The H3K27me3 demethylase UTX is a gender-specific tumor suppressor in T-cell acute lymphoblastic leukemia

Joni Van der Meulen¹, Viraj Sanghvi², Konstantinos Mavrakis², Kaat Durinck¹, Fang Fang³, Filip Mattheijssens¹, Pieter Rondou¹, Peter Vandenberghe⁴, Eric Delabesse⁵, Tim Lammens⁶, Barbara De Moerloose⁶, Björn Menten¹, Nadine Van Roy¹, Bruno Verhasselt⁷, Bruce Poppe¹, Yves Benoit⁶, Tom Taghon⁷, Ari Melnick³, Frank Speleman¹, Hans-Guido Wendel⁵* and Pieter Van Vlierberghe¹*

¹Center for Medical Genetics, Ghent University, Ghent, Belgium; ²Cancer Biology & Genetics, Memorial Sloan-Kettering Cancer Center, NY, USA; ³Department of Medicine, Weill Cornell Medical College, NY, USA; ⁴Centre for Human Genetics, University Hospital Leuven, Leuven, Belgium; ⁵INSERM U563, Toulouse, France; ⁶Department of Pediatric Hematology-Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium; ⁷Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium.

*Shared last authors

Short Title for Running Head
UTX is a tumor suppressor in T-ALL

Keywords
Leukemia, T-ALL, epigenetics, UTX, H3K27

Corresponding author:
Pieter Van Vlierberghe, PhD
Center for Medical Genetics Ghent (CMGG)
Ghent University Hospital
Medical Research Building (MRB), 2nd floor, room 120.032
De Pintelaan 185
9000 Ghent, Belgium
Tel: +32-9-3326950
Fax: +32-9-3324970
pieter.vanvlierberghe@ugent.be

Word count:
4195 words text, 122 words abstract, 5 figures, 4 supplemental figures & 7 supplemental tables, 42 references
KEY POINTS

- The H3K27me3 demethylase *UTX* is recurrently mutated in male T-ALL and escapes X-inactivation in female T-ALL blasts and normal T-cells.

- Loss of Utx contributes to T-ALL formation *in vivo*, and UTX inactivation confers sensitivity to H3K27me3 inhibition.
ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive form of leukemia that is mainly diagnosed in children and shows a skewed gender distribution towards males. Here, we report somatic loss-of-function mutations in the X-linked histone H3K27me3 demethylase UTX in human T-ALL. Interestingly, UTX mutations were exclusively present in male T-ALL patients and allelic expression analysis revealed that UTX escapes X-inactivation in female T-ALL lymphoblasts and normal T-cells. Notably, we demonstrate in vitro and in vivo that the H3K27me3 demethylase Utx functions as a bona fide tumor suppressor in T-ALL. Moreover, T-ALL driven by UTX inactivation exhibits collateral sensitivity to pharmacological H3K27me3 inhibition. All together, our results show how a gender-specific and therapeutically relevant defect in balancing H3K27 methylation contributes to T-cell leukemogenesis.
INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy that occurs in children and adolescents and is diagnosed more frequently in males than females. Current treatment schedules consist of intensified chemotherapy and are often associated with considerable side effects. T-ALL arises from a multistep oncogenic process in which different genetic alterations drive malignant transformation of immature T-cell progenitors. Several key oncogenic drivers mark particular molecular-genetic subgroups as demonstrated by genome-wide transcriptome studies on large cohorts of primary T-ALL samples. Activation of NOTCH signaling has been recognized as an oncogenic hallmark of T-ALL driven by activating NOTCH1 mutations and loss-of-function mutations targeting the E3-ubiquitin ligase FBXW7. Furthermore, a plethora of additional mechanisms of T-cell transformation have been elucidated involving an increasing number of T-ALL oncogenes and tumor suppressors.

Recent sequencing studies have identified the core components EZH2, EED and SUZ12 of the PRC2 complex, which mediates gene silencing through trimethylation of H3K27, as tumor suppressors in the pathogenesis of T-ALL. Moreover, conditional ablation of Ezh2 in mouse hematopoietic stem cells was shown to be sufficient for murine T-ALL development. The histone demethylase Ubiquitously Transcribed X Chromosome Tetratricopeptide Repeat Protein (UTX) counters the enzymatic activity of PRC2 by removing di- and trimethyl groups from H3K27. In 2009, somatic loss-of-function mutations targeting the UTX gene were identified in a variety of human tumors including multiple myeloma, esophageal and renal cancer. Recently, a general role for UTX as tumor suppressor in human cancer was further supported by the identification of recurrent inactivating UTX mutations in several leukemia and solid tumor cancer types.

In this report, we identified somatic loss-of-function mutations targeting the histone demethylase UTX in human T-ALL and provide in vitro evidence for its tumor suppressor function. Notably, our study reveals that the histone demethylase UTX can serve in vivo as a bona fide tumor suppressor in the molecular pathogenesis of T-ALL. Finally, we show that UTX mutant leukemias are more sensitive to treatment with an H3K27me3 inhibitor providing new opportunities for epigenetically targeted therapy in T-ALL.
METHODS

Collection of T-ALL patient and normal T-cell samples
A cohort of 35 bone marrow samples from primary T-ALL patients was collected from different medical institutes (UZ Ghent, Ghent, Belgium; UZ Leuven, Leuven, Belgium; Hôpital Purpan, Toulouse, France). This study was approved by the Medical Ethical Commission of Ghent University Hospital (Ghent, Belgium, B67020084745). Normal T-cells were obtained from human thymus tissue derived from female pediatric patients undergoing cardiac surgery. The thymic T-cells were obtained and used according to the guidelines of the Medical Ethical Commission of Ghent University Hospital (Belgium), and in accordance with the Declaration of Helsinki.

