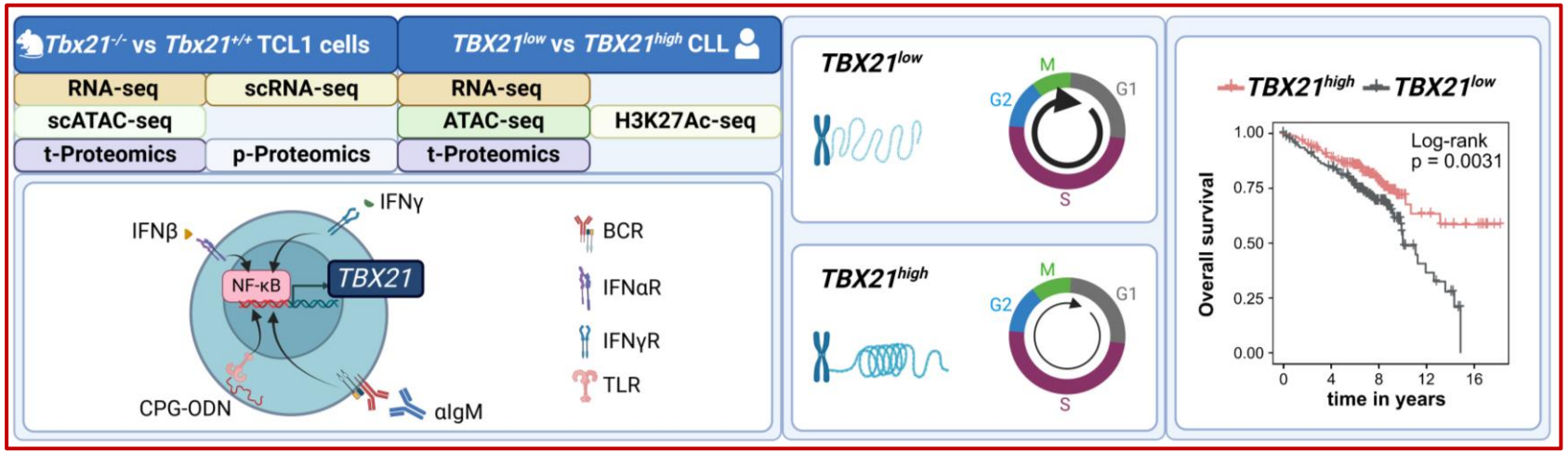
The Role of T-bet in Chronic Lymphocytic Leukemia (CLL)



Conclusions: **T-bet acts as tumor suppressor by enhancing interferon signaling and suppressing proliferation of malignant B cells, and its expression positively correlates with longer overall survival of patients with CLL.**

Roessner et al.

T-bet suppresses proliferation of malignant B cells in chronic lymphocytic leukemia

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Key points

- T-bet acts as tumor suppressor by enhancing interferon signaling and suppressing proliferation in malignant B cells
- T-bet expression in CLL cells is positively correlated with longer overall survival of patients with CLL

Abstract

The T-box transcription factor T-bet is known as a master regulator of T-cell response but its role in malignant B cells is not sufficiently explored. Here, we conducted single-cell resolved multi-omics analyses of malignant B cells from patients with chronic lymphocytic leukemia (CLL) and studied a CLL mouse model with genetic knockout of *TBX21*. We found that T-bet acts as a tumor suppressor in malignant B cells by decreasing their proliferation rate. NF-κB activity induced by inflammatory signals provided by the microenvironment, triggered T-bet expression which impacted on promoter proximal and distal chromatin co-accessibility and controlled a specific gene signature by mainly suppressing transcription. Gene set enrichment analysis identified a positive regulation of interferon signaling, and a negative control of proliferation by T-bet. In line, we showed that T-bet represses cell cycling and is associated with longer overall survival of CLL patients. Our study uncovers a novel tumor suppressive role of T-bet in malignant B cells via its regulation of inflammatory processes and cell cycling which has implications for stratification and therapy of CLL patients. Linking T-bet activity to inflammation explains the good prognostic role of genetic alterations in inflammatory signaling pathways in CLL.

Introduction

The T-box transcription factor T-bet encoded by *TBX21* is well known for its role in lineage commitment of $CD4^+$ T helper1 cells, effector functions of $CD8^+$ T cells, and differentiation of natural killer cells.[1-3] But extensive literature attributes an important role of T-bet also in B cells, mainly in the context of "age-associated" B cells (ABCs).[4-6] First described in aging mice, ABCs exist also in healthy humans, where they increase up to the age of 30, followed by a stabilization of their frequency.[6-8] An accumulation of ABCs was observed during infections, where T-bet^+ B cells contribute to protective immunity.[6,9-13] In addition, a rise in ABC numbers is observed in patients with humoral autoimmune diseases, such as systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis, Crohn disease, and Sjörgen syndrome, and also in adipose tissue during exacerbated metabolic disorders. Here, ABCs are linked to the production of auto-reactive antibodies and associated with worse clinical outcome.[5,6,8,14-22]

The expression of T-bet in B cells is induced via the activation of several signaling pathways, including toll-like receptor (TLR), B-cell receptor (BCR), CD40, and cytokine receptor signaling. These signals are provided by the microenvironment in lymphoid tissues, e.g. by bystander T cells.[6,10,23-26] Utilizing *Tbx21*-deficient B cells, an involvement of T-bet in immunoglobulin class switching and the generation of long-lived antibody-secreting B cells and their function in anti-viral control was observed in several mouse models.[9,10,26,27] Yang et al investigated a patient harboring a complete deficiency in T-bet and observed that T-bet is required for the generation of a $CD11c^{high}$ subset of ABC-like B cells, and is dispensable for memory and plasma cell generation and antiviral control.[26] T-bet expression was also detected in B-cell malignancies, including chronic lymphocytic leukemia (CLL), a disease of mature B cells with a highly heterogeneous course.[28,29] However, its role and potential pathological function remains largely unexplored.

Here, we explored the role of T-bet in CLL using single-cell resolved multi-omics analyses of patient samples and a CLL mouse model with *TBX21* knockout. We show that T-bet acts as a tumor suppressor in CLL by reducing proliferation and is associated with longer survival of patients.

Methods

Patient samples, mouse models, cell lines and published data sets

Patient and healthy, age-matched control samples were obtained after approval of study protocols by the local ethics committees according to the declaration of Helsinki, and after obtaining informed consent of patients. Patients met standard diagnosis criteria for CLL. Details are provided in Suppl. Table

1. The Eμ-TCL1 mouse model was the basis to generate *Tbx21*$^{-/-}$ TCL1 cells as previously described.[30] A list of all cell lines is provided in Suppl. Table 2, and an overview of published data sets used in this study in Suppl. Table 3.

A detailed description of all methods is provided in the Supplement.

Results

CLL cells have enhanced *TBX21* expression

We first compared *TBX21* expression levels in CLL cells and untransformed B cells of age-matched healthy controls which revealed higher T-bet transcript and protein levels in CLL cells (Figure 1A-B). This was also the case when comparing CLL cells with several developmental states of untransformed B cells, including mature CD5^{+} and memory B cells which have been suggested as the cellular origin of CLL (Figure 1C, Suppl. Figure 1A),[31,32] suggesting that high *TBX21* expression in CLL cells is disease-specific.

We next explored potential similarities between CLL cells and ABCs by analyzing typical ABC marker genes,[13,26,33] revealing a particularly high expression of *TBX21*, *ITGAX* (CD11c), and *FCRL5* accompanied by low expression levels of MS4A1 (CD20) (Figure 1D) in CLL cells, suggesting a phenotypical overlap with ABCs.

We further compared *TBX21* expression in genetically-defined, prognostic subgroups of CLL patients and did not observe major differences in cases with or without deletion of chromosome 13q (del13q) or somatic hypermutations of the *IGHV* gene locus, two commonly assessed prognostic features in CLL (Figure 1E). In contrast, patients with trisomy 12, associated with an intermediate prognosis,[34] showed a significantly higher expression of *TBX21*, whereas in patients with *ATM* mutation, a driver of more aggressive disease,[35] *TBX21* expression was significantly lower compared to cases without these aberrations.

To infer whether expression of *TBX21* is epigenetically imprinted in CLL cells, we compared chromatin accessibility by ATAC-seq, as well as H3K27-acetylation (H3K27ac) of the *TBX21* gene locus in CLL versus healthy control B cells.[36] This demonstrated that CLL cells not only show a higher transcriptional activity in *TBX21* but also higher signs of epigenetic activation and chromatin accessibility in comparison to B cells or B-cell subsets from healthy controls (Figure 1F, Suppl. Figure 1B-C).

Inflammatory signals drive *TBX21* expression in CLL cells via NF-κB activity

To explore signals that induce *TBX21* expression in CLL cells, we used data of CLL long-term cultures including so-called nurse-like cells and T cells,[37] as well as co-cultures of CLL cells with *in vitro* activated T cells.[38,39] In both these cultures, *TBX21* expression was induced in CLL cells (Figure 2A, Suppl. Figure 2A). By *in vitro* stimulation of CLL cells, we identified IFNγ, IFNβ, and CpG oligos as TLR9 ligand as *TBX21*-inducing signals, as well as combinations of these stimuli with BCR activation by αIgM (Figure 2B-C, Suppl. Figure 2B-C). These data suggest that the inflammatory milieu in CLL is responsible for the enhanced *TBX21* expression in the malignant B cells.[40,41]

A common feature of IFN-, TLR- and BCR-signaling is the subsequent activation of NF-κB. Therefore, we hypothesized that *TBX21* expression in CLL is induced via NF-κB.[42-44] In line, gene expression of *TBX21* was reduced in CLL patients during treatment with the BTK inhibitors ibrutinib[45,46] and acalabrutinib,[47] which among other signaling pathways are known to disrupt NF-κB activity[45,48] (Figure 2D-E, Suppl. Figure 2D). Further, ibrutinib-mediated reduction of NF-κB signaling prevented an inflammation-associated induction of T-bet *in vitro* (Figure 2F, Suppl. Figure 2E). In contrast, neither a 7-day treatment of CLL patients with the SYK inhibitor entospletinib (Suppl. Figure 2F) affecting downstream AKT signaling,[49-51] nor a 48-hour treatment of CLL cells *in vitro* with the Src inhibitor dasatinib altered the expression of *TBX21* (Suppl. Figure 2G).[52]

Next, we measured NF-κB p65 phosphorylation after stimulation with microenvironmental factors to experimentally validate the dependency of *TBX21* expression on NF-κB activity in CLL. As expected, CpG stimulation and the combination of αIgM and IFNγ showed increased total amounts of p65, but also higher levels of phosphorylation (Suppl. Figure 2H-I). Inhibition of NF-κB signaling by IKK-16 reduced basal T-bet expression and prevented the induction of T-bet in response to NF-κB-dependent microenvironmental signals in CLL cells (Figure 2G). In line, *Nfkbie*$^{-/-}$ TCL1 leukemic cells, which are derived from the Eμ-TCL1 mouse model of CLL and harbor hyperactive NF-κB signaling,[53] showed higher expression of T-bet than WT control cells (Figure 2H), confirming the importance of NF-κB in the induction of *TBX21* expression.

