-seq libraries, including kidney. Furthermore, analysis of splicing revealed no splice junctions for TET2b-specific exon 1 and exon 2 (SI Appendix, Fig. S1C and Dataset S2) (29). In contrast, TET2a was expressed in all tissues with highest expression in hematopoietic cells (Fig. 1C and D), establishing TET2a as the main and potentially only TET2 transcript in humans.

T-ALL can arise from any stage of T-cell development from early thymic progenitors (ETP) to differentiated single positive T cells (Fig. 1E, Upper). Revealing aberrant gene expression in T-ALL has been hampered by a lack of gene expression data for the normal progenitor T-cell (thymocyte) populations from which T-ALL derives. Using recently published RNA-seq data for the six canonical thymocyte subpopulations in humans (30), we could confirm that TET2 was highly expressed in all normal postnatal thymocyte populations isolated from children undergoing cardiac surgery (30). In contrast, compared to ETP, T-cell–committed primary thymocytes, and double positive primary thymocytes, TET2 expression was repressed (one-sample t test, P < 0.05) or silenced (transcript per million [TPM] < 1) in 71% (42/59) and 17% (10/59) of primary pediatric T-ALL, respectively (Fig. 1E, Lower) (31). A similar loss of TET2 expression was also observed in RNA-seq data of leukemic blasts from an independent cohort of 262 pediatric T-ALL patients (Fig. 1F) (32). Whereas TET2 and TET3 are known to have overlapping enzymatic function (1, 33), loss of TET2 was not associated with up-regulation of TET3 in T-ALL (SI Appendix, Fig. S1E). Thus, TET2 expression is aberrantly down-regulated or absent in the majority of pediatric T-ALL.

Our observations in primary T-ALL were supported by analysis of RNA-seq data for 20 T-ALL cell lines from the Cancer Cell Line Encyclopedia (CCLE) (34), which showed a similar distribution of TET2 down-regulation and silencing (Fig. 1G). Interestingly, reduced representation bisulfite sequencing (RRBS) data for 15 of these T-ALL cell lines (34) suggested that the TET2 promoter was hypermethylated in all but one cell line in which TET2 was silent (Fig. 1H) and showed a tendency toward higher methylation in cell lines in which TET2 expression was reduced (Fig. 1H and SI Appendix, Fig. S1D). Importantly, whole genome DNA methylation data for two independent cohorts (NNOPHO = 101, NALL IC-BFM = 109) of pediatric T-ALL patients (30, 35, 36) revealed hypermethylation of the TET2 promoter in a subset of patients (9/101 Nordic Society for Pediatric Hematology and Oncology [NOPHO]; 8/109 International Berlin-Frankfurt-Munster Study Group trials 2009 [ALL IC-BFM]) (Fig. 1I and J), suggesting one mechanism of TET2 silencing may be through aberrant DNA methylation. As with most molecular lesions in T-ALL, loss of TET2 did not associate with molecular subtype, prognosis, or key clinical parameters in a well-characterized cohort of 262 pediatric T-ALL patients (SI Appendix, Fig. S1I) (32).

Therefore, the tumor suppressor gene (TSG) TET2 is frequently silenced in T-ALL and may be a driver of T-ALL transformation and potential therapeutic target.