Murine and human T-ALL cell lines
The MOHITO T-ALL mouse cell line was cultured in RPMI-1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 20% FCS, glutamine (2 mM), penicillin (100 U/ml)-streptomycin (100 μg/ml), IL-7 (10 ng/mL) and IL-2 (5 ng/ml) (Peprotech, Rocky Hill, NJ, USA). Human T-ALL cell lines PEER, TALL-1 and PF-382 were obtained from the DSMZ repository (Braunschweig, Germany). The human ovarian adenocarcinoma cell line OVCAR-3 and the human colon adenocarcinoma cell line HT-29 were obtained from the ATCC repository (ATCC, Manassas, VA, USA). The cell lines were cultured in RPMI-1640 medium supplemented with 10% or 20% FCS, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) under controlled conditions (37°C, 5% CO2).

DNA and RNA isolation
DNA isolation was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). RNA isolation was performed using the miRNeasy mini kit with DNA digestion on-column (Qiagen). DNA and RNA concentration was measured on the NanoDrop 1000 Spectrophotometer. RNA quality was assessed using the Experion Automated Electrophoresis System according to the manufacturer’s instructions (Bio-rad Laboratories, Hercules, CA, USA). After RNA quality assessment, complementary DNA (cDNA) synthesis was performed using the iScript cDNA Synthesis Kit (Bio-rad Laboratories).

Sequencing analysis
We sequenced all coding exons of PTEN, PHF6 and UTX, and mutation hotspot regions for NOTCH1 and FBXW7 in 35 T-ALL patient samples by
Sanger sequencing. Primer sequences are noted in supplemental Table S1. The PCRx enhancer system (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used for NOTCH1 PCR reactions. FBXW7, PTEN, PHF6 and UTX amplification was performed using the KAPAq HotStart kit (Kapa Biosystems, Wilmington, MA, USA). For all reactions, the following PCR protocol was used: 95°C for 10 min, (96°C for 15 sec, 57°C for 1 min, then 72°C for 1 min) for 40 cycles, then 72°C for 10 min. PCR products were purified and analyzed using the Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Life Technologies).

Sanger sequencing of Utx, Ezh2, Suz12 and Eed in the MOHITO cell line, and Sanger sequencing of UTX, EZH2, SUZ12 and EED on human T-ALL cell lines was performed at the Geoffrey Beene Translational Oncology Core Facility at MSKCC.

Array Comparative Genomic Hybridization (array CGH)

T-ALL patient samples were profiled for copy number analysis on SurePrint G3 Human 4x180K CGH Microarrays (Agilent Technologies, Santa Clara, CA, USA). In brief, patient and control genomic DNAs were labeled using random prime labeling with Cy3 and Cy5 dyes (Perkin Elmer, Waltham, MA, USA), respectively. Next, hybridization was performed according to the manufacturer’s instructions (Agilent Technologies) followed by data-analysis using the in-house developed analysis tool arrayCGHbase.19

SNP genotyping

Genotyping and allelic expression analysis of SNPs rs181547731 and rs20539 located in the 3’ UTR and exon 20 of UTX respectively was performed in both genomic DNA (gDNA) and cDNA samples of 3 female T-ALL patients, 3 female normal T-cell donors and 2 female cancer cell lines OVCAR-3 and HT-29. The standard PCR and sequencing protocols were used as described above. Primer sequences are listed in supplemental Table S1.

Real-Time quantitative Polymerase Chain Reaction (qPCR)

The qPCR reaction was performed using the LightCycler 480 (Roche, Basel, Switzerland) and qPCR data was analysed by the ΔΔCt method using the in-house developed qPCR analysis program qBasePlus (Biogazelle, Gent, Belgium). Primers used to measure the expression of the genes of interest and reference genes are provided in supplemental Table S2.
Interleukin depletion assay

Short hairpin RNAs (shRNAs) were designed using the online program Designer of Small Interfering RNA (DSIR) followed by cloning into the retroviral MLS vector backbone, which also encodes for GFP co-expression. Next, the interleukin (IL-2/IL-7) dependent MOHITO cell line was transduced with shRNAs Utx and GFP percentage was evaluated using the GUAVA Flow Cytometer (Millipore, Billerica, MA, USA). Subsequently, MOHITO cells were depleted of IL-2 and IL-7 until less than 10% of viable cells were detected followed by rescue through supplementing IL-2 and IL-7 back in the media. After each round of interleukin depletion, the GFP percentage was measured. The sequences of the shRNAs are listed in supplemental Table S3.

T-ALL mouse model

Fetal liver cells were isolated at embryonic day 13-14 followed by retroviral transduction of shRNAs or empty control labeled with GFP (MLS backbone) and NOTCH1 labeled with mCherry (MIG backbone).\(^{20}\) Next, the transduced fetal liver cells were tail vein injected after lethal irradiation of mouse recipients. Subsequently, mouse recipients were monitored for leukemia onset by analysis of lymphoblast counts in blood smears and by physical appearance. By the time of leukemia formation, various tissues including spleen, lungs, liver, kidney and lymph nodes were fixed for histological evaluation. Furthermore, the lymphoblasts from spleen and thymus were mashed to single-cell suspensions and frozen in 10% DMSO. Survival data was analyzed using the Kaplan-Meier method and statistical significance was calculated using the log-rank (Mantel-Cox) test.