T-bet activity can be assessed by its target gene expression signature

The regulatory activity of T-bet on transcriptional programs in T cells is well described,[54] however transcriptional targets of T-bet in B cells are vastly unknown. Using a CRISPR/Cas9 approach, we generated *Tbx21*$^{-/-}$ TCL1 cells (Suppl. Figure 3A),[30] analyzed them by RNA-seq and mass spectrometry (MS) in comparison to WT control cells, and identified differentially expressed genes (DEGs) and proteins (Suppl. Table 4-5), respectively. We further stratified RNA-seq data of sorted CLL cells from patients according to their highest and lowest quartile of *TBX21* expression and analyzed DEGs in *TBX21*^{low} vs

TBX21^{high} cells (Suppl. Table 6).[55] We then identified the overlap of all DEGs and proteins in these data sets with proteins obtained from MS data of CLL patients that correlated with T-bet levels (Suppl. Table 7).[56] This integrated multi-omics analysis generated a list of 104 genes that displayed a positive (55 genes) or negative correlation (49 genes) with *TBX21* expression (Suppl. Figure 3B-C, Suppl. Table 8). Using this gene list, we explored whether T-bet activity was epigenetically imprinted in CLL by stratifying CLL patients according to their H3K27ac levels at the *TBX21* promoter, which correlated well with *TBX21* gene expression (Suppl. Figure 3D). We observed a clear correlation of differential gene expression and H3K27ac of T-bet-dependent genes in *TBX21*^{low} vs *TBX21*^{high} CLL cases, suggesting an epigenetic imprinting of T-bet activity in CLL (Suppl. Figure 3E). In addition, we independently validated our signature of T-bet-dependent genes in a published CLL dataset which confirmed that both the inducing and the repressing T-bet activity was higher in *TBX21*^{high} CLL (Suppl. Figure 3F).[57]

We next aimed to infer whether T-bet activity is specific to CLL cells or mirrored in B-cell subsets of healthy donors by comparing their expression of T-bet dependent genes using published transcriptome data.[32] Intriguingly, T-bet-dependent genes separated CLL samples from all other B-cell subsets, mature CD5$^+$ B cells being most similar to CLL cells (Figure 3A). We further calculated T-bet activity scores for induced and repressed genes, separately. Whereas no major differences in the inducing activity of T-bet were observed (Figure 3B), repressive activity of T-bet was highest in CLL, followed by mature CD5$^+$ B cells (Figure 3C). Correlation of T-bet activity scores and *TBX21* gene expression revealed a strong correlation of the repressive activity (Figure 3D), suggesting that T-bet acts as a silencing rather than activating transcription factor (TF) in CLL. A single-cell omics study recently defined an atlas of B cells in tonsils,[58] including a cell subset annotated as FCRL4$^+$/FCRL5$^+$ memory B cells that showed similarities to ABCs.[13,33,59,60] We hypothesized that this B-cell subset is controlled by *TBX21* and investigated T-bet activity scores across all B-cell subsets of the tonsil atlas. Of note, *TBX21* expression was highest in FCRL4$^+$/FCRL5$^+$ memory B cells (Figure 3E, Suppl. Figure 4A) which was accompanied by highest T-bet activity according to the CLL-defined target gene signature (Figure 3F). To confirm the robustness of this gene signature, we independently calculated T-bet activity in this data using the recently published pySCENIC tool.[61] This confirmed highest T-bet activity in FCRL4$^+$/FCRL5$^+$ memory B cells (Suppl. Figure 4B) and showed that the correlation of activity scores and *TBX21* gene expression was similar in both approaches (Suppl. Figure 4C-D). Of note, the CLL-defined T-bet activity was mainly driven by a gene module containing *NOTCH1, IRF9*, and *RUNX3* in FCRL4$^+$/FCRL5$^+$ memory B cells (Suppl. Figure 4E). Even though most T-bet-repressed genes in CLL showed also little expression in FCRL4$^+$/FCRL5$^+$ memory B cells, an exceptionally high expression of *BHLHE41* was observed in this subset, highlighting distinct regulatory activities of T-bet in malignant vs untransformed B cells.

In summary, we defined a T-bet-dependent gene expression signature in CLL cells, which allowed us to robustly assess T-bet activity in CLL cells.

T-bet regulates transcription via suppression of long-range chromatin interactions

To dissect the mechanisms of transcription regulation by T-bet, we generated scATAC-seq data of *Tbx21*$^{+/+}$ vs *Tbx21*$^{-/-}$ TCL1 cells with our TurboATAC protocol.[62] This protocol provides a very high Tn5 integration efficacy and yielded a mean of approximately 58,000 fragments per cell for a high coverage of regulatory elements. We analyzed the genomic location of accessible T-bet binding motifs associated with the T-bet-dependent gene set (Suppl. Figure 3C, and 5A-D). We identified T-bet binding motifs in 23 % of all peaks from the pseudo-bulk scATAC-seq data, subsequently referred to as T-bet peaks. Using the RWire software, we detected simultaneously accessible peaks in single cells between T-bet-dependent genes and T-bet peaks.[63,64] About 26 % of T-bet-dependent genes contained an ATAC peak with the T-bet binding motif at their promoter (Figure 4A). These T-bet promoter peaks showed a higher number of co-accessible links to distal peaks in *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells (Figure 4B). In contrast, there was no difference in the number of co-accessible links from T-bet-dependent gene promoters without T-bet peak between *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells. We conclude that promoter-bound T-bet represses regulatory interactions with distal genomic loci in *Tbx21*$^{+/+}$ TCL1 cells. In addition, we were able to link approximately 60 % of T-bet-dependent genes without a T-bet promoter peak to distal T-bet peaks by co-accessibility analysis of *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells (Figure 4A). The number of co-accessible links between T-bet-dependent genes and distal T-bet peaks increased in *Tbx21*$^{-/-}$ (n = 170) vs *Tbx21*$^{+/+}$ TCL1 cells (n = 151; Figure 4C). Roughly 15 % of co-accessible links were detected in both Tbx21$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells, however, most were unique to either *Tbx21*$^{-/-}$ or *Tbx21*$^{+/+}$ TCL1 cells. These findings point to a rewiring of distal gene regulation at T-bet-dependent genes in *Tbx21*$^{-/-}$ TCL1 cells. For 15 % of T-bet-dependent genes, no regulatory link to accessible T-bet binding motifs was detected (Figure 4A), which could reflect genes regulated indirectly by T-bet. An example of transcription regulation by T-bet that involves long-range interactions is given for *Nos1*, which is negatively regulated by T-bet (Figure 4D-E, bulk RNA-seq). Different T-bet-dependent regulatory mechanisms appear to be active when comparing 1 kb regions around the three transcriptional start sites (TSSs) of *Nos1* indicated as TSS1, TSS2 and TSS3 in Figure 4E: TSS1 is inaccessible in *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells, and does not contain a T-bet binding motif. TSS2 is moderately accessible in *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells, but does not contain a T-bet binding motif. It shows increased accessibility in *Tbx21*$^{+/+}$ TCL1 cells with additional and enhanced co-accessible links from the promoter to surrounding T-bet peaks. Thus, the lack of a T-bet binding motif at TSS2 is compensated by putative repressive interactions to distal regulatory T-bet peaks in *Tbx21*$^{+/+}$ TCL1

cells. TSS3 is highly accessible in both *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells and contains a T-bet motif. A co-accessible link to a downstream peak was found only in *Tbx21*$^{-/-}$ TCL1 cells. We conclude that the loss of repressive T-bet binding at TSS3 in *Tbx21*$^{-/-}$ TCL1 cells facilitates the formation of a regulatory link to a downstream peak.

In summary, T-bet controls the expression of most T-bet dependent genes via binding to cis-regulatory elements and modulating long-range interactions that enhance transcription of target genes as inferred from the co-accessibility analysis.

T-bet enhances interferon and represses cell cycle signatures in CLL

Next, we elucidated the function of *TBX21* in CLL cells with a gene set enrichment analysis (GSEA) of published transcriptome data of human CLL cells and our RNA-seq data of *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells. Differentially expressed gene sets (FDR < 0.05) were computed to identify *TBX21*-regulated pathways that are conserved across species. In total, 327 gene sets were differentially enriched in human and mouse, with 298 gene sets downregulated and 11 upregulated in *TBX21*$^{low/KO}$ cells (Figure 5A, Suppl. Figure 6A). Of interest, *TBX21*$^{low/KO}$ cells showed a lower abundance of interferon-associated pathways and an enrichment of a cell cycle signature (Figure 5A, Suppl. Figure 6B). KEGG pathway analysis using T-bet correlated genes and proteins derived from published data in CLL,[56] revealed that T-bet^{hi} CLL samples were enriched in B-cell, NF-κB, TLR and T_H17 pathways. In line with the GSEA results, *TBX21*$^{low/KO}$ samples were enriched in cell cycle pathways (Figure 5B). To confirm the positive regulation of interferon pathway activity by T-bet, we compared the expression of interferon-stimulated genes (ISGs) of *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells. At steady-state, we noted a higher expression of many ISGs in *Tbx21*$^{+/+}$ compared to *Tbx21*$^{-/-}$ TCL1 cells in our RNA-seq data (Figure 5C). Stimulation of these cells with IFNβ *in vitro* resulted in a stronger induction of ISGs in *Tbx21*$^{+/+}$ compared to *Tbx21*$^{-/-}$ cells (Figure 5D), confirming a positive regulation of interferon signaling in CLL by T-bet.