Vitamin C Toxicity in T-ALL Is Independent of TET Activity. Having revealed frequent repression or loss of TET2 expression in T-ALL, we next sought to test if vitamin C, a potent enhancer of TET activity, could rescue loss of TET2 by increasing the catalytic activity of the remaining TET enzymes. Indeed, decreased levels of global 5hmC are associated with worse prognosis in ATLL (11), and TET2 and TET3 have been shown to have redundant functions in both hematopoiesis and leukemogenesis (1, 33). To dissect the potential of vitamin C as a targeted therapy in T-ALL with loss of TET2, we obtained six T-ALL cell lines in which TET2 was expressed and unmethylated (SUP-T1, CCRF-CEM, JURKAT) or silent and methylated (LOUCY, TALL-1, DND-41) (Fig. 1G and H and SI Appendix, Fig. S1B). Expression status of all three TET genes and global 5hmC levels were assessed by qPCR and immuno-dot blot, respectively (SI Appendix, Fig. S2 A and B), showing no difference in TET1 and TET3 expression nor in 5hmC levels relative to TET2 status. Treatment of these cell lines with increasing vitamin C concentrations spanning physiological (10 to 100 μM) and pharmacological (300 to 10,000 μM) concentrations revealed similar cytotoxicity in all cell lines, albeit with a tendency toward increased toxicity in TET2-deficient cells (not significant) (Fig. 2A).
Fig. 1. TET2 is a novel tumor suppressor in T-ALL. (A) Mutations in TETs and IDHs in primary human T-ALL from a meta-analysis of nine studies including 854 patients. (B) Known TET2 isoforms, TET2a (red) and TET2b (blue). (C) Expression of TET2 isoforms in 27 healthy tissues from the Human Protein Atlas. (D) Expression of TET2 isoforms relative to housekeeping gene GAPDH in 25 human tissues. Data without error bars is based on RNA pools of three individuals each. (E) Upper) Schematic representation of human thymocyte development from ETP to CD4 or CD8 single positive T cells (CD4SP, CD8SP). (Lower) Expression of TET2a (red) and TET2b (blue) in the 6 human thymocyte subtypes (Left) and 59 primary T-ALL patients (Right). Dotted black line: silenced TET2 (TPM < 1), gray dotted line: repressed TET2 (one-sample t test, P < 0.05, compared to ETP, T-C, and DP primary thymocytes). T-C, T cell-committed; DP, double positive. (F) Expression of TET2 in primary human thymocytes and T-ALL patients from two independent studies. (G) TET2 expression in T-ALL cancer cell lines based on RNA-seq data from Broad Institute CCLE. (H-J) Methylation of the TET2 promoter (H) in T-ALL cell lines based on RRBS from CCLE and in primary human T-ALL patients from (I) NOPHO and (J) the ALL IC-BFM protocol including 20 samples from healthy human thymocytes (red). (C, F, and G) Dotted black line at TPM = 1 as cutoff for silencing. (H-J) Dotted black line at methylation = 0.2.
and SI Appendix, Fig. S2C). Furthermore, treatment with physiological vitamin C (100 μM) resulted in a pronounced increase in global 5hmC levels at 24 h, with a lower relative increase observed in TET2-deficient cells (Fig. 2B). Notably, a similar relative increase in global 5hmC levels was achieved with just 10 μM vitamin C as with elevated physiological concentrations (100 and 300 μM) (SI Appendix, Fig. S2 D and E), neither of which concentration was cytotoxic to any T-ALL cell line. The 5hmC levels at higher concentrations could not be reliably assessed due to cell death (SI Appendix, Fig. S2F). Importantly, increased 5hmC levels were not associated with increased expression of any TET gene (SI Appendix, Fig. S2G). Thus, supplementation of T-ALL cells with vitamin C at concentrations obtainable with oral administration (100 μM) resulted in a 2 to 10-fold increase in 5hmC levels but did not affect cell viability.

The observation that treatment with physiological vitamin C concentrations (100 μM) resulted in increased global 5hmC levels in T-ALL cells in the absence of toxicity suggested that vitamin C toxicity was not mediated by the TET enzymes (17, 18, 37, 38) (Fig. 2A and B). Vitamin C, however, can elicit toxicity through several pathways other than enhancement of TET activity (Fig. 2C). Having confirmed expression of the main vitamin C (SLC23A1 and SLC23A2) and dehydroascorbic acid (DHA; GLUT1) transporters in each T-ALL cell line (SI Appendix, Fig. S3A), we blocked transport through SLC23A1, SLC23A2, and GLUT1s with the inhibitor phloretin (30–40). As expected, the pharmacological inhibition of vitamin C uptake resulted in reduced 5hmC production compared with vitamin C alone (Fig. 2D and SI Appendix, Fig. S3 B and C). However, inhibition of vitamin C uptake did not reduce toxicity in T-ALL cell lines irrespective of TET2 status (Fig. 2E and SI Appendix, Fig. S3D).

The finding that vitamin C toxicity did not require its transport across the cell membrane, ruling out cytotoxicity through increased TET activity and DHA mediated loss of ATP (41, 42), suggested extracellular production of reactive oxygen species (ROS) as the potential source of cytotoxicity (Fig. 2C) (43, 44). Indeed, addition of vitamin C to culture medium resulted in increased levels of intracellular (SI Appendix, Fig. S4A) and total (intracellular + extracellular) ROS (Fig. 2F and SI Appendix, Fig. S4B), while supplementation with catalase, which decomposes H₂O₂ to H₂O and O₂, maintained ROS at background levels even at high (3,000 μM) vitamin C concentrations (SI Appendix, Fig. S4C). As expected, the consequent incorporation of 5-aza compared with DND-41 and LOUCY cells as evidenced by the lack of up-regulation of 5-aza-sensitive control genes in TALL-1 cells (SI Appendix, Fig. S5A). Importantly, treatment of TET2 wild-type (SUP-T1, CCRF-CEM, MOLT-3) and TET2-deficient (LOUCY, TALL-1, DND-41) cell lines with 5-aza also resulted in significantly higher cytotoxicity in cells lacking TET2 (Kolmogorov-Smirnov test, P = 0.013) (Fig. 2C and SI Appendix, Fig. S5B). Toxicity of 5-aza to TALL-1 cells (Fig. 3C) in the absence of TET2 up-regulation (Fig. 2B) shows that toxicity is not mediated by TET2 directly but that TET2 methylation is a marker of 5-aza sensitivity in T-ALL. Loss of TET2 compared to healthy thymocytes may result in the establishment of a distinct methylation profile that renders cells particularly sensitive to treatment with a global DNA-demethylating agent. Indeed, analysis of global methylation profiles in primary T-ALL cells revealed that TET2-silenced T-ALL cells have a distinct global methylation pattern (Fig. 3D) marked by extreme promoter hypermethylation but increased levels of 5-hydroxymethylcytosine (5hmC) (SI Appendix, Fig. S5C) and DNA demethylation renders cells particularly sensitive to treatment with a global DNA-demethylating agent, independent of reactivation of TET2 itself.