Immunophenotypic analysis

Surface marker analysis of murine tumor cells was performed using a BD LSR II flow cytometer with monoclonal antibodies CD3-APC (BD Biosciences, San Jose, CA, USA), CD8a-PECY7 (eBioscience, San Diego, CA, USA) and CD4-biotin (BD Biosciences). Data were analyzed with FACSDiva 6.1.2 software (BD Biosciences) and compensations were set using OneComp eBeads (eBioscience).

Histology

Histological analysis including Hematoxylin & Eosin (H&E) staining, Ki67 staining, H3K27me3 staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of the different mouse leukemias was performed at the Laboratory of Comparative Pathology of MSKCC.
Western Blotting

Cells were lysed using a lysis buffer containing 2% SDS, 62.5 mM Tris pH 6.8, 10% glycerol, 5% β-mercaptoethanol, supplemented with EDTA free protease inhibitor cocktail (Roche). Denatured samples were loaded on 10% or 7.5% gels (1h15min, 250V) (Bio-rad Laboratories) for electrophoresis, followed by immunoblotting on PVDF membranes using a wet blotting system (1h, 250V) (Bio-rad Laboratories). Membranes were incubated with primary and secondary antibody dilutions in 5% milk/TBST solutions, developed using SuperSignal West Dura Chemiluminescent Substrate (Pierce, Thermo Scientific, Rockford, IL, USA) and visualised on ChemiDoc-It imaging system 500 (UVP, Upland, CA, USA). Following antibodies were used: Utx (155 kDa, A302-374A, Bethyl Laboratories, Montgomery, Texas, USA), H3K27me3 (17 kDa, 9733, Cell Signaling, Danvers, MA, USA), EZH2 (95-100 kDa, 612666, BD Biosciences), tubulin (50 kDa, T5168, Sigma, St. Louis, MO, USA).

Gene expression arrays of murine T-ALL samples

RNA samples were profiled for gene expression analysis on SurePrint G3 Mouse 8x60K Microarrays according to the manufacturer’s instructions (Agilent Technologies). Normalization of gene expression data was done by quantile normalization using R. Background correction was done based on the dark corner probe group of the Agilent slides. Differential gene expression analysis was performed using fold change analysis and p-value calculation based on unpaired t-test. Biased gene set enrichment analysis (GSEA) was performed using a gene set consisting of differential probes between the Utx sh#1 and control murine leukemias, and evaluated against gene expression data of the Utx deficient MOHITO samples. Unbiased GSEA was executed against annotated gene sets of the Molecular Signatures Database (MSigDB). Gene expression data is accessible on ArrayExpress under accession number E-MTAB-2921.

Gene expression arrays of human T-ALL samples

Differential UTX expression analysis (unpaired t-test) was performed between female and male T-ALL patient samples on published gene expression data from Clappier et al. Journal of Experimental Medicine 2011. Patient gender was defined based on karyotype information. Gene expression data is accessible on ArrayExpress under accession number E-MTAB-59321.
**ChIP-sequencing**

Lymphoblasts from murine leukemias were cross-linked in 1% formaldehyde for 10 min on rotating platform in 37°C oven. Next, 2.5M of glycine (0.122M final concentration) was added for 5 min at 37°C to quench the cross-linking reaction. The cells were subsequently pelleted at 4°C for 5 min at 1750 rpm followed by three washing steps in cold PBS. The final pellet was stored at -80°C. Following H3K27me3 ChIP-procedure (H3K27me3: 17kDa, cat#6002, Abcam, Cambridge, UK) was performed as previously described (Beguelin et al. Cancer Cell 2013). 22 H3K27me3 ChIP-seq libraries were prepared using the Illumina ChIP-Seq sample kits based on the manufacturer. Libraries were validated using the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies) and 8-10pM was sequenced on HiSeq2000 sequencer (Illumina, San Diego, CA, USA). The raw data were aligned to mm9 genome using ELAND. Reads were normalizing for total ChIP-seq reads and quantified in 1 kb bins genome-wide. The enriched regions were identified as consecutive bins with read counts greater than 1 standard deviation of the genome-wide mean.

**Cell viability assay**

Murine and human T-ALL cell lines were treated with 3-Deazaneplanocin A hydrochloride (DZNep, Sigma) using a dilution series ranging from 50 nM to 100 μM. After 24 hours, cell viability was measured using the luminescence detection kit CellTiter Glo Cell Viability Assay (Promega, Madison, WI, USA).
RESULTS

Mutations recurrently target the H3K27me3 demethylase \emph{UTX} in male T-ALL patients

To identify a potential role for the H3K27me3 demethylase UTX in the molecular pathogenesis of T-ALL, we performed sequencing and copy number analysis in a series of 35 primary leukemia samples (10 female versus 25 male), including 25 pediatric and 10 adult T-ALL cases. We identified loss-of-function mutations in \emph{UTX} in 5 out of 35 (14.3\%) primary T-ALL patient samples (Fig. 1A, supplemental Table S4) including 3 frameshift and 2 in-frame insertion/deletion mutations. \emph{UTX} mutations were localized in a hotspot region within the catalytic Jumonji C (JmjC) domain of the protein (Fig. 1B) and sequencing of available remission material confirmed the somatic origin of these mutations (Fig. 1A). The somatic \emph{UTX} mutations in our patient population were exclusively identified in samples from male origin.