We further characterized effects of T-bet on chromatin accessibility and T-bet controlled TFs using our scATAC-seq of *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells and ATAC-seq data of *TBX21*^{low} vs *TBX21*^{high} CLL patients. This showed that *TBX21*$^{low/KO}$ cells harbor a higher accessibility of multiple chromatin sites (+500 % in TCL1 CLL and +200 % in human CLL cells; Figure 6A-B). Motif enrichment analyses inferred differential TF activities in *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells (Suppl. Figure 7A) and *TBX21*^{low} vs *TBX21*^{high} CLL cells from *IGHV*-unmutated (U-CLL) and mutated (M-CLL) patients (Suppl. Figure 7B). This analysis confirmed the mostly repressive transcriptional activity of T-bet in CLL. Overlap of differential binding motif enrichment of TCL1, U-CLL and M-CLL cells (Suppl. Figure 7C-D) identified an enrichment of 3 and a depletion of 9 binding motifs in *TBX21*^{high} compared to *TBX21*^{low} cells (Figure 6C, Suppl. Figure 7D). IRF, IRF and BATF co-

regulation, and type-1 interferon-sensitive response element (T1ISRE) motifs were enriched in *TBX21*^{high} cells (Figure 6C), which is in line with our results above showing that T-bet induces interferon signaling. In contrast, motifs of NF-κB p50, BORIS, CTCF, the B-cell TF E2A, and members of the POU TF family were enriched in *TBX21*^{low} cells. These TFs were shown to prevent spontaneous apoptosis in CLL,[65] and enhance proliferation of B cells,[66] which is in line with the negative correlation of T-bet with cell cycle observed by GSEA and KEGG analysis (Figure 5A and B).

In summary, our data show that T-bet maintains an inflammatory program, particularly type 1 interferon signaling, and represses cell cycle signatures in CLL cells.

T-bet suppresses cell proliferation

To validate the observed association of T-bet with cell cycle activity, we performed scRNA-seq of *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells. First, we detected a higher fraction of *Tbx21*$^{-/-}$ cells in G2/M or S phase in comparison to *Tbx21*$^{+/+}$ cells (Figure 7A, Suppl. Figure 8). Second, by analyzing KI-67 expression of TCL1 cells isolated from peritoneal cavity (PC), bone marrow (BM) and spleen (SPL) of leukemic mice, we observed a higher frequency of KI-67^{+} cells in *Tbx21*$^{-/-}$ compared to control mice (Suppl. Figure 9A). Third, phospho-proteomic analysis of *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells revealed an overrepresentation of AKT and PKC networks, as well as an enrichment of the CMGC kinase group in *Tbx21*$^{-/-}$ cells (Figure 7B, Suppl. Figure 9B-C). The latter included important mediators of cell cycle progression, such as CDK1, CDK2, and CDK5, which showed higher activity in the absence of T-bet. In support of that, 13 of in total 104 T-bet-dependent genes, all of which are repressed by T-bet, were associated with cell cycling (Suppl. Figure 3C), including *Bub1* which is involved in G1/S phase entry and cell-cycle progression.[67]

Next, we investigated published scRNA-seq data of CLL lymph nodes, in which CLL cells have been annotated to be either quiescent, activated, or proliferating.[68] Using our list of T-bet-dependent genes, we observed the lowest repressive T-bet activity in proliferating CLL cells (Figure 7C, Suppl. Figure 9D) which is in line with the proposed repressive activity of T-bet for cell cycle activity. Analysis of published data from cells of patients with monoclonal B lymphocytosis (MBL), a precursor state of CLL with a yearly progression rate of 1-2 % of cases,[69] revealed no differences in T-bet activity before and after progression to CLL (Suppl. Figure 10A), suggesting no major differences in cell cycle activity at the stage of disease progression.[70]

Finally, to experimentally validate the regulation of cell cycling by T-bet, we generated a CLL-like MEC-1 cell line and two B-cell lymphoma cell lines, OCI-Ly7 and U-2940, with inducible T-bet expression. Overexpression of T-bet upon doxycycline treatment (Suppl. Figure 10B-C) resulted in lower expansion

and proliferation rates in comparison to either GFP-transduced or non-treated control cells (Figure 7D, Suppl. Figure 10 D-E). Thus, T-bet plays a central role in suppressing proliferation of malignant B cells.
In summary, the omics data analyses of TCL1 and human CLL cells, followed by functional assays shows that T-bet activity inhibits malignant B-cell proliferation.

T-bet expression is a marker of good prognosis for CLL

A subset of patients with CLL suffers from disease transformation into the more aggressive Richter's syndrome (RS), which often resembles diffuse large B-cell lymphoma (DLBCL) and is associated with a poor outcome with short overall survival of less than a year. Recently, a novel mouse model mimicking the transformation of CLL cells into RS cells was published.[71] Using this model, we observed a higher expression of *Tbx21* in CLL *vs* RS cells (Suppl. Figure 11A). In addition, analysis of scRNA-seq data of paired CLL cells and transformed RS cells of 4 patients revealed the lowest repressive T-bet activity in a distinct cluster of proliferating RS cells (Suppl. Figure 11B).[72] Accordingly, comparing patient samples of CLL and other B-cell non-Hodgkin lymphoma (B-NHL) entities revealed the highest repressive T-bet activity in CLL and the lowest in DLBCL (Suppl. Figure 11C), which is in line with our observation that T-bet activity limits proliferation of malignant B cells.
We then assessed the prognostic impact of T-bet in CLL and stratified patients according to their T-bet expression levels acquired by RNA-seq or mass spectrometry in high and low expressing groups. CLL patients with high gene expression and protein levels of T-bet showed better outcome, specifically longer time to treatment and overall survival compared to cases with low expression levels (Figure 7E, Suppl. Figure 12A-D).[55-57] The longer treatment-free and overall survival of CLL patients with high T-bet expression was independent of their *IGHV* mutational status or *ZAP70* gene expression, which was confirmed by multivariate analysis (Figure 7F-G, Suppl. Figure 13A-D).
Altogether, our data identify T-bet as a novel prognostic marker in CLL. Mechanistically, this can be explained by T-bet driving inflammatory processes via IFN signaling and limiting proliferation of malignant B cells. These novel findings have implications for stratification and therapy of patients with CLL and likely other B-NHL.

Discussion

Our study provides evidence for a so far unexplored role of T-bet in CLL and provides insights into its transcriptional regulation and activity, as well as its prognostic role. We show higher T-bet expression in

CLL cells compared to B cells from healthy donors, and its induction by multiple factors within the tumor microenvironment via NF-κB and likely other pathways. We observed that T-bet acts mostly as a transcription repressor, and that its activity maintains IFN signaling and represses cycling of CLL cells. Therefore, targeting T-bet or its associated pathways might serve as a novel treatment strategy in CLL.

As T-bet expression is a feature of most ABCs,[73] we compared phenotypic and functional properties of CLL cells and ABCs to assess whether CLL cells might originate from this B-cell subset. Similarly as in ABCs, T-bet expression is induced in CLL cells by inflammatory signals, present in the micromilieu of CLL through BCR, TLR and IFN stimulation.[6,24,74] In addition, we detected the expression of several ABC marker genes in CLL but not non-malignant B cells. CLL cells are suggested to derive from self-reactive B-cell precursors, however do not secrete auto-antibodies, a typical feature of ABC.[6,75,76] Furthermore, whereas T-bet expression in ABCs is regulated by STAT signaling, we identified NF-κB as a key mediator of T-bet expression in CLL cells.[24] Thus, the regulatory properties of T-bet induction in malignant vs non-malignant B cells are likely distinct. To ultimately assess whether CLL cells originate from ABCs, further cell-of-origin modeling comparing different subsets of $CD11c^+$ untransformed B cells and CLL cells will be necessary.

Our *in vitro* and *in vivo* data show that BTK inhibitors reduce T-bet expression in CLL cells which is in line with these drugs' negative impact on NF-κB activity.[45,48] However, the overall clinical efficacy of BTK inhibition is clearly independent of the altered expression or activity of T-bet.

Type I interferons in the tumor microenvironment are known to suppress tumor growth, but in CLL, response to IFN differs in good *vs* bad prognostic subgroups. Whereas in low-risk patients, IFN signaling is associated with growth arrest, in aggressive CLL, IFN promotes tumor growth.[77] This might be explained by recent findings that suggest that aggressive forms of CLL are hypersensitive to autocrine IFN signaling.[78] In the light of our findings that T-bet induces IFN signaling and limits proliferation, this implies that the chronic inflammatory micromilieu that is mediated by T-bet activity in CLL, is a characteristic of indolent disease with a low proliferative rate, in contrast to a low T-bet activity in aggressive lymphoma such as Richter's syndrome. Of note, clinical IFNα treatment showed overall limited efficacy, but best responses were noted in a subset of previously untreated, early stage CLL,[79] which possibly could resemble CLL patients with high T-bet activity.

Our bulk ATAC-seq analysis of a large cohort of CLL patients combined with the scATAC-seq analysis of the CLL mouse model revealed a remarkable reduction in chromatin accessibility and subsequent gene expression by T-bet. This repressive activity of T-bet is in line with published data showing that T-bet represses specific gene programs in B cells.[27] Our findings show that T-bet has multiple modes of action to regulate target gene expression including direct promoter binding, but also control of regulatory long-

range interactions of enhancers. Our finding that T-bet deficiency is associated with an enrichment of binding sites for the POU TF family is in contrast with observations in human CD21^{low} ABC-like B cells that express T-bet and are enriched for POU binding sites in open chromatin regions,[8] highlighting again a difference between ABCs and CLL cells. Members of the POU TF family are required for progression of the cell cycle.[66,80] In accordance, the enhanced activity of POU TFs in *TBX21*$^{low/KO}$ CLL cells is associated with higher expression of cell cycle-associated gene signatures and higher cell proliferation rates. This is in line with and might explain the reduced overall survival of CLL patients with lower T-bet expression. Moreover, Penter *et al.* noted a reduced activity of POU TF family members in patients with CLL in comparison to the more aggressive Richter's syndrome,[81] which is consistent with our finding of a reduced T-bet activity in RS and in more aggressive types of lymphoma like DLBCL.

In summary, we show that T-bet is induced in CLL cells by microenvironmental signals present in lymphoid tissues via NF-κB signaling. It acts as tumor suppressor by maintaining IFN signaling and repressing cell cycling. As a consequence, T-bet expression levels are positively correlated with longer survival of CLL patients which has implications for clinical applications.

Towards this goal, specific immune stimulatory agents that lead to induction of T-bet in CLL cells need to be evaluated in preclinical models. Of note, such compounds likely also have a positive impact on other immune cells and thereby improve cancer-directed immune responses, which could result in an additive multi-target therapeutic efficacy.