5-Aza and Vitamin C Act Synergistically to Increase Expression of Methylated Genes and Endogenous Retroviruses. To shed light on the potential mechanisms of 5-aza sensitivity in TET2-silenced cells, we next sought to identify the transcriptional changes induced by 5-aza in T-ALL cell lines. Based on our observation that TET2 up-regulation in response to 5-aza (Fig. 2B) and the pronounced effect of vitamin C on TET activity (Fig. 2B), we additionally aimed to investigate vitamin C as a potential supplement to treatment with 5-aza. To this end, we treated two cell lines with silenced and methylated TET2 (LOUCY, DND-41) and two cell lines expressing TET2 (SUP-T1, JURKAT) with 2,000 nM 5-aza, 100 μM vitamin C, or a combination of both for 3 d followed by total RNA sequencing (Fig. 3A). A minimum of 66 million reads pairs of 150 bp each allowed for analysis of both coding and noncoding RNA (Dataset S3). The 5-aza induced expression of genes known to be silenced by DNA methylation (DAZL, GAGE1, PIWIL2, and XIST) (46, 47) as well as TET2 as determined by total RNA-seq (Fig. 4A) and qPCR (SI Appendix, Fig. S6A). As expected, treatment with 5-aza led to a global increase in gene expression in all cell lines (Fig. 4B) with up-regulated genes being enriched for both promoter hypermethylation (methylation > 0.7) and no expression and TP < 1) in untreated control samples (Fig. 4 C and D and Datasets S4 and S5). Thirty-six genes were exclusively up-regulated in both TET2-silenced cell lines, LOUCY and DND-41, but neither SUP-T1 nor JURKAT (SI Appendix, Fig. S6B and Dataset S6), among which were genes connected to promoter hypermethylation, tumorigenicity, and poor prognosis in a range of malignancies (48–52). As seen by others (53), up-regulated genes in response to 5-aza were not enriched for TSGs in any of the four cell lines analyzed (hyper-geometric test; |adjP| > 0.09 in all cell lines). Indeed, TSGs were significantly down-regulated by 5-aza treatment (Dataset S7). In stark contrast, we again observed up-regulation of TET2, an
Treatment with 3,000 μM vitamin C

A. Cell viability curve for T-ALL cell lines with expressed and silenced TET2 treated with increasing concentrations of VC for 24 h (Left) and boxplot showing cell viability for treatment with 3,000 μM VC compared to untreated control (Right).

B. Dot blot using an antibody against 5hmC in T-ALL cell lines treated with a H2O control or 100 μM VC for 24 h in triplicates (Left) or presence (+) of 100 μM phloretin for cell lines with expressed and silenced TET2 relative to untreated control samples (dotted line) (Right).

Treatment with VC can mediate its cytotoxic effect through three main pathways: 1) production of hydrogen peroxide (H2O2) and ROS during oxidation of VC to DHA, 2) energy crisis in cancers with a KRAS or BRAF mutation (yellow flash) after DHA is reduced back to VC intracellularly, or 3) cofactor of the TET enzymes (TET1-3) leading to increased enzymatic activity. GSH, glutathione.

Vitamin C acting through loss of ATP: colorectal cancer, gastric cancer

Vitamin C acting through TETs: AML, melanoma, bladder cancer

Vitamin C acting through H2O2 and ROS: lymphoma, breast cancer, pancreatic cancer, ovarian cancer, glioblastoma, myeloma, non-small-cell lung cancer

C. Vitamin C acting through loss of ATP: colorectal cancer, gastric cancer

D. Vitamin C acting through H2O2 and ROS: lymphoma, breast cancer, pancreatic cancer, ovarian cancer, glioblastoma, myeloma, non-small-cell lung cancer

E. Cell viability curves for T-ALL cell lines treated with increased concentrations of VC with or without 100 μM phloretin for cell lines with expressed TET2 (Left) or silenced TET2 (Right). Biological triplicates for three different cell lines each (nexpressed = 9 and nsilenced = 9).

F. Boxplot showing levels of total (intracellular and extracellular) ROS created in response to treatment of T-ALL cell lines with VC at increasing concentrations relative to an untreated control (dotted line); pyocyanine used as a positive control.

G. Intracellular and extracellular ROS created in response to treatment of T-ALL cell lines with 3,000 μM VC without and with the addition of catalase for each cell line individually (Left) and box plot representing all cell lines (Right) relative to an untreated control (dotted line).