Equal dosage of chromosome X genes between male and female cells is regulated by random inactivation of one copy of the X chromosome in female cells. However, previous studies have shown that some genes, including \emph{UTX}\textsuperscript{23}, can escape chromosome X-inactivation in certain tissues. Notably, we analyzed two silent SNPs, rs181547731 situated in the 3’ UTR and rs20539 situated in exon 20 of \emph{UTX}, in lymphoblasts from 3 female T-ALL patients and confirmed bi-allelic expression of \emph{UTX} in female T-ALL lymphoblasts (Fig. 1C, supplemental Fig. S1A). In agreement, \emph{UTX} expression is elevated in female T-ALL patient samples in comparison with male T-ALL patient cases (student t-test, p=0.001, female: n=5, male: n=20) (supplemental Fig. S1B).\textsuperscript{21} In addition, we were able to demonstrate that \emph{UTX} is also able to escape X-inactivation in normal T-cells derived from female donors (supplemental Fig. S1C) and in 2 female cancer cell lines, the ovarian adenocarcinoma cell line OVCAR-3 and the colon adenocarcinoma cell line HT-29 (supplemental Fig. S1D).

In T-ALL, a wide variety of oncogenic lesions cooperate to induce T-cell transformation. To identify genetic lesions that co-occur with loss of \emph{UTX} in T-ALL, we classified our cohort into the known molecular genetic subgroups based upon the presence of genetic defects and/or aberrant expression of transcription factor oncogenes (Fig. 1D).\textsuperscript{3-5} Furthermore, we screened our patient population for alterations targeting T-ALL specific oncogenes and tumor suppressors (Fig. 1D). This analysis showed that \emph{UTX} mutations co-occur with aberrant expression of the \emph{TLX3} oncogene, activating \emph{NOTCH1} mutations, and mutations or deletions targeting the putative chromatin reading factor \emph{PHF6} (Fig. 1D). Of note, no deletions were identified in the second H3K27me3 eraser and UTX family member \emph{Jumonji D3} (JMJD3).
Utx acts as a tumor suppressor in T-ALL

To functionally validate the role of Utx as a putative tumor suppressor in the context of malignant T-cell transformation, we used the interleukin (IL2/IL7) dependent murine T-ALL cell line MOHITO. We transduced MOHITO cells with shRNAs against Utx and an empty vector control, and assessed knockdown efficiency using Western blot analysis (Fig. 2A). Both Utx shRNA#1 and Utx shRNA#3 mediate a clear decrease in Utx protein levels. Next, we examined if knockdown of Utx can provide an oncogenic advantage in vitro to the MOHITO cells. First, MOHITO cells were partially transduced with Utx shRNA#1, Utx shRNA#3 or empty vector control (GFP co-expression). After successive rounds of interleukin depletion that lead to a drop in cell viability, GFP enrichment was assessed as an indicator of tumor promoting activity (Fig. 2B). Notably, murine tumor cells infected with functional Utx shRNA#1 or Utx shRNA#3 showed significant GFP enrichment over MOHITO cells infected with an empty vector control (Fig. 2C) indicating that Utx loss grants oncogenic properties to the MOHITO cells.

Next, we used a bone marrow transplant mouse model of NOTCH1-induced T-ALL to study the in vivo role of Utx in the pathogenesis of T-ALL. Leukemic onset in mouse recipients receiving fetal liver cells with enforced NOTCH1 expression occurred around 55 days (n=14, mean latency = 54 days) (Fig. 3B). Infection of fetal liver cells with Utx shRNA#1 together with activated NOTCH1 resulted in an acceleration of T-ALL onset (n=7, p<0.0001, mean latency = 29 days, Fig. 3B) confirming the in vitro oncogenic properties observed in the T-ALL MOHITO cells. Introduction of Utx shRNA#3 provided a milder effect on T-ALL latency (n=4, p=0.02, mean latency = 43.5 days; Fig. 3B).

In all murine tumors, histological analysis revealed aggressive leukemia phenotypes marked by absence of apoptosis, high proliferation and infiltration of lymphoblasts in different organs (supplemental Fig. S2A). In addition, evaluation of the shRNA mediated Utx knockdown confirmed loss of Utx expression in vivo in the NOTCH1 T-ALL mouse model (Fig. 3C and supplemental Fig. S2B). Notably, immunohistochemistry and Western blot analysis of H3K27me3 revealed that the H3K27me3 levels between Utx driven and NOTCH1 driven control murine T-cell tumors were largely comparable (Fig. 3C and supplemental Fig. S2C). Immunophenotypic characterization of mouse leukemias indicated an earlier T-cell maturation arrest for tumors driven by loss of Utx as compared to NOTCH1 control tumors (Fig. 3D and supplemental Table S5-S7). The NOTCH1 only controls were typically CD4+CD8+ double positive. In contrast, immunophenotypic marker expression in Utx driven tumors was more heterogeneous including cell populations that lacked both CD8 and CD4 surface expression (Fig. 3D).
and supplemental Table S5-S7). Hence, the demethylase Utx is able to restrain T-ALL development in vivo.