Conflict of interests

CS received research funding from Genmab. AVD received consulting fees from Abbvie, AstraZeneca, BeiGene, Bristol Meyers Squibb, Genentech, GenMab, Incyte, Janssen, Lilly Oncology, MEI Pharma, Nurix, Oncovalent, Pharmacyclics and TG Therapeutics and has ongoing research funding from Abbvie, AstraZeneca, Bayer Oncology, Bristol Meyers Squibb, Cyclacel, Lilly Oncology, MEI Pharma, Nurix and Takeda Oncology. AW received research support from Pharmacyclics LLC, an AbbVie Company, Acerta Pharma, a member of the Astra-Zeneca group, Merck, Nurix, Verastem and Genmab. JAB received research funding from Pharmacyclics LLC and BeiGene; served on the advisory board for Janssen, Gilead, TG Therapeutics, Pharmacyclics LLC, BeiGene, and Novartis.

Author Contributions

PMR conceptualized the study, designed, and performed experiments, analyzed, and interpreted data, generated figures and wrote the manuscript. IS analyzed and interpreted data, generated figures and wrote parts of the manuscript. VC analyzed and interpreted data, generated figures. RJ and HB performed experiments, analyzed data, and generated figures. RMB, PB, TR performed bioinformatics analyses and generated figures. JB and HB performed experiments and analyzed data. LA, MC, SC, AMV, MS, MM and SRR performed experiments. AB provided experimental materials. SAH, MZ, CS, HK, TN, PMB, FC, ETH, MS, DH performed bioinformatics analyses or performed LC-MS based analyses. DY, JK, AVD, MB, KH, CS, SS, AW provided patient samples, clinical information, or datasets. JPM provided expertise as well as materials for scATAC-seq. JAB, DGE, PL provided oversight and logistic and budget support for the generation of experimental materials. SD provided patient samples and clinical information, access to patient data and intellectual feedback to the manuscript. IMS, KR provided oversight, budget support, intellectual feedback and wrote parts of the manuscript. MS supervised the study, provided logistic and budget support, and wrote the manuscript. All the authors read, reviewed, and revised the manuscript.

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Graphical abstract was created with BioRender.com.

Data availability

Bulk gene expression data were deposited at the ArrayExpress repository of the European Bioinformatics Institute (https://www.ebi.ac.uk/biostudies/arrayexpress/studies) with the accession number E-MTAB-13030. The scRNA-seq and scATAC-seq data are available from the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) under accession code GSE234226. Previously published sequencing data that were used in the analysis are listed in Suppl. Table 3. GEO data from GSE22529,[82] GSE36907,[32] GSE50572[38] were analyzed derived from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). Gene expression of *Tbx21* in a mouse model of matched CLL and Richter's syndrome (GSE186137) was analyzed after transfer of cells in WT animals.[71]

Code availability

Custom code used is available from GitHub at https://github.com/RippeLab/RWire-IFN and, https://github.com/tnaake/TBET_in_CLL and https://github.com/massonix/Tbet_in_CLL.

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Figure Legends

Figure 1: *TBX21* expression is higher in CLL in comparison to healthy B cells

A) Gene expression of *TBX21* in CLL cells (n = 41) and B cells of age-matched donors (n = 11). *p* values were obtained by unpaired t-test. B) Flow cytometric analysis of T-bet levels in CLL cells (n = 20) and B cells of age-matched donors (n = 5). *p* values were obtained by unpaired t-test. C) Gene expression of *TBX21* in normal B-cell subsets (n = 5-7) and CLL cells (n = 10). Bar limits indicate mean expression; error bars indicate sem. *p* values were obtained by one-way ANOVA controlling the FDR using the Benjamini-Hochberg (BH) method. D) Expression of ABC marker genes in CLL cells (n = 10) and normal B-cell subsets (n = 5-7). High (green) and low (blue) expression in ABCs is depicted on the right. E) Analysis of the association between *TBX21* gene expression and the presence of specific driver genetic alterations. Point estimates with 95 % confidence intervals were calculated for the whole CLL cohort and *IGHV* subtypes using two-sided t-tests and controlling the FDR using the BH method. The point estimates represent the difference between the mean *TBX21* expression in individuals with CLL with and without each corresponding alteration. The point estimates are color-coded based on false discovery rate (FDR). The Oncoprint shows the association of genetic driver alterations with higher or lower expression of *TBX21*, along with additional clinical information such as the *IGHV* status, time to first treatment and patient status (treated/untreated). Samples are ordered from lower to higher *TBX21* gene expression. MBL cases are excluded from this analysis. Genetic driver alterations are depicted using distinct colors corresponding to the alteration type. The number of samples with mutations as well as the percentage of mutated samples over the whole cohort is shown on the right. The analyzed dataset consisted of gene expression microarrays from 364 CLL samples.[55] F) Chromatin landscape of *TBX21* showing the median ATAC-seq, H3K27ac ChIP-seq and positive strand RNA-seq levels from 7 CLL patients and 15 samples from 4 different normal B-cell subpopulations (naïve, germinal center and memory B cells, and plasma cells).

Figure 2: Inflammatory signals by activated T cells drive *TBX21* expression in CLL cells via NF-κB

A) Log2-transformed *TBX21* gene expression of CLL cells (n = 5) cultured alone, after co-culture with CD40L-expressing fibroblasts, or *in vitro* activated autologous T cells. *p* values were obtained by RM-one way ANOVA and controlling the FDR using the Benjamini-Hochberg (BH) method. B-C) Flow cytometric analysis of T-bet levels in CLL cells. B) CLL PBMCs (n = 7) or C) purified CLL cells (n = 11) were stimulated with various cytokines and combinations thereof. Induction of T-bet expression was compared to medium control. *p* values were obtained by one-sample t-tests and Wilcoxon signed-rank tests and controlling the FDR using the BH method. D) Flow cytometric analysis of T-bet levels in CLL cells of

patients prior to ibrutinib treatment, and after 3 and 6 months of ibrutinib (n = 8). *p* values were obtained by RM-one way ANOVA and controlling the FDR using the BH method. E) *TBX21* gene expression of CLL cells of patients prior to acalabrutinib treatment, and after 1 and 6 months of acalabrutinib (n = 20). *p* values were obtained by RM-one way ANOVA and controlling the FDR using the BH method. F-G) Flow cytometric analysis of T-bet levels in CLL cells after stimulation of CLL PBMCs with various cytokines and combinations thereof. F) Cells (n = 7) were stimulated in the presence of vehicle control or ibrutinib. Quantification displays log2FC in comparison to vehicle control. *p* values were obtained by RM-one way ANOVA and controlling the FDR using the BH method. G) Purified CLL cells (n = 8) were stimulated in the presence of vehicle control or the NF-κB-inhibitor IKK-16. Quantification displays log2FC in comparison to vehicle control. *p* values were obtained by Friedman's test and controlling the FDR using the BH method. H) T-bet levels of two individual TCL1 CLL clones harboring a hyperactive NF-κB signaling (*Nfkbie*$^{-/-}$) in comparison to WT controls as analyzed by flow cytometry (n = 3 technical replicates).

Figure 3: T-bet has lineage separating properties in CLL

A) Expression of T-bet-dependent genes in normal B-cell subsets (n = 5-7) and CLL cells (n = 10). B-C) Activity scores of T-bet were calculated based on B) induced and C) repressed genes individually for normal B-cell subsets and CLL cells. *p* values were obtained by One-way ANOVA and controlling the FDR using the Benjamini-Hochberg (BH) method. D) Correlation of *TBX21* gene expression and T-bet activity. *p* values were obtained by Pearson correlation testing. E-F) Analysis of B-cell subsets of the human tonsil atlas with a representation of T-bet expression in different clusters. F) T-bet activity scores in human tonsillar B cells calculated based on induced genes by T-bet in CLL. *P* values were obtained by Mann-Whitney testing.

Figure 4: T-bet regulates gene transcription mainly via distal chromatin co-accessibility in CLL cells

A) T-bet-mediated regulation of T-bet-dependent genes. Promoter with an ATAC peak containing a T-bet binding motif, blue; co-accessible link of promoter to distal T-bet peak within 1 Mb, orange; no link to T-bet motif peak, grey. **B)** Number of co-accessible links from T-bet-dependent gene promoters without (left) or with (right) ATAC peak with T-bet binding motif within a 1 Mb window in *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells. Whiskers represent standard error of biological replicates (n = 2). **C)** Overlap of co-accessible links from T-bet-dependent genes with distal T-bet peaks within 100 kb in *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells. Co-accessible links from biological replicates were merged. **D)** Gene expression of *Nos1* in *Tbx21*$^{-/-}$ (n = 6) and *Tbx21*$^{+/+}$ TCL1 cells (n = 5) from bulk RNA-seq data. **E)** Co-accessibility in *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells at the T-bet-dependent gene *Nos1* region. Browser tracks and co-accessible links from

biological replicates were merged. Top: browser tracks of pseudo-bulk chromatin accessibility from single cells. Middle: 2 kb regions around peaks from pseudo-bulk chromatin accessibility with no accessibility change (grey), significantly higher accessibility in *Tbx21*$^{+/+}$ TCL1 cells (black) and significantly higher accessibility in *Tbx21*$^{-/-}$ TCL1 cells (blue); T-bet binding motif positions and gene annotation in black. Bottom: co-accessible links between peaks at *Nos1* promoters and distal peaks in *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells. Promoters of *Nos1* (1 kb around TSSs) are marked in red.

Figure 5: T-bet is required for interferon signaling in CLL cells

A) GSEA of RNA-seq of *Tbx21*$^{-/-}$ (n = 6) vs *Tbx21*$^{+/+}$ (n = 6) TCL1 cells and *TBX21*^{low} vs *TBX21*^{high} CLL cells was performed, and commonly regulated gene sets are depicted. NES, normalized enrichment score. B) KEGG pathway analysis of RNA-seq and mass spectrometry data of T-bet^{low} vs T-bet^{hi} CLL cells. NES, normalized enrichment score. C) Basal expression of interferon-stimulated genes (ISGs) in *Tbx21*$^{-/-}$ compared to *Tbx21*$^{+/+}$ TCL1 cells as analyzed by RNA-seq. D) Purified *Tbx21*$^{-/-}$ or *Tbx21*$^{+/+}$ TCL1 cells were stimulated *in vitro* with IFNβ. Log2FC of ISG expression in comparison to medium control as analyzed by RT-qPCR. *p* values were obtained by multiple t-tests and controlling the FDR using the Benjamini-Hochberg method.

Figure 6: T-bet acts as a silencing transcription factor in CLL cells

A)-B) Differential chromatin accessibility in A) *Tbx21*$^{-/-}$ vs *Tbx21*$^{+/+}$ TCL1 cells as analyzed by scATAC-seq, and B) *TBX21*^{low} vs *TBX21*^{high} CLL cells as analyzed by ATAC-seq (FDR 0.05). Numbers of up- and downregulated peaks are indicated. C) Motif enrichment analysis of ATAC-seq data was performed individually for U-CLL, M-CLL and TCL1 cells. Commonly enriched motifs are displayed.