H. Cell viability of T-ALL cell lines after treatment with 3,000 μM VC without and with the addition of catalase for each cell line separately (Left) and all cell lines combined (Right). Percentage of untreated control.

Fig. 2. Cytotoxicity of vitamin C (VC) is caused by ROS independently of TET activity in vitro. (A) Cell viability curve for T-ALL cell lines with expressed and silenced TET2 treated with increasing concentrations of VC for 24 h (Left) and boxplot showing cell viability for treatment with 3,000 μM VC compared to untreated control (Right). (B) Dot blot using an antibody against 5hmC in T-ALL cell lines treated with a H2O control or 100 μM VC in the absence (-) or presence (+) of 100 μM phloretin.

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5-Aza induces TET2 expression and targets TET2-silenced cells. (A) Schematic representation of experimental setup. T-ALL cell lines with silenced or expressed TET2 were treated with 5-aza, vitamin C, or a combination of both for 3 d followed by removal of treatment and a recovery period of 5 d. (B) Expression (qPCR) of TET2 relative to housekeeping gene GAPDH in T-ALL cell lines with expressed (SUP-T1, JURKAT) and silenced (LOUCY, DND-41, TALL-1) TET2 after treatment with 500 nM or 2000 nM 5-aza for 72 h or after treatment followed by recovery without treatment for 5 d. No Ct value in one or more than one biological replicate indicated as no detection by qPCR (+). (C) Cell viability curve for T-ALL cell lines with wild-type (3 cell lines) and silenced (3 cell lines) TET2 treated with increasing concentrations of 5-aza for 72 h, comparison by Kolmogorov-Smirnov test (Left). Boxplot showing cell viability for treatment with 500 and 2000 nM 5-aza compared to untreated control; two-tailed, unpaired Student’s t test; ns, P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 (Right), n = 3 for each cell line. (D) Multidimensional scaling (MDS) plot including the 1,000 most variable CpGs for the ALL IC-BFM 2009 protocol cohort including healthy primary thymocytes (EPIC array). (E) Number of methylated CpGs in CpG islands (Left) and intergenic regions (Right) in the ALL IC-BFM 2009 cohort including healthy primary thymocytes (EPIC array). (D and E) Status of TET2 promoter methylation indicated by color.

Figure 3. 5-Aza induces TET2 expression and targets TET2-silenced cells. (A) Schematic representation of experimental setup. T-ALL cell lines with silenced or expressed TET2 were treated with 5-aza, vitamin C, or a combination of both for 3 d followed by removal of treatment and a recovery period of 5 d. (B) Expression (qPCR) of TET2 relative to housekeeping gene GAPDH in T-ALL cell lines with expressed (SUP-T1, JURKAT) and silenced (LOUCY, DND-41, TALL-1) TET2 after treatment with 500 nM or 2000 nM 5-aza for 72 h or after treatment followed by recovery without treatment for 5 d. No Ct value in one or more than one biological replicate indicated as no detection by qPCR (+). (C) Cell viability curve for T-ALL cell lines with wild-type (3 cell lines) and silenced (3 cell lines) TET2 treated with increasing concentrations of 5-aza for 72 h, comparison by Kolmogorov-Smirnov test (Left). Boxplot showing cell viability for treatment with 500 and 2000 nM 5-aza compared to untreated control; two-tailed, unpaired Student’s t test; ns, P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 (Right), n = 3 for each cell line. (D) Multidimensional scaling (MDS) plot including the 1,000 most variable CpGs for the ALL IC-BFM 2009 protocol cohort including healthy primary thymocytes (EPIC array). (E) Number of methylated CpGs in CpG islands (Left) and intergenic regions (Right) in the ALL IC-BFM 2009 cohort including healthy primary thymocytes (EPIC array). (D and E) Status of TET2 promoter methylation indicated by color.

established TSG in numerous hematopoietic malignancies, in both LOUCY and DND-41 cells (Fig. 4A and SI Appendix, Fig. S6A), highlighting the exceptionality of TET2 silencing and repression of retroviruses (Fig. 4F) including HERVs previously reported to be activated in response to 5-aza such as HERV-Fc1, LTR12E, MER70A, and MER48 (21). While vitamin C alone did