**Transcriptional programs driven by loss of Utx in murine T-ALL models**

To gain more insight in the oncogenic mechanisms mediated by loss of Utx, we performed gene expression profiling of NOTCH1 control tumors and mouse leukemias driven by Utx inactivation. Supervised gene expression analysis (FC>1.5, p<0.05; Fig. 4A) showed that Utx deficient mouse tumors are associated with a specific gene expression signature. Utx leukemias are characterized by down regulation of the T-ALL tumor suppressor genes *Ect2l* and *Nf1* and activation of the T-ALL oncogene *Tal1* (Fig. 4A). We could confirm down and up regulation of Utx regulated genes using qPCR analysis, some representative examples are shown in supplemental Fig. S4. Furthermore, Gene Set Enrichment Analysis (GSEA) in the mouse tumors driven by loss of Utx showed enrichment of gene sets linked to early T-cell lymphocytes (p<0.01; Fig. 4B; supplemental Fig. S3A) and *LYL1* neighbouring genes in T-ALL (p=0.02; supplemental Fig. S3B), in line with the early immunophenotypic arrest observed in these murine leukemias (Fig. 3D).

Next, we performed H3K27me3 chromatin immunoprecipitation (ChIP) sequencing analysis on murine tumor material, to evaluate if genes down regulated in Utx driven driven mouse leukemias show differential H3K27me3 levels at their promoters. In total, 51 genes out of 258 down regulated genes (19.7%) show an enhanced H3K27 trimethylation at their promoter regions in at least 2 Utx sh#1 samples compared to 2 control samples. In comparison, 1801 genes out of 39430 genes (4.5%) on the gene expression array demonstrate enrichment of H3K27me3 at their promoter region (hypergeometric test, p=6.9058e-19, log=-41.8168). Hereby, the genes that are down regulated after Utx knockdown have statistically more H3K27me3 enrichment as compared to the general number of H3K27me3 enrichment over all genes. Notably, the ChIP-sequencing analysis revealed an accumulation of H3K27me3 at promoter regions of genes with putative tumor suppressor activity like *Pcgf2*, *Lzts2*, *Dock4*, *Pura*, *Col2a1*, *Sic26a* in the Utx tumors (Fig. 4C and supplemental Fig. S3C).

To further explore the relationship between the genetic program mediated by the in vivo transplant model and the in vitro MOHITO model, we performed GSEA of gene expression signatures associated with Utx knockdown in MOHITO cells. Interestingly, this analysis revealed a significant enrichment of genes down regulated upon loss of Utx in the mouse leukemias in Utx deficient MOHITO cells (p=0.007, 82 of 252 probes in core enrichment, Fig. 4D).
**Loss of Utx provides a selective pressure towards DZNep treatment**

As loss of UTX will affect the genome-wide distribution of H3K27me3, we hypothesized that decreased UTX levels would render leukemia cells more vulnerable to treatment with 3-Deazaneplanocin A (DZNep), an epigenetic compound that specifically targets H3K27me3\(^3\)\(^4\). Treatment of three T-ALL cell lines with DZNep demonstrated enhanced sensitivity of the UTX mutant cell line PF-382 as compared to the PEER cell line and the EZH2 mutant cell line TALL-1 (Fig. 5A-D). Moreover, TALL-1 cells were strongly resistant to DZNep treatment even at 100 μM presumably because of very low H3K27me3 levels (Fig. 5C-D). As expected, DZNep treatment of PF-382 and PEER resulted in decreased levels of H3K27me3\(^3\)\(^4\) as determined by Western blot (Fig. 5E). In parallel, we confirmed that MOHITO cells showed enhanced sensitivity towards DZNep treatment upon Utx knockdown as compared to control cells (Fig. 5F). Notably, although control and Utx knockdown MOHITO cells show comparable baseline levels of H3K27me3, DZNep treatment induced a stronger decrease in H3K27me3 expression in the Utx knockdown samples as compared to the empty vector controls (Fig. 5G). Hence, UTX deficient T-ALL shows collateral sensitivity to H3K27me3 inhibition.
DISCUSSION

A role for UTX as tumor suppressor was initially postulated in several human tumors including multiple myeloma, esophageal and renal cancer. Notably, UTX deletions and mutations have also been identified in patients with the Kabuki syndrome, a rare congenital anomaly syndrome. Interestingly, six Kabuki patients have been reported that developed different types of cancer suggesting an increased susceptibility to cancer for Kabuki patients carrying abnormalities in UTX. In this report, we identified gender-restricted somatic loss-of-function mutations targeting the histone demethylase UTX in male T-ALL patients and demonstrate its tumor suppressor function.

Random inactivation of one copy of the X-chromosome in female cells is the mechanism that ensures equal dosage of chromosome X genes between male and female cells. However, some genes can escape chromosome X-inactivation and show bi-allelic expression in normal female tissues. Previous studies demonstrated that UTX can escape chromosome X-inactivation in normal female tissues and accordingly shows higher expression in female embryonic stem cells and female tissues derived from brain, liver, neurons and sexual organs in comparison with the male counterpart. The fact that UTX escapes chromosome X-inactivation in normal cells has important implications for gender-related tumor susceptibility. Indeed, given the previously established role for UTX as a tumor suppressor in human cancer, inactivation of only one single UTX copy in males will contribute to tumor development. In contrast, female cells are protected against such single copy loss of UTX because they still express UTX from the second allele (Supplemental Fig. S1A).

