Regulatory mechanisms and context-dependent roles of TAL1 in T-cell acute lymphoblastic leukemia

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy derived from thymic T-cell precursors. Approximately 40-60% of T-ALL cases exhibit aberrant overexpression of the TAL1 oncogenic transcription factor. Here, we provide a comprehensive view of the TAL1-induced transcriptional program in human T-ALL cells using a rapid protein degradation system coupled with integrative approaches. Our study demonstrates that TAL1 targets can be classified into several groups, each of which exhibits unique gene expression kinetics, chromatin features, and regulatory mechanisms. Group A genes are highly dependent on TAL1, many of which are not expressed in normal T cells or TAL1-negative T-ALL cells, representing an oncogenic TAL1 signature. The TAL1 complex predominantly activates group A genes. TAL1's effect is not replaceable with its regulatory partners GATA3 or RUNX1. In contrast, group B genes, many of which are generally expressed across different T-ALL subgroups, exhibit densely-connected chromatin-chromatin interactions and demonstrate the collaborative roles played by TAL1 with other transcription factors. Interestingly, TAL1 cooperates with NOTCH1 to regulate gene expression in TAL1-positive T-ALL cells, whereas it potentially antagonizes the NOTCH1-MYC pathway and leads to lethality in TAL1-negative/TLX3-positive cells, demonstrating the context-dependent roles of TAL1.

Introduction

TAL1 is an essential regulator of hematopoiesis that plays crucial roles in the maintenance of hematopoietic stem cells (HSC) and progenitor cells as well as in the specification and maturation of erythro-megakaryocyte lineage.[1-3] During normal T-cell development, TAL1 and its binding partner LMO2 are silenced, which enables the formation of E-protein dimers (e.g., E2A and HEB), which facilitate the expression of genes required for T-cell differentiation, such as RAG recombinases (*RAG1* and *RAG2*) and pre-T-cell receptor subunit (*PTCRA*).[4,5] This stage-specific regulation of TAL1, LMO2 and E-proteins is important for T-cell development.

TAL1 and LMO2 have also been implicated as oncogenes in T-cell acute lymphoblastic leukemia (T-ALL), a malignancy derived from immature T cells.[6,7] TAL1 is ectopically overexpressed in 40-60% of human T-ALL, while LMO2 or a functionally-redundant protein, LMO1, is often overexpressed with TAL1. TAL1 forms a complex with the E-proteins, LMO1/2 and GATA3 in T-ALL cells. Overexpression of TAL1 and LMO1/2 or inhibition of E-proteins activity leads to blocked differentiation and initiation of T-cell leukemogenesis in mouse models.[8,9] Thus, one of the main oncogenic mechanisms of TAL1 involves the induction of differentiation arrest. On the other hand, TAL1 can also induce a unique set of genes in human T-ALL, such as *ALDH1A2*,[10] which has not been observed in mouse models. Notably, in many human T-ALL cases, the expression of TAL1 is driven by genetic mutations in an enhancer that can be activated by TAL1 and its regulatory partners (GATA3 and RUNX1) via an autoregulatory loop, forming the core regulatory circuit (CRC).[11,12]

Another prevalent oncogenic transcription factor in T-ALL is NOTCH1. The NOTCH pathway is constitutively activated due to activating mutations of *NOTCH1* and/or inactivating mutations of *FBXW7*, which is observed in over 50% of T-ALL.[13] NOTCH1 transcriptionally induces the expression of the *MYC* oncogene,[14] thereby promoting cell proliferation, metabolism and survival. In the *Tal1* transgenic mouse,

Notch1 mutations were frequently observed in T-ALL cells,[15] suggesting that there is a selective advantage to acquire *Notch1* mutation for the clones initiated by *Tal1* and thus these two factors cooperate in the transformational process. TAL1 and NOTCH1 regulate different sets of genes, but they share the same targets, such as *GIMAP*, in T-ALL cells.[16] However, the relationship and roles of TAL1 and NOTCH1 in the regulation of shared targets have not been fully elucidated.

Additionally, it has been established that the expression of TAL1 and LMO1/2 identifies a specific subgroup of T-ALL cells that is distinct from other subgroups expressing *TLX1*, *TLX3* or *HOXA* or early T-cell progenitor (ETP)-ALL.[7,17-19] These T-ALL subgroups exhibit distinct mutational landscapes and different gene expression signatures (called "Type A abnormalities").[17] Each of these subgroups also shows preferential relationship of a specific signaling pathway (called "Type B abnormalities", e.g., PI3K-AKT with TAL1, and JAK-STAT with TLX3) and is also associated with a specific differentiation stage (e.g., late cortical stage with TAL1). These relationships suggest that TAL1 may require a specific cellular context and collaborating partners to exert oncogenicity.