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Fig. 4. 5-Aza and vitamin C act synergistically to increase expression of methylated genes and HERVs. (A) Expression of TET2 and selected methylated genes in response to treatment with 2,000 μM 5-aza and 100 μM vitamin C for 72 h in T-ALL cell lines with expressed (SUP-T1, JURKAT) and silenced (LOUCY, DND-41) TET2. Differential expression based on total RNA sequencing of biological duplicates shown as log (2) fold change relative to an untreated control. (B) Number of differentially expressed genes in TET2-expressing and TET2-deficient cells when treated with 2,000 μM 5-aza and 100 μM vitamin C for 72 h. (C) Base-resolution promoter DNA methylation in untreated T-ALL cell lines for genes that become up-regulated following treatment with 2,000 μM 5-aza and 100 μM vitamin C for 72 h. (D) Expression (TPM) in untreated T-ALL cell lines for genes that become up-regulated following treatment with 2,000 μM 5-aza and 100 μM vitamin C for 72 h. (E) Venn diagrams showing overlap of significantly up-regulated genes [log (2) fold change > 0.5, padjusted < 0.05] in four T-ALL cell lines treated with 2,000 μM 5-aza only or 5-aza plus 100 μM vitamin C. (F) Number of differentially expressed HERVs [log (2) fold change > 0.5, padjusted < 0.05] in TET2-expressing and TET2-deficient cells when treated with 2,000 μM 5-aza and 100 μM vitamin C for 72 h. (G) Expression of interferon type I and antiviral response genes in response to treatment with 2,000 μM 5-aza with and without 100 μM vitamin C for 72 h in T-ALL cell lines. Expression shown as log (2) fold change of biological duplicates relative to an untreated control. Dotted line shows log (2) fold change of 0.5. (C and D) Fisher’s exact test, Benjamini-Hochberg, FDR-corrected P, ns, P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
not lead to a change in HERV expression, the addition of vitamin C to 5-aza treatment had a profound additive effect on global HERV up-regulation as previously found by others (21). Adding vitamin C to 5-aza treatment resulted not only in a higher number of differentially expressed HERVs (Fig. 4F) but also a general increase of HERV expression in all T-ALL cell lines except JURKAT (SI Appendix, Fig. S6D), which was even more pronounced than the effect observed for genes (Fig. 4B and SI Appendix, Fig. S6C). Notably, LOUCY cells that had the weakest reactivation of TET2 (Fig. 4A) were the only cell line that did not show an increased number of up-regulated HERVs (Fig. 4F) in response to supplementing 5-aza treatment with vitamin C.

Other studies have shown treatment with hypomethylating agents such as 5-aza and the resulting up-regulation of HERV's to lead to an interferon type I response (21, 53–56) with the strongest reaction seen to occur as many as 24 h after treatment (55). Even though our analysis is limited by a comparatively short timeframe, we observed increased expression of selected genes involved in double-stranded RNA sensing (IFIHI, IFIR), antiviral response (MX2, OASL), and antigen presentation (MAGEA4B, MAGEA4) (Fig. 4G) after only 3 d of treatment. While the addition of vitamin C to 5-aza resulted in a clear increase in differentially expressed genes and HERVs, it had no effect on tetradoxin cell line, independently of expression of TET2 (SI Appendix, Fig. S6E).

Taken together, a physiologically relevant concentration of vitamin C had a strong synergistic effect in combination with 5-aza on both genes and HERV expression.

Discussion

T-ALL patients are typically treated with a regime of intense chemotherapy. This approach often fails to prevent relapse and can result in high levels of treatment-related toxicity and death (57). Thus, a key challenge to improving prognosis in T-ALL is identification and targeting of novel pathways involved in pathogenesis and relapse. Here, we show that expression of the TSG TET2 is repressed or silenced in 71% and 17% of primary T-ALL, respectively. As TET2 is highly expressed in all progenitor T-cell populations from which T-ALL arises, loss of TET2 expression in T-ALL is aberrant and does not reflect expression in the cell of origin. The frequency of TET2 repression or loss in T-ALL even exceeds the frequency (30% to 65%) of TET2 loss-of-function mutations observed in angioimmunoblastic T-cell lymphoma in adults (58–60). TET2 mutations are common in blood cells in the general adult population and appear to confer an increased risk for development of hematopoietic malignancies as opposed to acting as the genetic lesion driving transformation (15). It is tempting to speculate that in pediatric malignancies, which have a substantially lower (5 to 10 fold) mutational burden than adults (61, 62), increased risk of transformation may be more readily conferred by alteration of TSG or oncogene expression than through genetic lesions.

As with all dioxygenases, the activity of the TET enzymes can be enhanced with vitamin C (63, 64) by facilitating rapid recycling of molecular iron required for TET catalytic activity (63, 65). The observation of overlapping and often redundant functions of TET2 and TET3 (1, 33, 66) in murine T-cell development suggested that enhancement of TET3 activity in TET2-deficient cells may mimic TET2 recovery (18). Moreover, vitamin C resulted in reduced tumorigenic potential of bone marrow–derived cells from tet2−/− mice but not cells from tet2−/−/tet3−/− double knockouts (18), again supporting a redundant function for Tet2 and Tet3 in hematopoiesis (1). Indeed, aberrant hematopoietic stem cell (HSC) renewal observed in a tet2−/− mouse model was rescued by vitamin C treatment and closely mimicked genetic restoration of Tet2 (18). Similarly, vitamin C was shown to maintain normal Tet2 activity in HSCs in vivo, thereby decreasing HSC turnover and malignant transformation (17). Here, we observed both toxicity and an increase in 5hmC production in six T-ALL cell lines upon addition of vitamin C. However, 5hmC production was independent of toxicity in vitro, which was due to production of extracellular ROS. These results are in line with early studies on vitamin C toxicity in a range of cancer cell lines (43, 44) but may fail to capture more complex cytotoxic effects of vitamin C that may occur in vivo. Any direct cytotoxic effects of increased 5hmC production in response to vitamin C treatment may require long-term treatment regimens to allow the increased 5hmC to result in replication-dependent DNA demethylation and subsequent transcriptional reprogramming.