Most notably, T-ALL is characterized by a skewed gender distribution with a 3:1 male to female ratio. In contrast to other X-linked tumor suppressor genes in T-ALL (PHF6 and RPL10), UTX escapes chromosome X-inactivation in female T-ALL blasts, suggesting that females are protected against single copy loss of one UTX allele. Therefore, UTX is the first X-linked tumor suppressor gene that might partially explain the skewed gender distribution in T-ALL towards males on a genetic level.

The PRC2 complex mediates H3K27 methylation and therefore counteracts UTX activity. Tri-methylation of lysine 27 on histone 3 is a chromatin state that is usually associated with transcriptional gene repression. Depending on the tissue-type and cellular context, the PRC2 complex can function as an oncogene (for example in B-cell lymphoma) or tumor suppressor gene (for example in T-ALL). In addition, Ezh2 loss was sufficient to induce murine T-ALL development further supporting a tumor suppressive activity for EZH2 in the context of T-ALL.
Given our finding that the H3K27me3 modulator UTX is targeted by loss-of-function alterations in primary T-ALL, we explored its tumor suppressor activity using *in vitro* and *in vivo* perturbation model systems. First, Utx knockdown provided murine leukemia cells with an oncogenic advantage after interleukin depletion using the IL2-IL7 dependent MOHITO culture system. Second, loss of Utx resulted in significant acceleration of leukemia onset in a NOTCH1-induced T-ALL mouse model. In addition, differences in tumor immunophenotype between Utx driven mouse leukemias and control tumors suggests that the tumor suppressor activity of Utx could depend on the maturation arrest or the cell of origin during murine T-cell transformation. For the first time, these functional *in vitro* and *in vivo* data firmly establish Utx as a *bona fide* tumor suppressor involved in murine T-cell transformation.

Transcriptional profiling of the *in vivo* transplant model revealed that loss of Utx drives a unique gene expression signature in NOTCH1 driven mouse leukemias. Interestingly, in a previous study we could demonstrate that shRNA mediated knockdown of Nf1, one of the down regulated genes after Utx knockdown, is also able to induce a strong acceleration in T-ALL onset in the NOTCH1 T-ALL mouse model. Hence, Nf1 loss seems to mimic the oncogenic effect of Utx loss in the same *in vivo* leukemic model system.

Notably, an important part of the gene expression profiles of the Utx knockdown samples most likely reflects the immunophenotypic differences observed between control and Utx deficient tumors. However, ChIP-seq analysis confirmed that some of the transcriptional differences are associated with accumulation of H3K27me3 at the promoter regions and therefore most probably reflect direct consequences of loss of Utx during malignant T-cell transformation.

Epigenetic drugs are currently developed and evaluated in different cancer subtypes. Since we identified UTX as a *bona fide* tumor suppressor gene in the pathogenesis of T-ALL, we hypothesized that loss of UTX would render leukemia cells more vulnerable to treatment with particular chromatin modifying drugs. As loss of UTX is associated with enhanced H3K27me3 levels, we explored the effect of DZNep, an epigenetic compound that specifically targets the H3K27me3 mark. Previous studies reported a strong apoptotic effect after DZNep administration in cancer cells whereas normal cells were not affected. Importantly, in our study, we showed in both murine and human *in vitro* T-ALL model systems that loss of UTX/Utx renders the leukemic population more sensitive to DZNep treatment. In contrast, the EZH2 mutant T-ALL cell line TALL-1 seems resistant to DZNep treatment. Importantly, such proof of principle data should be further validated in a preclinical setting in which H3K27me3 modifying agents are formally tested in well-characterized genetic animal models of T-ALL.
In conclusion, our results provide new insights into the pathogenesis and gender distribution in T-ALL. Furthermore, we identified *UTX* as an X-linked tumor suppressor gene and showed that Utx can act as a *bona fide* tumor suppressor in T-ALL. All together, our study reveals how maintaining and pharmacologically restoring the precise balance of H3K27 methylation can restrain T-cell leukemia.
ACKNOWLEDGEMENTS

We would like to thank the members of the Wendel lab and the members of the Speleman lab for experimental support and discussion during this research project; and Aline Eggermont for excellent technical assistance. The Geoffrey Beene Translational Oncology Core Facility at MSKCC for the sequencing support and the Flow Cytometry Facility at MSKCC for cell sorting assistance. The Memorial Sloan Kettering (MSK) animal facility and Research Animal Resource Center (RARC) for assistance with mouse experiments and the Laboratory of Comparative Pathology for histological analysis.

This work is supported by the Fund for Scientific Research (FWO) Flanders (postdoctoral grants to P.V.V., P.R. and T.T., PhD grant to J.V.d.M., B.P. and P.V. are Senior Clinical Investigators of FWO-Flanders, Odysseus grant to P.V.V and T.T. and project grants G.0198.08, G.0564.13N, G.0550.13N and G.0869.10N to F.S., G065614, 3GA00113N and G.0C47.13N to P.V.V. and G0B2913N and 3G002711 to T.T.; the Flemish Liga against Cancer (VLK) (PhD grant to J.V.d.M. and postdoctoral grant to F.M.); the Agency for Innovation by Science and Technology (IWT) (PhD grant to K.D.); the GOA-UGent (grant no. 12051203 to F.S.); Belgian Foundation against Cancer, the Cancer Plan from the Federal Public Service of Health (F.S.); the Children Cancer Fund Ghent (F.S.); the Belgian Program of Interuniversity Poles of Attraction IUAP; the Belgian Foundation Against Cancer (project grant 2010-187 to T.L., 365O9110 to F.S.); the NCI R01-CA142798-01 (H-G.W.) and U01CA105492-08 (H-G.W.); the Leukemia Research Foundation (H-G.W.). H-G.W. is a Scholar of the Lymphoma & Leukemia Society, the American Cancer Society and the Geoffrey Beene Cancer Center. A.M. is supported by LLS SCOR 7006-13 and LLS TRP 6141-14, and the Burroughs Wellcome Foundation.