Figure 7: T-bet represses cell cycling in CLL cells and predicts good outcome

A) scRNA-seq of *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells was performed, and single cells were annotated according to their cell cycle phase. B) Phospho-specific mass spectrometry analysis of *Tbx21*$^{-/-}$ and *Tbx21*$^{+/+}$ TCL1 cells was performed, and kinase networks enriched in *Tbx21*$^{-/-}$ TCL1 cells are displayed. C) T-bet activity score based on repressed genes was calculated from scRNA-seq data of CLL lymph node samples annotated according to their proliferation status. D) MEC-1 cell lines with inducible overexpression of T-bet or GFP as control were generated. Using doxycycline, overexpression was induced and expansion of GFP^{+} control cells and T-bet-overexpressing MEC-1 cells was analyzed by CellTiter Glow proliferation assay (n = 4 biological replicates á 3 technical replicates). *p* values were obtained by unpaired t-test of the means of biological replicates. E-G) RNA-sequencing of CLL patient samples was performed at diagnosis. CLL patients were stratified according to *TBX21* mRNA abundance using the maxstat rank statistics-based cutoff. E) overall survival of *TBX21*^{high} vs *TBX21*^{low} CLL patients was analyzed. F) CLL patients were stratified according to *TBX21* mRNA abundance using the maxstat rank

statistics-based cutoff and their *IGHV* mutational status (M = mutated vs UM = unmutated). Overall survival of M-*TBX21*^{high} vs M-*TBX21*^{low} vs UM-*TBX21*^{high} vs UM-*TBX21*^{low} CLL patients was analyzed. G) CLL patients were stratified according to *TBX21* mRNA abundance using the maxstat rank statistics-based cutoff and their *ZAP70* gene expression level (*ZAP70*^{high} vs *ZAP70*^{low}). Overall survival of *ZAP70*^{high}-*TBX21*^{high} vs *ZAP70*^{high}-*TBX21*^{low} vs *ZAP70*^{low}-*TBX21*^{high} vs *ZAP70*^{low}-*TBX21*^{low} CLL patients was analyzed. *p* values were obtained by log-rank testing.

Figure 1

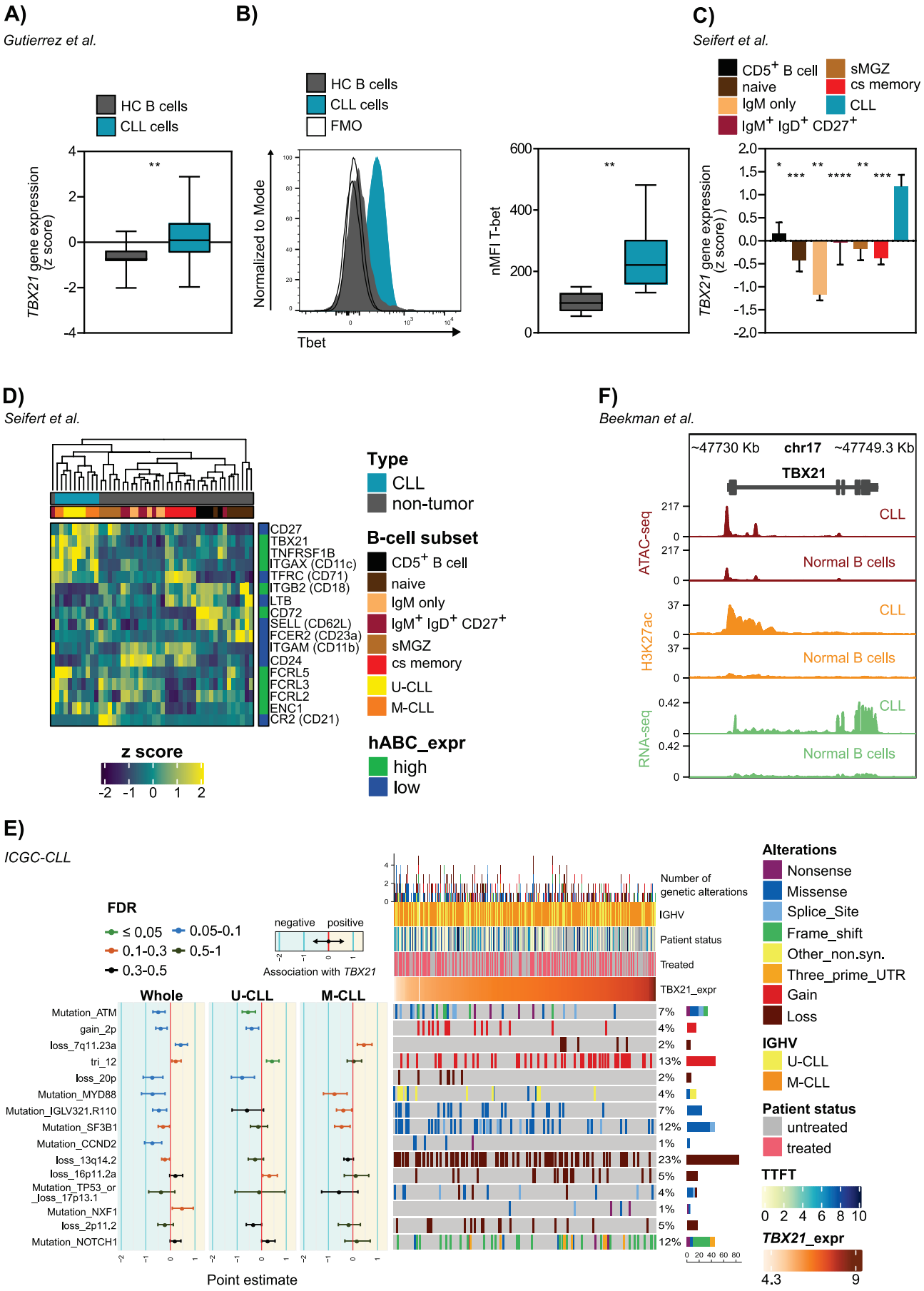

A)
Gutierrez et al.
HC B cells
CLL cells
TBX21 gene expression (z score)
B)
HC B cells
CLL cells
FMO
Normalized to Mode
Tbet
nMFI T-bet
C)
Seifert et al.
CD5+ B cell
naive
IgM only
IgM+ IgD+ CD27+
sMGZ
cs memory
CLL
TBX21 gene expression (z score)
D)
Seifert et al.
CD27
TBX21
TNFRSF1B
ITGAX (CD11c)
TFRC (CD71)
ITGB2 (CD18)
LTB
CD72
SELL (CD62L)
FCER2 (CD23a)
ITGAM (CD11b)
CD24
FCRL5
FCRL3
FCRL2
ENC1
CR2 (CD21)
z score
-2 -1 0 1 2
Type
CLL
non-tumor
B-cell subset
CD5+ B cell
naive
IgM only
IgM+ IgD+ CD27+
sMGZ
cs memory
U-CLL
M-CLL
hABC_expr
high
low
F)
Beekman et al.
~47730 Kb
chr17
~47749.3 Kb
TBX21
ATAC-seq
H3K27ac
RNA-seq
CLL
Normal B cells
E)
ICGC-CLL
FDR
≤ 0.05
0.05-0.1
0.1-0.3
0.5-1
0.3-0.5
negative
positive
Association with TBX21
Whole
U-CLL
M-CLL
Mutation_ATM
gain_2p
loss_7q11.23a
tri_12
loss_20p
Mutation_MYD88
Mutation_IGLV321.R110
Mutation_SF3B1
Mutation_CCND2
loss_13q14.2
loss_16p11.2a
Mutation_TP53_or_loss_17p13.1
Mutation_NXF1
loss_2p11.2
Mutation_NOTCH1
Point estimate
Number of genetic alterations
IGHV
Patient status
Treated
TBX21_expr
7%
4%
2%
13%
2%
4%
7%
12%
1%
23%
5%
4%
1%
5%
12%
Alterations
Nonsense
Missense
Splice_Site
Frame_shift
Other_non.syn.
Three_prime_UTR
Gain
Loss
IGHV
U-CLL
M-CLL
Patient status
untreated
treated
TTFT
0 2 4 6 8 10
TBX21_expr
4.3
9