As TET2 silencing was typically associated with hypermethylation of its promoter region, we next explored the possibility of restoring TET2 expression using the nonspecific DNA demethylating agent 5-aza. Indeed, 5-aza treatment resulted in robust and stable re-expression of TET2 in two of three TET2-silenced T-ALL cell lines (LOUCY and DND-41) even after removal of 5-aza, supporting a role for DNA methylation in TET2 suppression. This is a critical finding as we have previously shown that most promoter hypermethylation in cancer occurred at genes that 1) were already silent in the tissue from which the cancer derived (67) and 2) consequently were not re-expressed upon treatment with 5-aza (68). Here, our findings suggest that DNA demethylating agents can rescue aberrant silencing of the key tumor suppressor, TET2 in T-ALL cells. Importantly, 5-aza was significantly more toxic to all T-ALL cell lines in which TET2 was silenced. However, re-expression of TET2 was not required to elicit increased toxicity in all cell lines (TALL-1), suggesting that DNA methylation alterations associated with loss of TET2 may render cells more sensitive to the DNA demethylation effects of 5-aza. Indeed, T-ALL cell lines lacking TET2 showed a distinct hypermethylation phenotype, which was also observed in primary T-ALL cells.

Total RNA-seq of 5-aza–treated cells confirmed up-regulation of methylated genes in all cell lines, including TET2 in LOUCY and DND-41 cells. A general enrichment of apoptotic pathways was not observed, consistent with the highly pleiotropic effect of 5-aza. However, several well-characterized apoptosis-related (e.g., DNA damage sensing) genes were specifically up-regulated in TET2-silenced cells. For instance, DDIA, a known suppressor of apoptosis (69), was induced by 5-aza treatment exclusively in LOUCY and DND-41 but not in TET2-expressing cells. Upon closer inspection, DDIA was highly expressed in untreated SUP-T1 and JURKAT while being silenced in TET2-deficient cells. The lack of expression of DDIA might play a role in sensitizing TET2-silenced cells to treatment with 5-aza, which induces DNA damage.

As expected, treatment of T-ALL cell lines with a physiologically relevant concentration of vitamin C (100 μM) resulted in little change in global gene or HERV expression. However, treatment with 100 μM vitamin C and 5-aza in combination resulted in a marked synergistic effect. Moreover, the synergistic effect of vitamin C was most notable in DND-41 cells, in which TET2 re-expression had been most pronounced, suggesting an additive effect of 5-aza–mediated DNA demethylation and TET2-mediated DNA demethylation. Alternatively, reduction of global DNA methylation levels by 5-aza may render the T-ALL epigenome more responsive to the enhancing effect of vitamin C on histone demethylases, since vitamin C has been shown to act as a cofactor for several Junonji-catalytic-domain–containing histone demethylases such as KDM2A-B (H3K36me), KDM3A (H3K9me), KDM4A-E (H3K36me and H3K9me), and KDM6A-B (H3K27me) (70). A similarly pronounced synergistic effect was observed in HERV expression, with a clear association between TET2 re-expression and treatment effect size. Whereas further experiments are required to determine the molecular underpinnings of 5-aza toxicity in TET2-silenced T-ALL cells, our results clearly identify TET2 as
a potential tumor suppressor and, importantly, a therapeutic target in T-ALL.

Materials and Methods

Meta-analysis for mutations in Primary T-ALL. Data for mutations in T-ALL from whole exome sequencing, whole genome sequencing, or targeted exome sequencing were collected and summarized from nine recently published studies (Dataset S1), resulting in a dataset including 584 patients.

Mutations in T-ALL Cell Lines. Information on mutations in T-ALL cell lines was collected from Broad Institute's CCLE, and the functional impact of missense mutations was assessed using the PROVEAN Web Server Tool (71) and the mutation assessor online tool (72). A consensus on the functional impact of a missense mutation was reached based on the PROVEAN and SIFT score (both from PROVEAN tool) and the combined functional score from mutation assessor.

RNA-Seq Transcriptome Quantification from Publicly Available Data. Transcriptome mapping was performed using Salmon v0.12.0 (–gBias-seqBias-numBootstrap=100–validateMappings) (73) using known Refseq transcripts (NM, * and NR, * ) from the GRCh38.p12 assembly (GCF_000001405.38_GRCh38.p12_rna.fna.gz) as a reference. Library type options were Human Protein Atlas (27); IU, TARGETY T-ALL; ISR, CCLE: ISR. We did not observe any effect of adapter or quality trimming on abundance estimates in our data and analyzed fastq files directly. Abundance estimates were converted to h5 format using Wasabi (https://github.com/COMBINE-lab/wasabi), and data normalization and differential expression analysis was performed using likelihood ratio tests and gene-level P value aggregation as implemented in Sleuth (74, 75). Fetal RNA-seq samples for expression analysis were performed using likelihood ratio tests and gene-level P value aggregation as implemented in Sleuth (74, 75).