AUTHORSHIP

CONFLICT OF INTEREST

No potential conflicts of interest.
REFERENCES


FIGURE LEGENDS

Figure 1. UTX mutations in human T-ALL

(A) DNA sequencing chromatogram showing a UTX mutation in the gDNA of a male primary T-ALL patient sample. The mutation is absent in remission material of the same patient. (B) Graphical representation of the localization of genetic lesions in the UTX protein structure (TPR = tetratricopeptide repeat, JmjC = Jumonji C). In-frame deletion/insertion mutations are depicted in orange circles, frameshift mutations in blue circles. (C) Genotyping and allelic expression analysis of SNP rs181547731 in gDNA and cDNA derived from female T-ALL lymphoblasts. (D) Graphical representation of the different mutations (depicted by dark orange rectangles) and deletions (depicted by light orange rectangles) present in a set of T-ALL oncogenes and tumor suppressor genes in 35 primary T-ALL patient samples. The different T-ALL subgroups include TAL-LMO, TLX3, TLX1, HOXA and patients for which the subgroup is unknown. The age subgroups include children (age ≤ 15 years, depicted in dark red rectangles) and adults (age > 15 years, depicted in light red rectangles). Male and female T-ALL patient samples are presented in dark blue and light blue rectangles, respectively.

Figure 2. Knockdown of Utx augments the oncogenic activity in the murine T-ALL cell line MOHITO

(A) Western blot analysis of GFP sorted MOHITO knockdown samples containing 3 different hairpins against Utx compared to empty vector control. Blots were incubated with antibodies against Utx and tubulin. Expression levels were normalized to tubulin levels and compared to the vector control. (B) Graphical illustration of the interleukin depletion assay in the IL2-IL7 dependent MOHITO cell line. MOHITO cells are partially transduced with vectors encoding shRNAs against Utx and co-expressing GFP, after which the GFP percentage is measured by FACS analysis before and after successive rounds of interleukin depletion. (C) GFP percentage measured at Day 0, and after 1st and 2nd depletion rounds in Utx sh#1 and Utx sh#3 knockdown samples and controls. The depletion assay was repeated twice. The GFP enrichment was statistically significant after the 1st depletion for Utx sh#1* and Utx sh#3** and after the 2nd depletion for Utx sh#1*** compared to empty vector control respectively (unpaired t-test: *p=0.005, **p=0.049, ***p=0.008).
Figure 3. Loss of Utx accelerate leukemia development in a NOTCH1-induced T-ALL mouse model

(A) Graphical illustration of NOTCH1-induced T-ALL mouse model. Fetal liver cells are partially transduced with vectors encoding NOTCH1 (ICN) (co-expressing mCherry) and the shRNA or empty vector control (co-expressing GFP) followed by tail vein injection in lethally irradiated mouse recipients and monitoring of leukemia onset. (B) Kaplan-Meier curves and log-rank (mantel-cox) analysis show accelerated leukemia onset in Utx sh#1 (n=7, p<0.0001, blue) and Utx sh#3 (n=4, p=0.02, red) mice as compared to empty vector (n=14, black) mouse recipients. (C) Western blot analysis of Utx and H3K27me3 in a representative Utx sh#1 mouse leukemia sample. Utx protein levels and H3K27me3 levels are quantified by Image J, normalized to tubulin levels, and compared to expression levels in control mice. (D) Immunophenotypical FACS analysis of CD4 and CD8 T-cell markers in the Utx sh#1, Utx sh#3 and empty vector control mouse leukemias. A representative example of each subtype is depicted.

Figure 4. Gene networks regulated by Utx in T-ALL

(A) Differentially expressed genes (FC>1.5; p<0.05) between Utx knockdown and control murine leukemias are represented in a heat map. A selection of genes is shown in rows and each column represents one individual mouse leukemia sample. The scale bar shows color-coded differential expression from the mean in standard deviation units with red indicating higher levels and blue lower levels of expression. (B) Unbiased GSEA of gene expression signatures associated with murine leukemias driven by loss of Utx or NOTCH1 only vector controls. Gene sets involving early T-lymphocytes (p<0.01) are significantly enriched in Utx driven leukemias. (C) H3K27me3 ChIP-seq profiles at 2 specific gene loci (Lzts2 and Pcgf2) in murine leukemias driven by loss of Utx or NOTCH1 only vector controls. (D) GSEA of transcripts significantly down regulated upon Utx knockdown in murine leukemias (FC>1.5, p<0.05, 252 probes) in gene expression signatures obtained from MOHITO samples driven by loss of Utx. Heatmap displays the TOP25 leading edge of this gene set in Utx driven MOHITO samples.