Figure 2

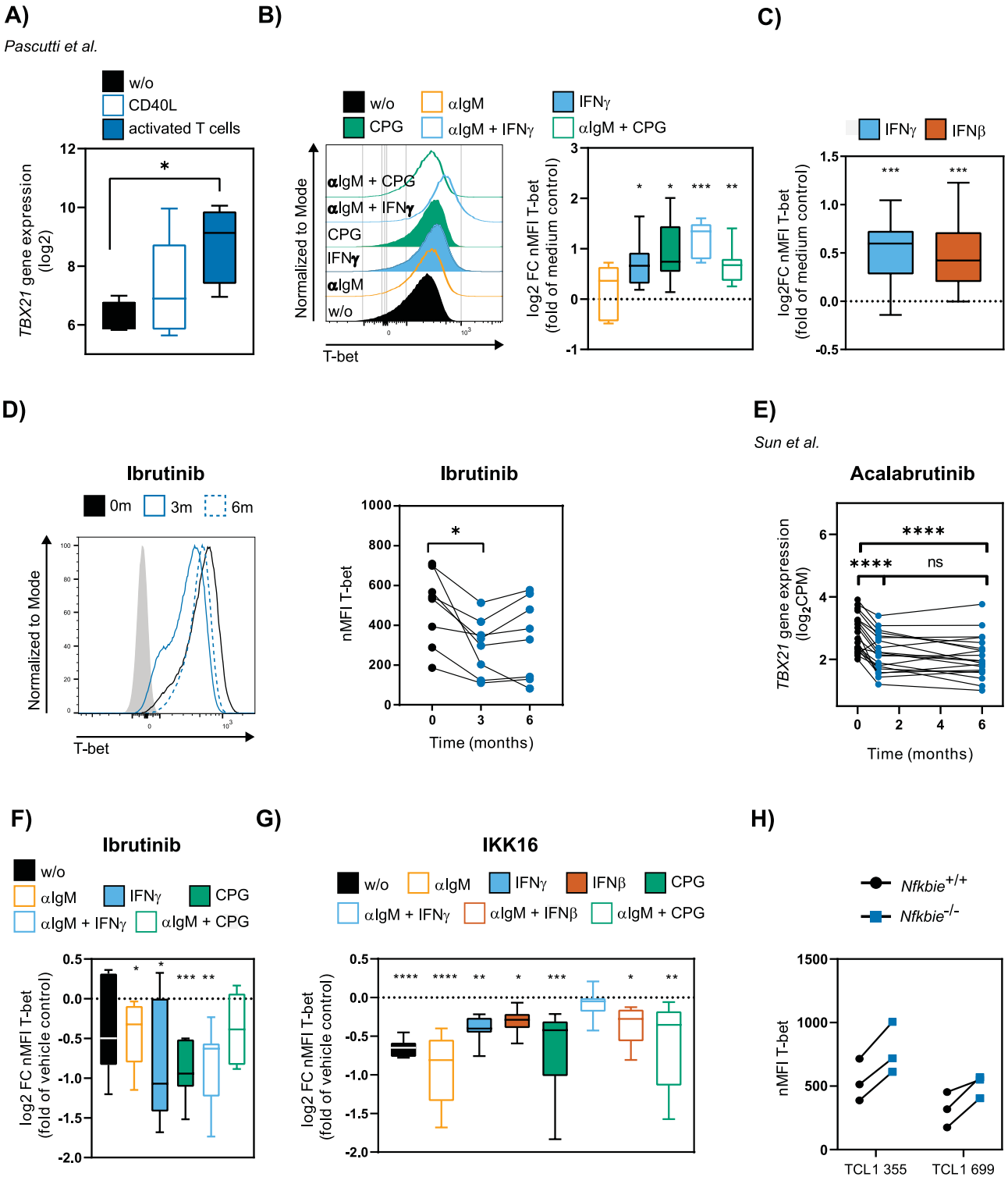

A)
Pascutti et al.
w/o
CD40L
activated T cells
TBX21 gene expression (log2)
B)
w/o
αIgM
CPG
αIgM + IFNγ
IFNγ
αIgM + CPG
αIgM + CPG
αIgM + IFNγ
CPG
IFNγ
αIgM
w/o
Normalized to Mode
T-bet
log2 FC nMFI T-bet (fold of medium control)
C)
IFNγ
IFNβ
log2FC nMFI T-bet (fold of medium control)
D)
Ibrutinib
0m
3m
6m
Normalized to Mode
T-bet
Ibrutinib
nMFI T-bet
Time (months)
E)
Sun et al.
Acalabrutinib
TBX21 gene expression (log2CPM)
ns
Time (months)
F)
Ibrutinib
w/o
αIgM
IFNγ
CPG
αIgM + IFNγ
αIgM + CPG
log2 FC nMFI T-bet (fold of vehicle control)
G)
IKK16
w/o
αIgM
IFNγ
IFNβ
CPG
αIgM + IFNγ
αIgM + IFNβ
αIgM + CPG
log2 FC nMFI T-bet (fold of vehicle control)
H)
Nfkbie+/+
Nfkbie-/-
nMFI T-bet
TCL1 355
TCL1 699

Figure 3

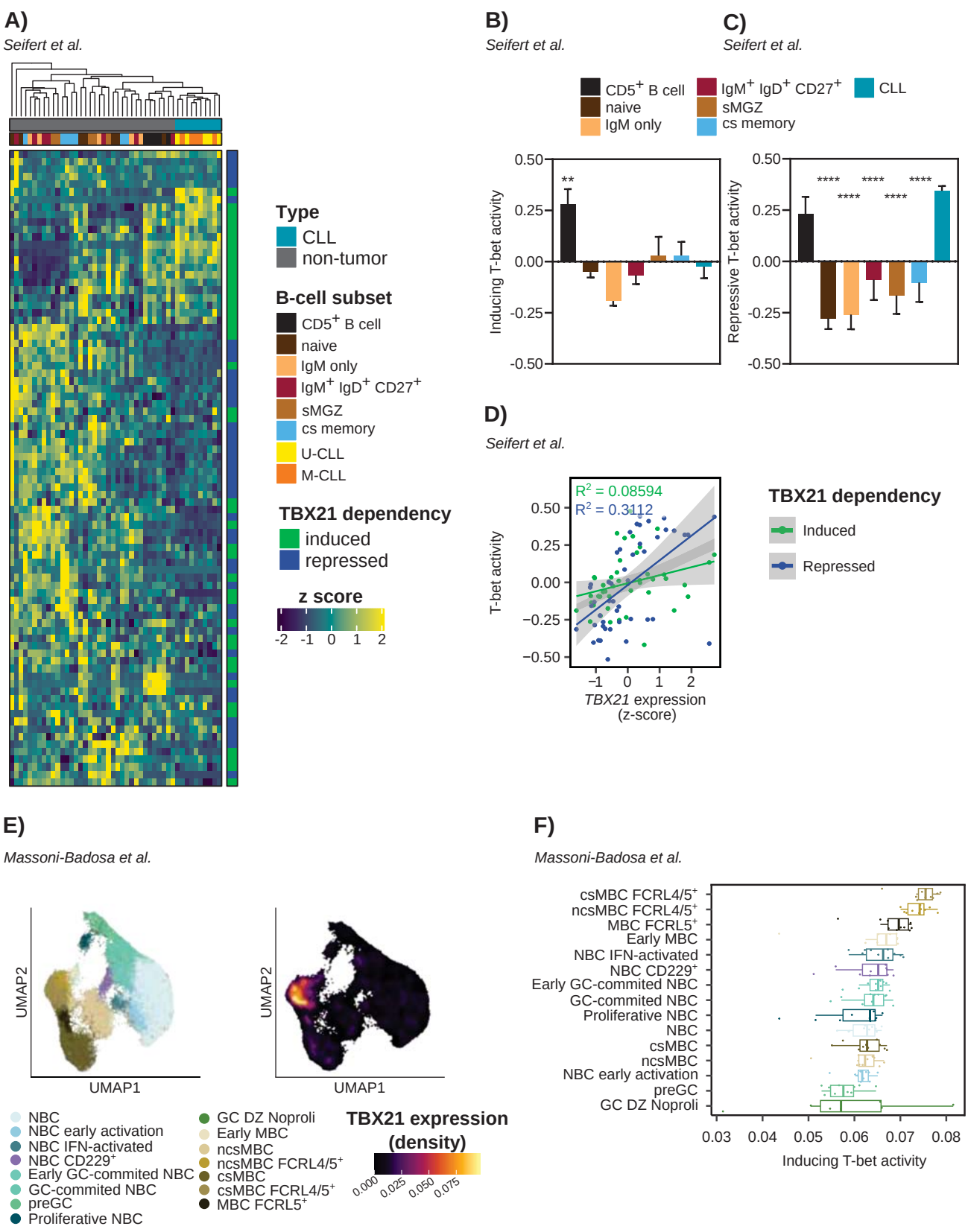
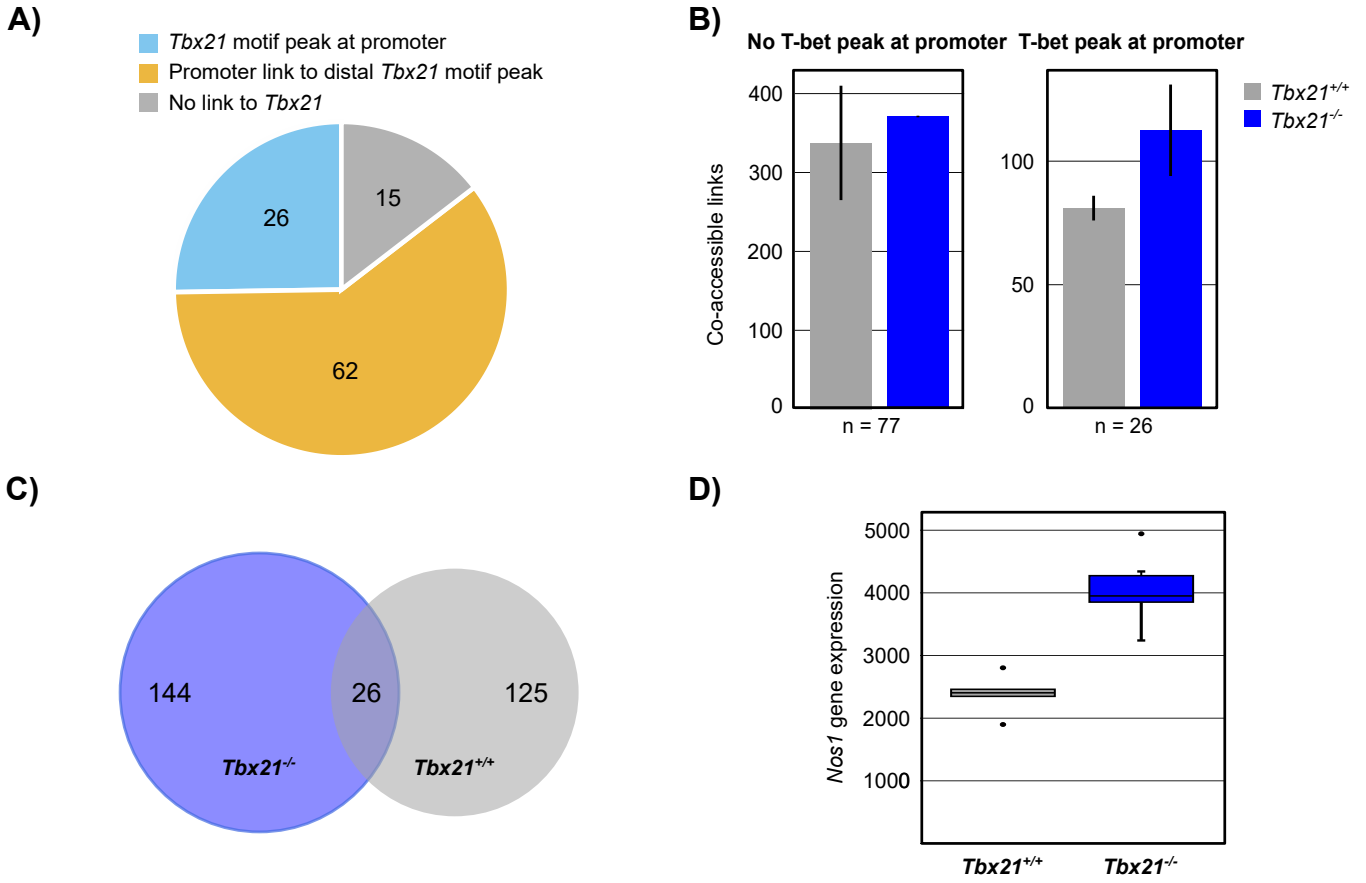