Abundance estimates were converted to h5 format using Wasabi (https://github.com/COMBINE-lab/wasabi), and data normalization and differential expression analysis was performed using likelihood ratio tests and gene-level P value aggregation as implemented in Sleuth (74, 75). Fetal RNA-seq samples for expression analysis were performed using likelihood ratio tests and gene-level P value aggregation as implemented in Sleuth (74, 75).

Cell Culture. Human peripheral blood mononuclear cells were isolated from buffy coats by healthy donors using Lymphoprep (Axis-Shield) and further separated based on CD4 and CD8 expression levels. CD4+ T-cells were isolated from the CD4 negative fraction, CD8+ T-cells were enriched by staining with fluorescently labeled antibodies against CD3+ (BioLegend, 4368814) at 37 °C for 1 h at room temperature. Membranes were washed twice in TBS-Tween and once in TBS before visualizing with the help of Clarity ECL Western Substrates (Bio-Rad). Quantification of signals for 5hmC was done using the image-processing software ImageJ (NIH). Antibody specificity was confirmed by bloting 10 ng of DNA standards with unmodified, methylated, or hydroxymethylated cytosine (GeneTex, GTX400004) with each blot.

DNA and RNA Extraction. DNA and RNA was extracted using the Quick-DNA/ RNA Kit (Zymo Research, D7001) according to the manufacturer’s instructions. Nucleic acid concentration was determined by nanodrop.

Conversion of RNA to cDNA and qPCR. The 200 ng (cell lines) or 100 ng (primary tissues) RNA was treated with dsDNase I (Thermo Fisher, EN0771) for 2 min at 37 °C and converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) at 37 °C for 2 h followed by inactivation for 5 min at 85 °C. cDNA was diluted to a volume of 200 μL of which 5 μL was used for qPCR. qPCR was carried out in technical triplicates or quadruplicates (5-aza-treated cell lines) with the help of Fast Universal PCR Master Mix (Thermo Fisher Scientific, 4352042) using TaqMan probes (Thermo Fisher Scientific), as indicated in Dataset S8. qPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following temperature profile: initial denaturation at 95 °C for 20 s and 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Data were analyzed using the ΔCt or ∆∆Ct method as appropriate with GAPDH as housekeeping gene. For analysis of TET2 expression in human tissues (3D8+ T-cells) or 5 (peripheral blood mononuclear cells, naïve CD4+ T-cell, CD4+ T-cells), biological replicates were analyzed. Additionally, TET2 expression was assessed in three samples of commercially available bone marrow (biochain, ATR1234024; Zygren, HR-704; TakaRa, 636591) and a panel of 20 tissues (Ambion, AM6000), including pooled samples from three individuals each.

Immunofluorescence. Immunofluorescent images of the TET2 genomic region (±5 kb) was acquired from the UCSC genome browser. RNA-seq read lop100way scores of the TET2 genomic region (±5 kb) was acquired and preprocessed using functional normalization (79) as implemented in R/minfi (80), and a general probe mask was applied to fil-

450k and EPIC Methylation Array Analysis. Pediatric T-ALL data from NOPHO (35) was acquired and preprocessed using functional normalization (79) as implemented in R/minfi (80), and a general probe mask was applied to filter out bad probes (81). TET2 promoter location was defined as Refseq NM_001127208.2 TSS extended 1 kb upstream and downstream.

Cell Culture Reagents and Treatment. 105 cells (for treatment with 5-aza) were treated in 1 mL RPMI medium. Following, 100 μL cell suspension per well was given into a 96-well plate in technical triplicates and incubated at 37 °C with 5% CO2 in a humidified incubator at 37 °C with 5% CO2. 105 cells (for vitamin C, phloretin, and catalase treatment) (BioLegend, 421301) and Propidium iodide (BioLegend, 640906) were dissolved in RPMI medium, and aliquots were stored at −20 °C. 5-Aza (Sigma-Aldrich, A1287) was dissolved in RPMI medium and used at a concentration of 600 U/mL. Phloretin (ACROS Organics, 307651000) was dissolved in RPMI medium, and aliquots were stored at −20 °C for up to 2 mo. The 1 × 106 cells (for vitamin C, phloretin, and catalase treatment) or 1.5 × 105 cells (for treatment with s-Aza) were treated in 500 μL compound diluted in RPMI medium at 37 °C for 24 h (vitamin C, phloretin, catalase) or 72 h (s-Aza, s-Aza plus vitamin C) with refreshment of treatment after 24 and 48 h. Treatments with distilled H2O and DMEM were used as controls for vitamin C and phloretin treatment, respectively.

