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# Tet2 is a tumor suppressor in the pre-leukemic phase of T-cell acute lymphoblastic leukemia

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**Abstract:**
TET2-mediated DNA demethylation plays a pivotal role in regulating pre-leukemic clonal expansion in acute myeloid leukemia (AML), where TET2 mutations are also linked to AML progression. However, its function in other types of leukemias, including T-cell acute lymphoblastic leukemia (T-ALL), remains unclear. Here, we used two different T-ALL mouse models to study the possible tumor suppressor role of Tet2 in pre-leukemic T-ALL. Overexpression of Tet2 resulted in a mild but significant increase in T-ALL latency in the immature CD2-Lmo2tg T-ALL mouse model, but no effect on survival was observed in the mature Lck-Cretg/+ Ptenfl/lf T-ALL mouse model. In contrast to the pre-leukemic thymocytes from CD2-Lmo2tg mice, Lck-Cretg/+ Ptenfl/fl thymi do not display self-renewal suggesting that the anti-leukemic effect of Tet2 occurs mainly in the pre-leukemic phase of T-ALL. In conclusion, we demonstrated that the Tet2 tumor suppressor function is dependent on the differentiation stage of T-ALL and limited to the pre-leukemic phase.

**Conflict of interest:** No COI declared

**COI notes:**

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## *Tet2* is a tumor suppressor in the preleukemic phase of T-cell acute lymphoblastic leukemia

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Running Title: *Tet2* in preleukemic T-ALL

For original data, please contact steven.goossens@ugent.be

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T-cell acute lymphoblastic leukemia (T-ALL) is a rare but aggressive leukemia affecting the T-cell lineage. During the malignant transformation of immature T-cells, a clonal expansion of progenitor cells is selected via the gradual accumulation of advantageous genetic mutations and epigenetic changes (1). Alterations in the DNA methylome are suggested as one of the potential initiating mechanisms that allow this gradual oncogenic transformation to occur (2). During DNA methylation, a methyl group is covalently attached to a cytosine in CpG-rich dinucleotide sequences by DNA methyltransferases (DNMTs) (3). It is a plastic and reversible process, where active demethylation is catalyzed by the ten-eleven translocation (TET) oxydase enzymes (4,5). *Tet2* knockouts are not only predisposed to myeloid malignancies, but are also prone to develop lymphoid malignancies, including T-ALL (6,7). Although *TET2* mutations in T-ALL are rare (0.7%), *TET2* is transcriptionally repressed or silenced in 71% and 17% of T-ALL, respectively. This silencing is often associated with hypermethylation of the *TET2* promotor (8,9). Based on these observations, restoration of the TET2 tumor suppressor function was proposed as a potential effective therapeutic strategy.

Long-lived pre-leukemic stem cells (pre-LSCs) were uncovered as an initiating event in various blood-born cancers (10). Pre-LSCs are phenotypically normal, but molecularly poised to transform. Their self-renewing capacity allows clonal expansion and subsequent acquisition of extra oncogenic driver mutations, eventually leading to the onset of a full-blown leukemia. Of interest, these pre-LSCs have a competitive advantage compared to untransformed progenitors and are more chemo/radio-resistant (11-12). As a result, they are considered to reflect the population that potentially can give rise to relapse (13-15). In T-ALL, the existence of a pre-LSC population has first been described and identified in a spontaneous T-ALL mouse model overexpressing LMO2, *CD2-Lmo2*$^{tg/+}$ mice (16-18). Unlike the thymus of normal mice, which is continuously replenished by progenitors from the bone marrow, the preleukemic thymus of *CD2-Lmo2*$^{tg/+}$ mice is self-sustaining from young age and many months before leukemia development. Similar thymocyte transplantation experiments, to demonstrate presence of a pre-LSC self-renewing thymocyte population, have not been reported for other spontaneous T-ALL mouse models. To investigate presence/absence of pre-LSCs in an unrelated second T-ALL model, thymocytes from *Lck-cre*$^{tg/+}$*Pten*$^{fl/fl}$ mice (19) or their Cre-negative wildtype (WT) littermate controls were transplanted into sublethal irradiated syngeneic Ly5.1 (CD45.1+) recipients. *CD2-Lmo2*$^{tg/+}$ thymocytes and WT bone marrow cells were used as a positive control. Via flow cytometric analysis, we analyzed how many of the donor versus recipient cells contributed to the repopulation of the thymus 6 weeks after transplantation. In contrast to CD45.2+ *CD2-Lmo2*$^{tg/+}$ thymocytes, none of the CD45.2+ *Lck-cre*$^{tg/+}$*Pten*$^{fl/fl}$ thymocytes could be detected in the recipient mice, demonstrating the absence of a preleukemic phase in the *Lck-cre*$^{tg/+}$*Pten*$^{fl/fl}$ model (Fig 1A).