A)
Seifert et al.
Type
CLL
non-tumor
B-cell subset
CD5+ B cell
naive
IgM only
IgM+ IgD+ CD27+
sMGZ
cs memory
U-CLL
M-CLL
TBX21 dependency
induced
repressed
z score
-2 -1 0 1 2
B)
Seifert et al.
Inducing T-bet activity
0.50
0.25
0.00
-0.25
-0.50
**
C)
Seifert et al.
CD5+ B cell
naive
IgM only
IgM+ IgD+ CD27+
sMGZ
cs memory
CLL
Repressive T-bet activity
****
D)
Seifert et al.
R2 = 0.08594
R2 = 0.3112
T-bet activity
TBX21 expression
(z-score)
TBX21 dependency
Induced
Repressed
E)
Massoni-Badosa et al.
UMAP2
UMAP1
NBC
NBC early activation
NBC IFN-activated
NBC CD229+
Early GC-commited NBC
GC-commited NBC
preGC
Proliferative NBC
GC DZ Noproli
Early MBC
ncsMBC
ncsMBC FCRL4/5+
csMBC
csMBC FCRL4/5+
MBC FCRL5+
TBX21 expression
(density)
0.000
0.025
0.050
0.075
F)
Massoni-Badosa et al.
csMBC FCRL4/5+
ncsMBC FCRL4/5+
MBC FCRL5+
Early MBC
NBC IFN-activated
NBC CD229+
Early GC-commited NBC
GC-commited NBC
Proliferative NBC
NBC
csMBC
ncsMBC
NBC early activation
preGC
GC DZ Noproli
0.03
0.04
0.05
0.06
0.07
0.08
Inducing T-bet activity

Figure 4


A)
Tbx21 motif peak at promoter
Promoter link to distal Tbx21 motif peak
No link to Tbx21
26
15
62
B)
No T-bet peak at promoter
T-bet peak at promoter
Co-accessible links
400
300
200
100
0
n = 77
100
50
0
n = 26
Tbx21+/+
Tbx21-/-
C)
144
26
125
Tbx21-/-
Tbx21+/+
D)
Nos1 gene expression
5000
4000
3000
2000
1000
Tbx21+/+
Tbx21-/-

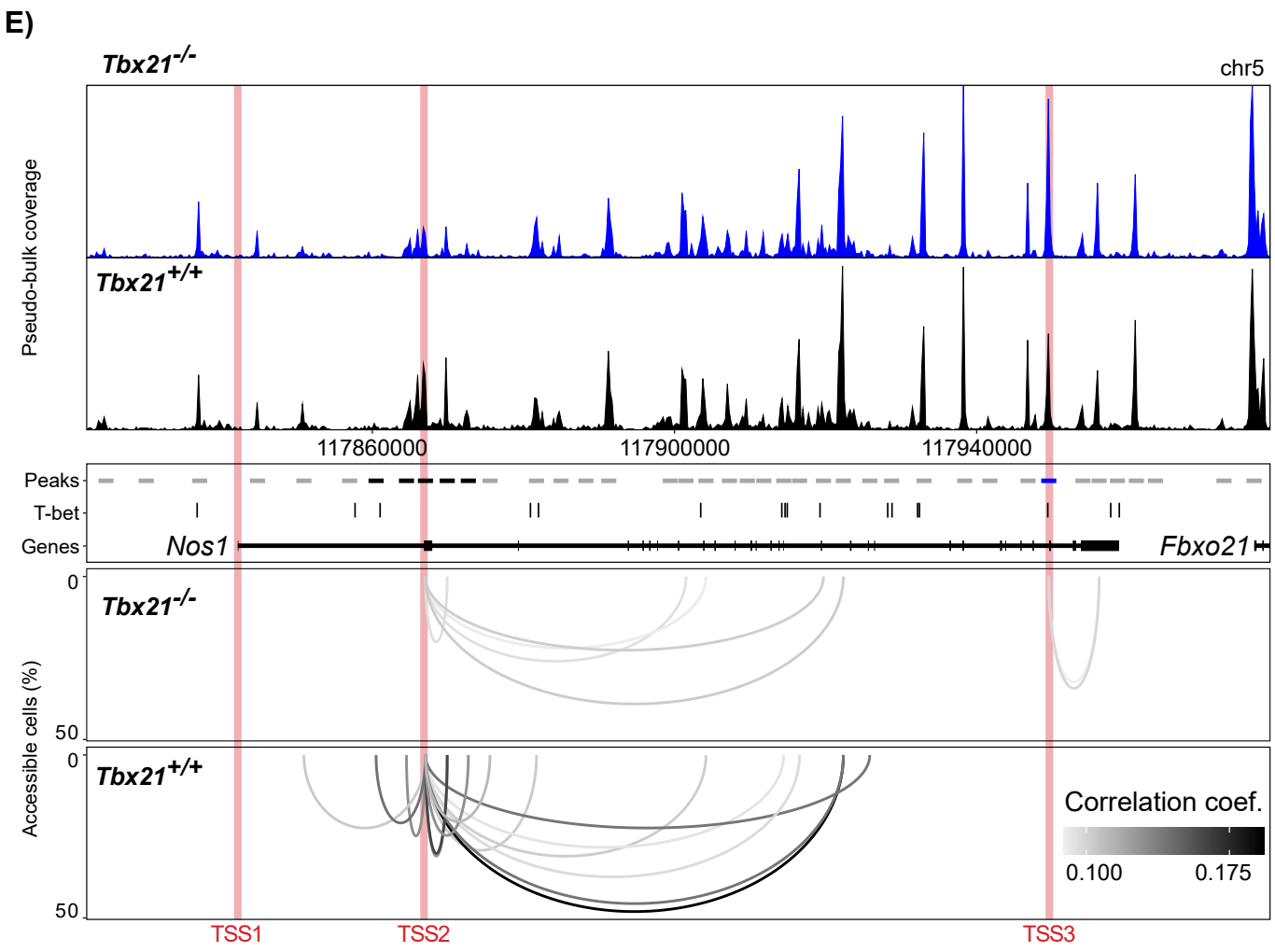

E)
Tbx21-/-
chr5
Pseudo-bulk coverage
Tbx21+/+
117860000
117900000
117940000
Peaks
T-bet
Genes
Nos1
Fbxo21
0
Tbx21-/-
50
Accessible cells (%)
0
Tbx21+/+
50
Correlation coef.
0.100
0.175
TSS1
TSS2
TSS3

Figure 5

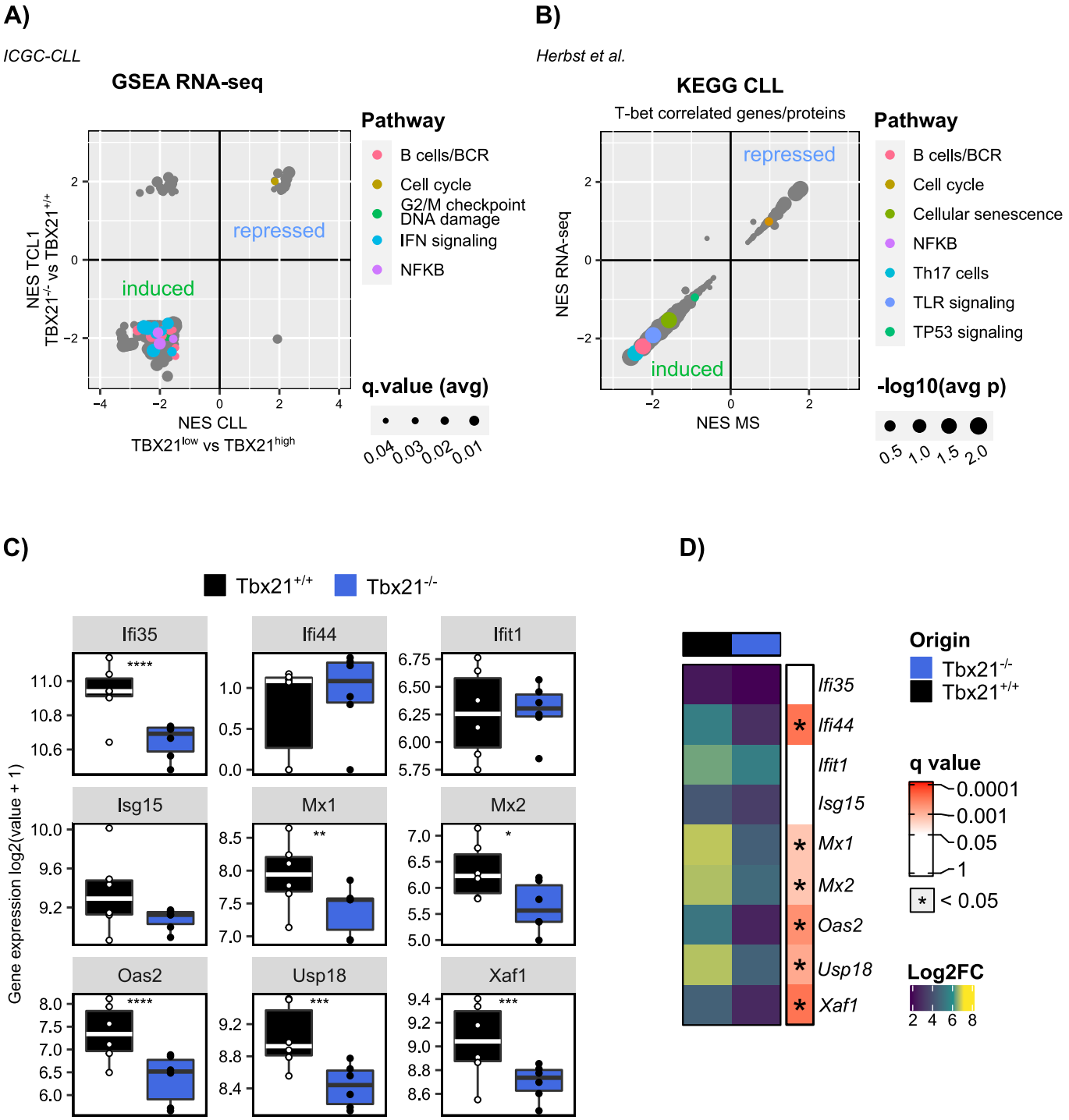

A)
ICGC-CLL
GSEA RNA-seq
repressed
induced
NES TCL1
TBX21-/- vs TBX21+/+
NES CLL
TBX21low vs TBX21high
Pathway
B cells/BCR
Cell cycle
G2/M checkpoint
DNA damage
IFN signaling
NFKB
q.value (avg)
0.04
0.03
0.02
0.01
B)
Herbst et al.
KEGG CLL
T-bet correlated genes/proteins
repressed
induced
NES RNA-seq
NES MS
Pathway
B cells/BCR
Cell cycle
Cellular senescence
NFKB
Th17 cells
TLR signaling
TP53 signaling
-log10(avg p)
0.5
1.0
1.5
2.0
C)
Tbx21+/+
Tbx21-/-
Ifi35
Ifi44
Ifit1
Isg15
Mx1
Mx2
Oas2
Usp18
Xaf1
Gene expression log2(value + 1)
D)
Ifi35
Ifi44
Ifit1
Isg15
Mx1
Mx2
Oas2
Usp18
Xaf1
Origin
Tbx21-/-
Tbx21+/+
q value
0.0001
0.001
0.05
1
* < 0.05
Log2FC
2 4 6 8