Figure 5. Utx driven cell lines are more sensitive for H3K27me3 inhibition

(A) Genomic DNA sequencing chromatograms representing 2 missense mutations in the EZH2 gene detected in the T-ALL cell line TALL-1. (B) DNA sequencing chromatogram representing a nonsense mutation in the UTX gene detected in the human T-ALL cell line PF-382. (C) Western blot analysis of EZH2, H3K27me3 and tubulin in the human T-ALL cell lines PF-382, TALL-
1 and PEER. EZH2 protein and H3K27me3 levels are quantified by Image J, normalized to tubulin levels. (D) Luminescence-based viability assay after 24 hours (24h) of DZNep administration in 3 different T-ALL cell lines (TALL-1: black, PEER: red, PF-382: green) using a range of DZNep concentrations from 0 to 100μM. The experiment was done using 6 replicates, and repeated two times independently. The viability score for each concentration was significantly different between the 3 T-ALL cell lines (*Kruskal-Wallis test, p<0.0001). (E) Western blot analysis of H3K27me3 and tubulin 24h after DZNep administration (1000nM & 500nM) in 2 different T-ALL cell lines (PEER: red, PF-382: green). H3K27me3 levels are quantified by Image J and normalized to tubulin levels. DZNep treated samples are compared to expression levels in untreated samples. (F) Luminescence-based viability assay after 24h of DZNep administration in Utx knockdown and control MOHITO samples (vector: black, Utx sh#1: green). The experiment was done in fourfold (unpaired t-test: *p<0.0001). (G) Western blot analysis of H3K27me3 and tubulin 24h after DZNep administration (1000nM & 500nM) in Utx knockdown and control MOHITO samples (vector: black, Utx sh#1: green). H3K27me3 levels are quantified by Image J, normalized to tubulin levels. DZNep treated samples are compared to expression levels in untreated samples.
Figure 1

A. **UTX** c.3337ins(CTACC)  
   p.Val1113Leufs*8

B.  
   - TPR repeats
   - JmjC domain

C. rs181547731  
   3'UTR **UTX**

D.  
   - **age**
   - **sex**
   - **NOTCH1**
   - **FBXW7**
   - **PHF6**
   - **PTEN**
   - **UTX**
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>Utx</th>
<th>Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utx sh#1</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Utx sh#2</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Utx sh#3</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

B

shRNAs (GFP labeled) against Utx

Partial transduction in the interleukin dependent MOHITO cell line

Evaluation of GFP% before/after interleukin depletion

C

GFP % vs. Day 0, 1st depletion, 2nd depletion

- **: p < 0.01
- ***: p < 0.001
Figure 3

A

Pregnant mouse → Fetal liver cells → Stem cell transfer → Leukemia

ICN shRNA/Vector

B

Percent survival vs. Days

ICN + Utx sh#1 (p<0.0001)
ICN + Utx sh#3 (p = 0.02)
ICN + Vector

C

Utx
H3K27me3
Tubulin

1 0.5
1 1.02

ICN + Vector ICN + Utx sh#1

D

CD4 vs. CD8

ICN + Vector
ICN + Utx sh#1
ICN + Utx sh#3

6.6 93.4
51 29.2
17.2 38.5

0 0
6.8 12.9
15.4 28.9
Figure 4

A Gene set: down regulated in Utx sh#1 murine leukemias

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vector</th>
<th>Utx sh#1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ect2l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nf1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hdac5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pcgf2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ltz2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pura</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppp1r13b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dock4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rps6ka2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dapk1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnk2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col2a1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slc26a4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bbl11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bin1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cux2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rn39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tran1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cacng4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cnn3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fam102a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcf711</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ilsa1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pak3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prt1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cpeb4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccdc88a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angp12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wwtr1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fam83d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anln</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sqtst1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epgn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uba2c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crkl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerpn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Map2k6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vang1l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamc2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt5a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sfrn1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ttnk2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pik1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tpl1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nly1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neddf9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccnd2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdk2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dlgap5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccnf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aurk6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tal1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B Gene set: EARLY_T_LYMPHOCYTE_DOWN
Vector controls vs Utx sh#1 murine leukemias

Enrichment score (ES)

<table>
<thead>
<tr>
<th>Vector</th>
<th>Utx sh#1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.1</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

C Vector control leukemias
Utx sh#1 leukemias

D Gene set: down regulated in Utx sh#1 murine leukemias
Vector controls vs Utx sh#1 MOHITO samples

Enrichment score (ES)

<table>
<thead>
<tr>
<th>Vector</th>
<th>Utx sh#1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>0.07</td>
</tr>
</tbody>
</table>
**Figure 5**

(A) **TALL-1**
- EZH2
c.2139A>G  
p.I713M
- EZH2
c.1680C>A  
p.N560K

(B) **PF-382**
- UTX
c.3835C>T  
p.R1279X

(C) **EZH2**
- H3K27me3
- TUBULIN

(D) **DZNep 24h**

(E) **H3K27me3**
- TUBULIN

(F) **DZNep 24h**
- Utx sh#1
- Vector

(G) **H3K27me3**
- TUBULIN
The H3K27me3 demethylase UTX is a gender-specific tumor suppressor in T-cell acute lymphoblastic leukemia

Joni Van der Meulen, Viraj Sanghvi, Konstantinos Mavrakis, Kaat Durinck, Fang Fang, Filip Matthijssens, Pieter Rondou, Peter Vandenberghe, Eric Delabesse, Tim Lammens, Barbara De Moerloose, Björn Menten, Nadine Van Roy, Bruno Verhasselt, Bruce Poppe, Yves Benoît, Tom Taghon, Ari Melnick, Frank Speleman, Hans-Guido Wendel and Pieter Van Vlierberghe

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.