Currently, detailed molecular insights are lacking in how thymocytes can gain this aberrant self-renewal and whether altered DNA methylation plays a pivotal role in this initiation event. Of interest, in our previous study (20) we already demonstrated that *CD2-Lmo2*$^{tg/+}$ and *Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$ murine T-ALLs have significantly different DNA methylation profiles. To investigate a potential role for *Tet2* in pre-LSCs, we started with an expression analysis for *Tet2* in thymocytes isolated from preleukemic *CD2-Lmo2*$^{tg/+}$ and WT littermate mice at the age of 8 and 24 weeks. *Tet2* mRNA levels

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were significantly downregulated after 8 weeks and almost completely absent after 24 weeks (Fig 1B). A similar *Tet2* mRNA expression analysis was performed in preleukemic *Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$ thymocytes. Since these mice develop T-ALL faster (Supplemental Fig 1), we could only collect thymocytes of non-leukemic *Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$ mice at the age of 8 weeks. In contrast to the *CD2-Lmo2*$^{tg/+}$ mice, no difference in *Tet2* levels could be observed between WT and *Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$ mice (Fig 1C). Based on these findings, we hypothesized that a gradual loss of *Tet2* during the preleukemic expansion of self-renewing *CD2-Lmo2*$^{tg/+}$ pre-LSCs may contribute to the progression of overt leukemia in this model.

To functionally investigate the *Tet2* tumor suppressor role in T-ALL initiation, we developed a conditional *Tet2* gain-of-function mouse model using our previously reported transgenic pipeline (Fig 2A) (21). We crossed this newly developed *R26-Tet2*$^{tg}$ with the *CD2-iCre* transgenic line (22), expressing the *Cre* recombinase from the common lymphoid progenitor stage onward. In the resulting *R26-Tet2*$^{tg/tg}$;*CD2-iCre* mice, Cre-mediated deletion of the floxed transcriptional stop cassette enabled expression of a bicistronic mRNA encoding for TET2 and an eGFP/luciferase fusion reporter in lymphoid progenitors and their progeny (Fig 2A). Next, we crossed the *R26-Tet2*$^{tg}$ with the *CD2-Lmo2*$^{tg/+}$ T-ALL model (18) and generated an aging cohort of *CD2-Lmo2*$^{tg/+}$ animals without (Cre$^{-}$,*CD2-Lmo2*$^{tg/+}$*R26-Tet2*$^{tg/tg}$*,* hereafter named *Lmo2)* and with *Tet2* overexpression (*CD2-iCre*$^{tg/+}$*CD2-Lmo2*$^{tg/+}$*R26-Tet2*$^{tg/tg}$*,* hereafter named *Lmo2-Tet2*) to evaluate the effects of TET2 gain on T-ALL initiation. Mice were sacrificed when signs of hematological malignancy were observed. *Lmo2-Tet2* mice showed a significant delay in T-ALL formation (median survival of 266 days) compared to *Lmo2* mice (median survival of 201 days), which confirmed the role of TET2 as tumor suppressor in T-ALL (Fig 2B). *Tet2* overexpression in *Lmo2-Tet2* leukemia samples was confirmed by qPCR (Fig 2C) and was in line with gain of *GFP* (Supplemental Fig 2A) and loss of the transcriptional stop cassette (Supplemental Fig 2B). Luciferase activity (Supplemental Fig 2C-D) confirmed the conditional expression of the transgene transcript in *Lmo2-Tet2* mice. Endogenous *Tet2* mRNA levels did not differ between both groups (Supplemental Fig 2E), indicating that the observed increase in T-ALL latency is exclusively caused by the *Rosa26*-driven *Tet2* overexpression.
Next, we wondered if TET2 could act as a tumor suppressor in a mouse model that lacks pre-LSCs. For this, we crossed the *R26-Tet2*$^{tg/tg}$ mice *with the Lck-cre*$^{tg/+}$*Pten*$^{fl/fl}$ T-ALL model and generated an aging cohort of mice with (*Lck-Cre*$^{tg/+}$*Pten*$^{fl/lf}$*R26-Tet2*$^{tg/tg}$, hereafter named *Pten-Tet2)* or without *Tet2* overexpression (*Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$*R26-Tet2*$^{wt/wt}$, hereafter named *Pten)*. No significant difference in overall survival was observed (Figure 2D)*,* although gain of *Tet2* mRNA expression was confirmed (Figure 2E) in obtained leukemia samples*.* Similar as for the *LMO2* aging cohort, the increased *Tet*2 mRNA levels corresponded with gain of the *GFP-Luciferase* reporter gene and loss of the STOP codon (Supplemental Fig 3A-D) in the *Pten-Tet2* mice. Endogenous *Tet2* levels did not differ between both groups (Supplemental Fig 3E).