Isolation and Sorting of Primary Immune Cells. peripheral blood mononuclear cells were isolated from buffy coats by healthy donors using Lymphoprep (Axis-Shield) and further separated based on CD4 with magnetic beads (CD4+ T-cell isolation kit, Miltenyi Biotec, 130-096-533). The CD4-positive fraction was used as-is, and from the CD4 negative fraction, CD8+ cells were enriched by staining with fluorescently labeled antibodies against CD3+ (Anti-CD3, Pacific Blue, clone UCHT1, BioLegend, 300418) and CD8+ (Anti-CD8-APC, clone RPA-T8, BD, 553569) followed by fluorescence-activated cell sorting. Naïve CD4+ T-cells were isolated with the help of a Naïve CD4 T-Cell Isolation Kit (Miltenyi Biotec, 130-096-533).

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the manufacturer's instructions. After 10 min of treatment, ROS was detected by measuring fluorescence (excitation: 488 nm, emission: 520 nm) in a Spark 10M plate reader (Tecan). Treatment with 500 μM Pyocyanin was used as a positive control for ROS creation.

**Flow cytometry assay (detects intracellular ROS).** In a similar fashion as the plate reader assay, 1 × 10^5 cells in a volume of 200 μL were treated with the desired concentration of vitamin C in flow cytometry tubes. After 30 min of treatment intracellular ROS was detected by fluorescence with a Gallios Flow Cytometer (Beckman Coulter). Treatment with 500 μM Pyocyanin was used as a positive control for creation of ROS.

**RNA-Seq Library Preparation.** RNA for sequencing underwent in-column treatment with 5 U DNase I (Zymo Research, E1010) during RNA extraction. Only RNA samples with an RNA integrity number > 8 as shown by 2100 Bioanalyzer (Agilent) were chosen for RNA sequencing (average RIN score > 9.8). The 400 ng of total RNA was treated with 500 U DNase I (Promega) during RNA extraction. Treatment with 500 U DNase I (Zymo Research, E1010) during RNA extraction.

**Cytomoter (Beckman Coulter).** Treatment with 500 U DNase I (Promega) during RNA extraction. Treatment intracellular ROS was detected by fluorescence with a Gallios Flow Cytometer (Beckman Coulter). Treatment with 500 μM Pyocyanin was used as a positive control for creation of ROS.

**DNA methylation analysis following treatment with S-aza and vitamin C.** RRSB for T-ALL cell lines was acquired from Broad Institute's CCLE (34). Promoter regions were defined as 1 kb upstream region of the TSS. Enrichment of methylated genes was tested using a Fisher's exact test by comparing the ratio of methylated (CpG methylation > 0.7) genes and unmethylated genes (CpG methylation < 0.3) against the same ratio among all expressed genes. **Gene enrichment analysis.** Enrichment of TSGs and cancer testis antigens (CTA) was assessed by hypergeometric tests, using “phyper,” in R. The TSG gene set was acquired from the NCGC.0 database (85) by selecting for all TSG marked genes found in the CTA gene set in the CCLE database (86). HERV analysis. To quantify expression of HERV families the RNA-seq samples were aligned using STAR v2.6.0c (76). The genomic position of all HERVs was acquired from the HERV database (69). Reads in HERVs were counted using “featurecounts” from the Rsubread package with the parameter “primary” to only count primary alignments. The individual HERV counts were then loaded into R and summarized over 563 families using the match function.

**RNA-Seq Data Analysis.** Total RNA-seq expression analysis. Transcriptome mapping was performed using Salmon v3.1 (25). Brias-seqBootstrap 100–validateMappings (62) using known Refseq transcripts (NM.* and NR.*) from the GRCh38.p12 assembly (GCF_00001405.38_GRCh38.p12_rna-fna.g2). As a reference. Based on our findings (Fig 1 B–D and SI Appendix, Fig S1C) the TET2 isoform TET2b (NM_017628) was excluded from the Salmon index generation. Abundance estimates were converted to h format using Wasabi (https://github.com/COMBINE-lab/wasabi) followed by data normalization and differential expression analysis performed using Wald tests implemented in Sleuth (63, 64). Differentially regulated genes were defined as qval < 0.05 (Wald test: BH, false discovery rate [FDR]-corrected P), with up-regulated and down-regulated defined as b (beta from sleuth) > 0.5 and b < −0.5 respectively. Overlap between cell line gene sets were explored using the VennDiagram (83) and UpSetR (84) packages in R.

**Data Availability.** The RNA-seq data reported in this paper have been deposited in the ArrayExpress database, https://www.ebi.ac.uk/arrayexpress/ (accession no. E-MTAB-10512) (87). All code for the RNA-seq analysis is available on github (https://github.com/Olof_Rundquist/TET2_RNA_seq_analysis).
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