Figure 6

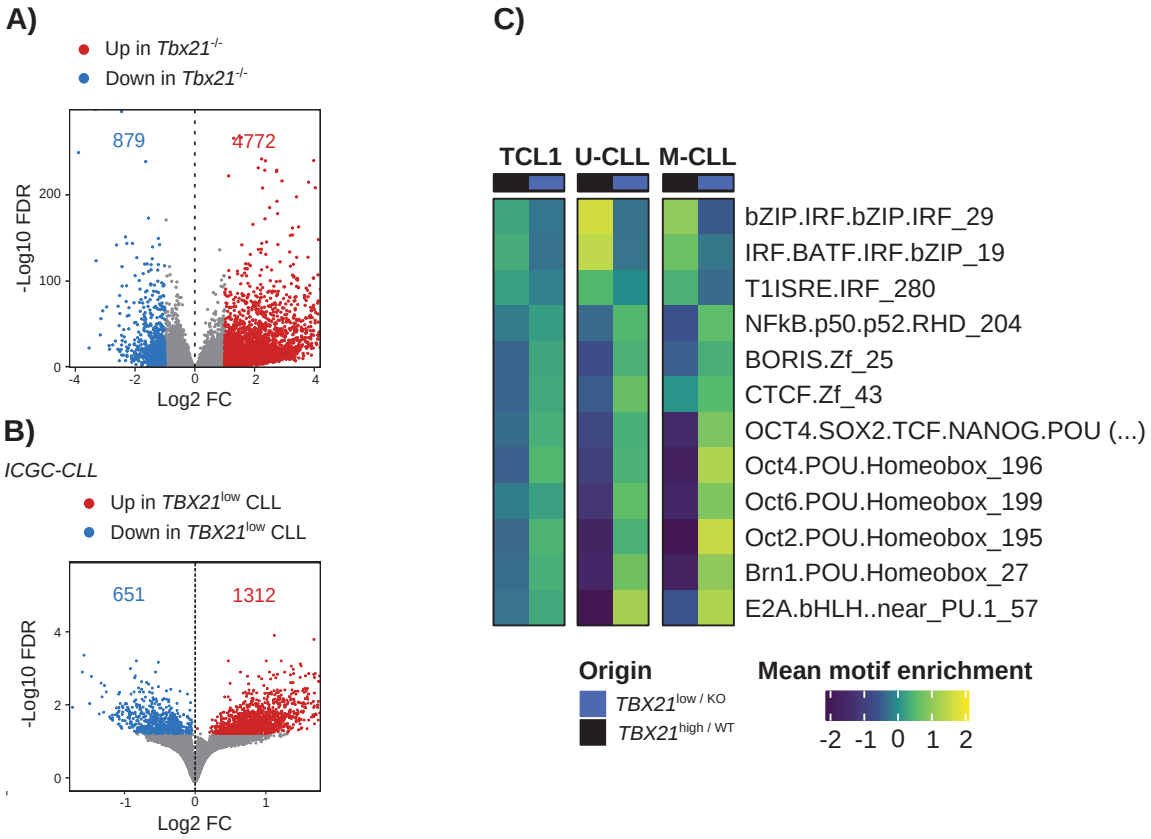
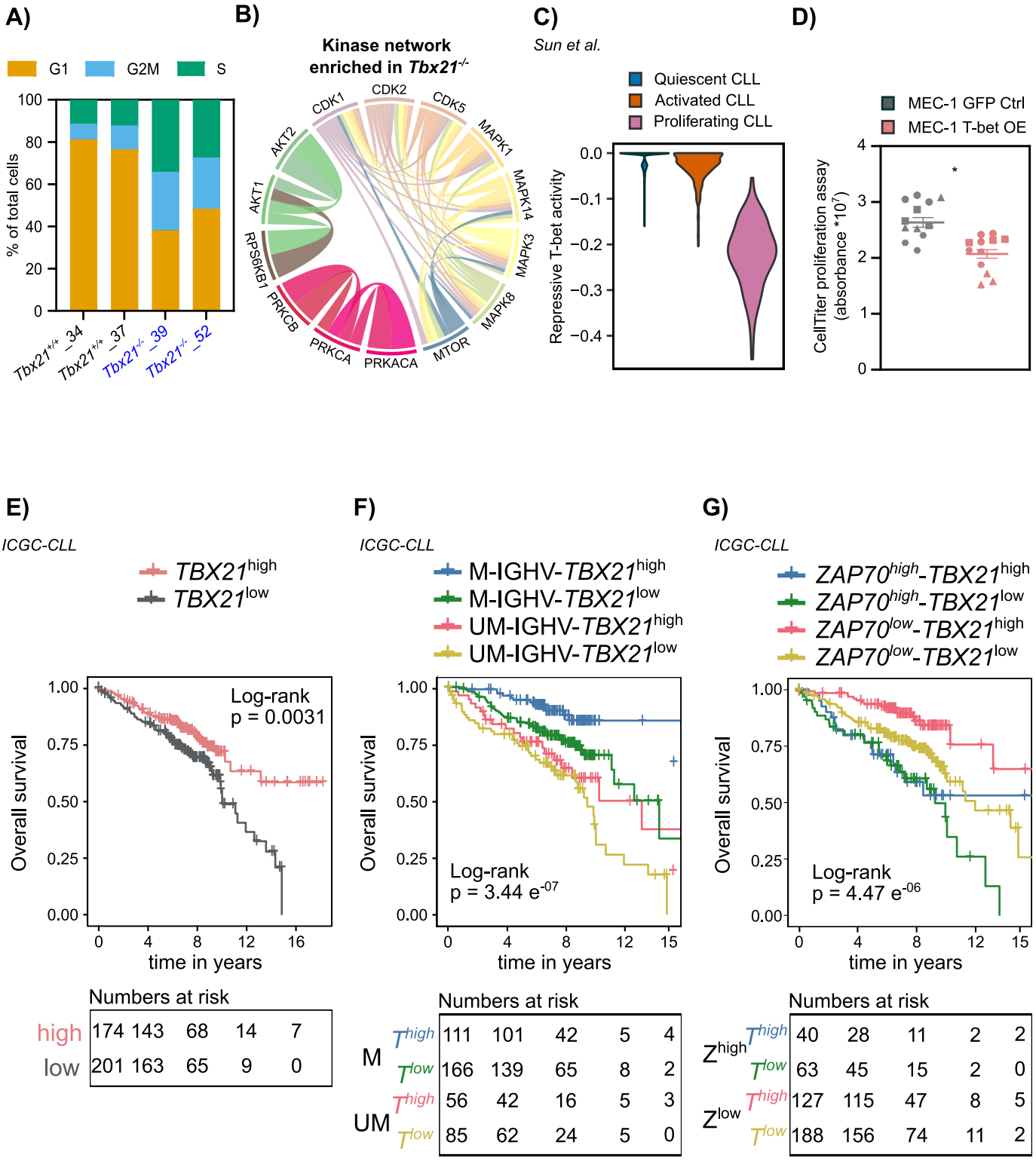

A)
Up in Tbx21-/-
Down in Tbx21-/-
879
4772
-Log10 FDR
Log2 FC
B)
ICGC-CLL
Up in TBX21low CLL
Down in TBX21low CLL
651
1312
-Log10 FDR
Log2 FC
C)
TCL1
U-CLL
M-CLL
bZIP.IRF.bZIP.IRF_29
IRF.BATF.IRF.bZIP_19
T1ISRE.IRF_280
NFkB.p50.p52.RHD_204
BORIS.Zf_25
CTCF.Zf_43
OCT4.SOX2.TCF.NANOG.POU (...)
Oct4.POU.Homeobox_196
Oct6.POU.Homeobox_199
Oct2.POU.Homeobox_195
Brn1.POU.Homeobox_27
E2A.bHLH..near_PU.1_57
Origin
TBX21low / KO
TBX21high / WT
Mean motif enrichment
-2 -1 0 1 2

Figure 7


A)
G1
G2M
S
% of total cells
100
80
60
40
20
0
Tbx21+/+ _34
Tbx21+/+ _37
Tbx21-/- _39
Tbx21-/- _52
B)
Kinase network
enriched in Tbx21-/-
CDK1
CDK2
CDK5
MAPK1
MAPK14
MAPK3
MAPK8
MTOR
PRKACA
PRKCA
PRKCB
RPS6KB1
AKT1
AKT2
C)
Sun et al.
Quiescent CLL
Activated CLL
Proliferating CLL
Repressive T-bet activity
0.0
−0.1
−0.2
−0.3
−0.4
D)
MEC-1 GFP Ctrl
MEC-1 T-bet OE
CellTiter proliferation assay
(absorbance *10^7)
4
3
2
1
0
*
E)
ICGC-CLL
TBX21high
TBX21low
Overall survival
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 0.0031
0
4
8
12
16
time in years
Numbers at risk
high 174 143 68 14 7
low 201 163 65 9 0
F)
ICGC-CLL
M-IGHV-TBX21high
M-IGHV-TBX21low
UM-IGHV-TBX21high
UM-IGHV-TBX21low
Overall survival
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 3.44 e-07
0
4
8
12
15
time in years
Numbers at risk
M Thigh 111 101 42 5 4
M Tlow 166 139 65 8 2
UM Thigh 56 42 16 5 3
UM Tlow 85 62 24 5 0
G)
ICGC-CLL
ZAP70high-TBX21high
ZAP70high-TBX21low
ZAP70low-TBX21high
ZAP70low-TBX21low
Overall survival
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 4.47 e-06
0
4
8
12
15
time in years
Numbers at risk
Zhigh Thigh 40 28 11 2 2
Zhigh Tlow 63 45 15 2 0
Zlow Thigh 127 115 47 8 5
Zlow Tlow 188 156 74 11 2