Currently, the tumor suppressor role of TET2 in T-ALL has only been studied in knockout models and rescue experiments using vitamin C or 5-azacitidine (9). Here, for the first time, we investigated the effects of gain of TET2 in T-ALL making use of genetically overexpressing mouse models. We demonstrated a tumor suppressor role of TET2 in the preleukemic phase of T-ALL, while no effects were seen on T-ALL

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maintenance. The difference in the effect of *Tet2* overexpression between the *Pten* and *Lmo2* T-ALL mouse models can be correlated with our previous observations and reported differences in the DNA methylation profile of these two models (20). The observed decrease in *Tet2* mRNA levels in preleukemic *Lmo2* mice, is associated with an increase in methylation at CpG islands, while this is not seen in the *Pten* model (20). Based on these findings, we hypothesize that a critical TET2 threshold is necessary to protect preleukemic cells from obtaining secondary mutations that in the end will lead to the formation of T-ALL.

All in vivo experiments were approved by the ethical committee for animal experimentation of the Faculty of Medicine and Health Sciences of Ghent University.

## Authorship

Conceptualization: SDC, JR, DC, SG, PVV; Methodology: SDC, BL, STS; Formal analysis: SDC, JR; Investigation: SDC, BL, STS; Resources: TT, DC, TP, SG, PVV; Data curation: SDC; Writing original draft: SDC, JR, TP, SG, PVV; Writing - review and editing: SDC, JR, BL, STS, TT, DC TP, SGS; Visualization: SDC; Supervision: SG, PVV; Project administration: SDC, SG, PVV; Funding acquisition: SG, PVV.

**The authors declare no conflict of interest.**

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## Figure legends

### Figure 1

(A) %CD45.2 in thymi of sublethal irradiated CD45.1 mice injected with bone marrow of wild type mice (n=4), thymus of wild type mice (n=4), thymus of *CD2-Lmo2*$^{tg/+}$ mice (n=4) and thymus of *Lck-Cre*$^{tg/+}$ *Pten*$^{fl/lf}$ mice (n=4)

(B) Normalized *Tet2* mRNA expression in thymi of WT (n=4) and *CD2-Lmo2*$^{tg/+}$ (n=4) mice at 8 (p=0.0286) and 24 weeks (p=0.0286) of age

(C) Normalized *Tet2* mRNA expression in thymi of WT (n=4) and *Lck-Cre*$^{tg/+}$*Pten*$^{fl/lf}$ (n=4) mice at 8 weeks of age (p=0.2900)

### Figure 2

(A) Schematic overview of breeding scheme resulting in *CD2-iCre*$^{tg/+}$*;R26-Tet2*$^{tg/tg}$ mice

(B) Aging cohort of *CD2-Lmo2*$^{tg/+}$ (=*Lmo2)* (n=13, median survival 201 days) and *CD2-iCre*$^{tg/+}$*CD2-Lmo2*$^{tg/+}$*R26-Tet2*$^{tg/tg}$ *(=Lmo2-Tet2)* (n=13, median survival 266 days) mice (p=0.0226)