In this study, we provide a comprehensive view of the TAL1-induced transcriptional program and mechanisms in human T-ALL cells using an integrative approach. TAL1 targets can be classified into several groups, each of which exhibits unique chromatin features and regulatory mechanisms.

Methods

Cell samples

Human T-ALL cell lines were cultured in RPMI-1640 medium (BioWest) supplemented with 10% fetal bovine serum (BioWest) in a humidified incubator containing 5% CO_2 at 37°C.

dTAG-mediated depletion and gene overexpression

The degradation tag (dTAG) system was established following methods described previously.[20] *TAL1* and *LMO1* cDNA were overexpressed by lentivirus or retrovirus infection in T-ALL cells. Detailed methodologies can be found in the *Online Supplementary Appendix* and *Online Supplementary Table S1*.

Cell growth assay

The cells were seeded into 96-well plates and cell viability was measured based on luminescence observed via a CellTiter Glo assay (Promega) using a Promega GloMax-Multi plate reader (Promega).

Western blot analysis

Cell pellets were lysed in RIPA buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Roche). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio–Rad). Bound antibodies were visualized using ECL Prime Western blotting reagents (GE Healthcare) with an ImageQuant LAS500 imager (GE Healthcare).

Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted using a NucleoSpin RNA Extraction Kit (Macherey-Nagel) and converted into cDNA using an EvoScript Reverse Transcriptase Kit (Roche). The mRNA expression levels were measured using Power SYBR Green PCR Master Mix (Roche) in a QuantStudio5 Real-Time PCR System (Thermo Fisher Scientific). Design and Analysis software 2.4.3 was used to analyze data. The primer sequences are listed in *Online Supplementary Table S2*.

Sequencing analyses

Detailed methodologies and dataset availability can be found in the *Online Supplementary Appendix* and *Online Supplementary Tables S3*-5.

RNA sequencing

Total RNA was extracted using QIAzol lysis reagent (Qiagen) and cleaned using an RNeasy Kit (Qiagen). RNA samples were treated with TuRBO DNase (Ambion). Strand-specific library construction and sequencing of 100 bp paired-end reads on the DNBSEQ platform were performed by BGI Genomics.

Cut-and-run assay

Cut-and-run sequencing was performed utilizing a Cutana Cut-and-Run assay kit (EpiCypher). Cells were incubated with CUTANA pA/G-Mnase and chromatin fragments were purified utilizing a Cutana DNA purification kit. Libraries were generated using a NEBNext Ultra II DNA Library Prep Kit and sequenced on an Illumina NovaSeq at GENEWIZ Azenta Life Sciences.

Chromatin immunoprecipitation sequencing

HA-Tag chromatin immunoprecipitation (ChIP) sequencing was performed as previously described.[21]

Hi-chromatin immunoprecipitation sequencing

Crosslinked cells were lysed in Hi-C buffer, sonicated and immunoprecipitated with H3K27ac antibody (Abcam). PCR library amplification was performed on DNA tagmented with Tn5 enzyme (Nextera). Library was sequenced with 100X100 paired-end reads on the DNBseq platform by BGI Genomics.

4C-sequencing

4C-sequencing was performed according to a protocol described previously.[22]

ATAC sequencing

ATAC sequencing was performed according to protocols described previously.[23] The amplified library was purified with AMPure XP beads (Beckman Coulter) and sequenced on an Illumina NovaSeq at GENEWIZ Azenta Life Sciences.

Statistical analysis and reproducibility

All statistical analyses were performed using GraphPad Prism software. *P* value less than 0.05 was considered statistically significant. Student's two-tailed *t* test was used for experiments with equal sample sizes.