(C) *Tet2* mRNA expression in thymi of *Lmo2* (n=12) and *Lmo2-Tet2* mice (n=11)(p=0.0045)
(D) Aging cohort of *Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$*R26-Tet2*$^{wt/wt}$ (=*Pten)* (n=11, median survival 75 days) and *Lck-Cre*$^{tg/+}$*Pten*$^{fl/fl}$*R26-Tet2*$^{tg/tg}$ (=*Pten-Tet2)* (n=11, median survial 87 days) mice (p=0.4508)
(E) *Tet2* mRNA expression in thymi of *Pten* (n=10) and *Pten-Tet2* (n=10) mice (p=0.0138)

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Figure 1

Figure 1

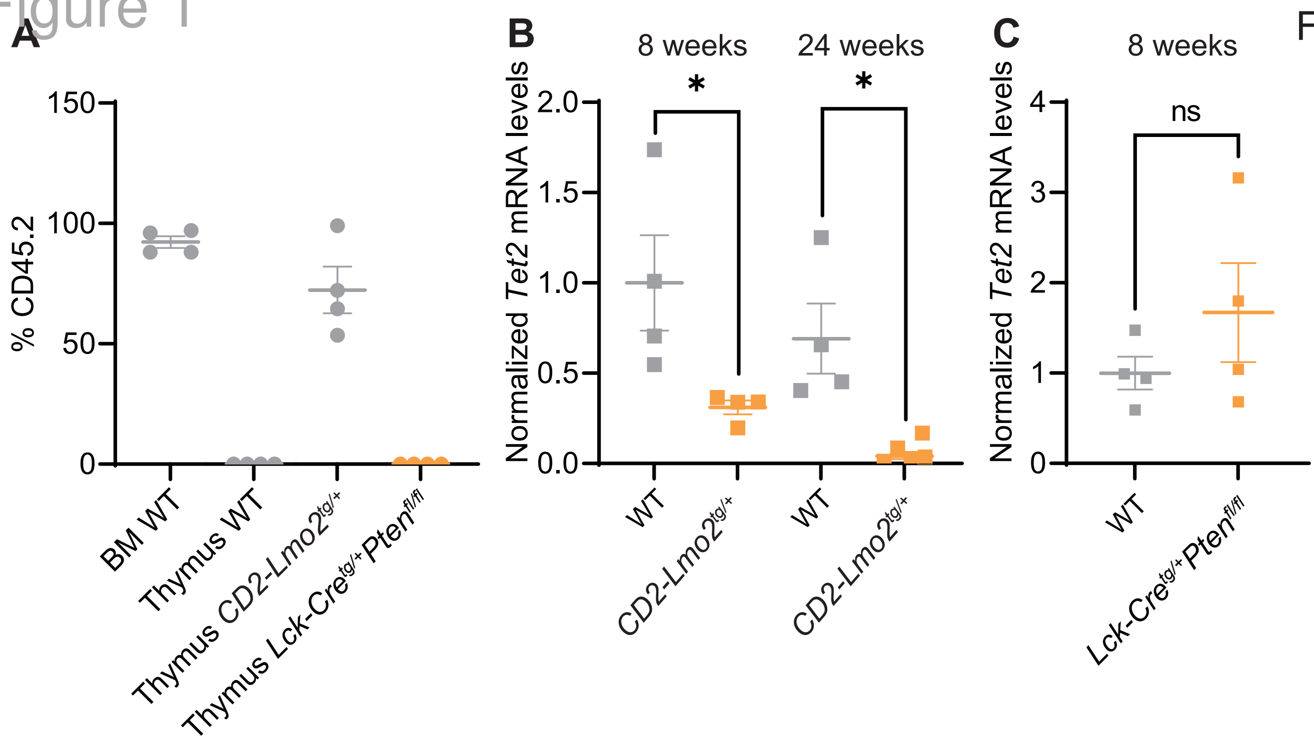


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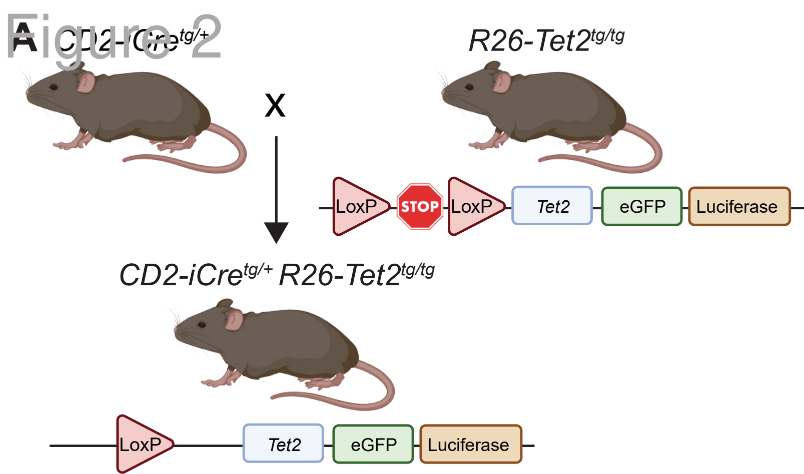
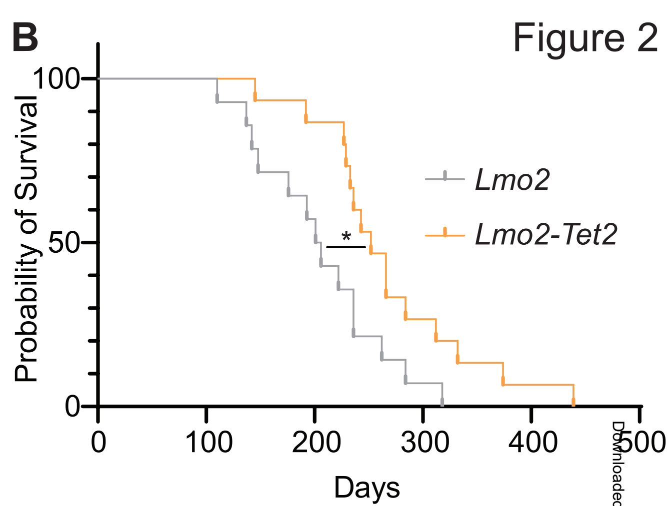
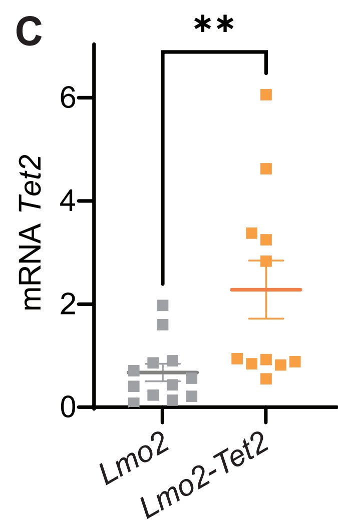
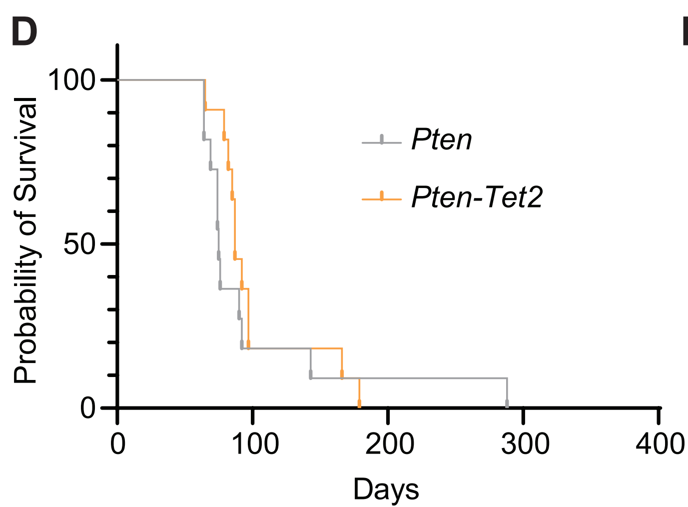
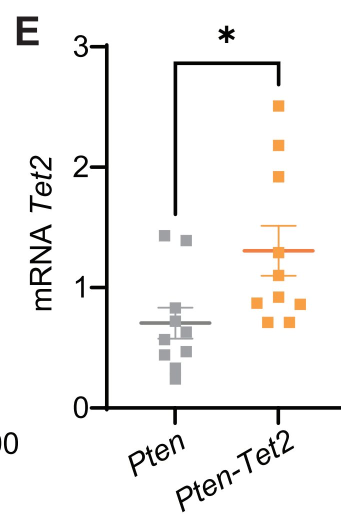
Figure 2

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