Results

TAL1 differentially regulates gene expression in human T-cell acute lymphoblastic leukemia cells

In order to profile the overall pattern of gene regulation induced by TAL1, we first established a degradation tag (dTAG) system using a representative T-ALL cell line (Jurkat), in which the target protein was fused to a FKBP12^{F36V} domain[20] (Figure 1A). The addition of dTAG-13 small-molecule can induce the interaction of the FKBP12^{F36V}-TAL1 fusion protein and the CRBN E3 ligase complex, leading to ubiquitination and protein degradation. This system shows several advantages in terms of kinetics and degree of depletion compared to shRNA- or CRISPR/sgRNA-mediated gene knockout/knockdown which typically take a few days to produce an effect. Hence, we used the dTAG system to analyze short-term effects after complete depletion of TAL1. Successful knock-in of *FKBP12*^{F36V} into the C-terminus of the *TAL1* gene was validated by genomic polymerase chain reaction (PCR) (*Online Supplementary Figure S1A*). Complete TAL1 protein degradation was observed after treatment with dTAG-13 within 2 hours and persisted for 72 hours (Figure 1B). Cell viability was not affected in a short-term culture over 5 days (*Online Supplementary Figure S1B*); thus, the non-specific effects resulting from cell death were considered minimal. This result also suggests that although TAL1 serves as an oncoprotein by initiating the transformational process, it is not the sole driver in the transformed cells because Type B abnormalities can also sustain cell survival and proliferation. In order to identify the mRNA kinetics of direct TAL1 targets, we then performed a RNA-sequencing analysis at different time points after dTAG-13 treatment. Differential expression analysis (P<0.05, log2-fold change 0.3) revealed that TAL1 depletion affected a small number of genes at 1-72 hours, instead of globally regulating transcription (Figure 1C; *Online Supplementary Table S6*). TAL1 deletion also did not affect the status of RNA polymerase II phosphorylation (*Online Supplemental Figure S1C*).

From this analysis, we observed changes in gene expression for several known TAL1 targets. For instance, *ALDH1A2*, *NKX3-1*, *miR-223*, *ARID5B* and *GIMAP* family genes, which had been demonstrated to be directly regulated by TAL1,[10,16,24-26] were significantly downregulated at multiple time points, confirming that FKBP12^{F36V}-tagged TAL1 protein retains original function. Importantly, we found two distinct patterns of gene expression changes among the downregulated gene pool, termed "group A" and "group B" genes (Figure 1C-E). Group A genes, including *ALDH1A2*, *PI16* and *SIX6*, showed a rapid response to TAL1 degradation, with some targets significantly downregulated within 4 hours (log2-fold change ≤-0.7 and P<0.05) (Figure 1D). The magnitude of the downregulation of the group A genes was also more pronounced, with a decrease exceeding 50% within 72 hours, and was further downregulated in a time-dependent manner. Gene expression changes were independently validated using quantitative reverse-transcription PCR (qRT-PCR) (*Online Supplementary Figure S1D*). In contrast, group B genes, including *RUNX1*, *GIMAP2* and *MYB*, did not show the rapid or high degree of gene downregulation as the group A genes (-0.7< log2-fold change <-0.3, P<0.05) (Figure 1E). Additionally, the expression level of the group B genes remained unchanged at several time points. This result indicated that group A gene expression was highly dependent on TAL1, while group B gene expression was regulated in a different manner.

In addition, we identified "group C" genes as those that were upregulated after TAL1 degradation (log2-fold change ≥0.3, P<0.05) (Figure 1F). These genes showed slower kinetics during expression changes: they were upregulated more significantly after 24 hours, and many were further upregulated throughout 72 hours. These findings suggested that group C gene expression was indirectly regulated by TAL1. This group included *RAG1*, *RAG2* and *PTCRA*, which has been known to be positively regulated by E-proteins and thus negatively regulated by TAL1.[27,28]

TAL1 predominantly induces the expression of a set of genes that are normally silenced in T cells

In order to analyze whether group A, B and C genes exhibit different features during transcriptional regulation, we utilized ATAC-sequencing and ChIP-sequencing methods to analyze chromatin and epigenetic states as well as the DNA binding of transcription factors in T-ALL and normal hematopoietic cells. We selected representative genes from each group for downstream analyses.

We first focused on group A genes. Given that their expression was found to be highly reliant on TAL1 expression, we assumed that TAL1 is the dominant factor affecting the initial or multiple processes in transcriptional regulation. Interestingly, ATAC-sequencing data revealed that the chromatin status at many group A gene loci, such as *ALDH1A2* and *SIX6*, was not open in normal hemtopoeitic stem cell (HSC), progenitors or T-cell compartments (Figure 2A; *Online Supplementary Figure S2A*). Two transcriptional start sites (TSS) of *PI16* gene showed a different pattern: TSS 2 was open in HSC and multi-potent progenitor (MPP) while TSS1 was open in common lymphoid progenitor (CLP)

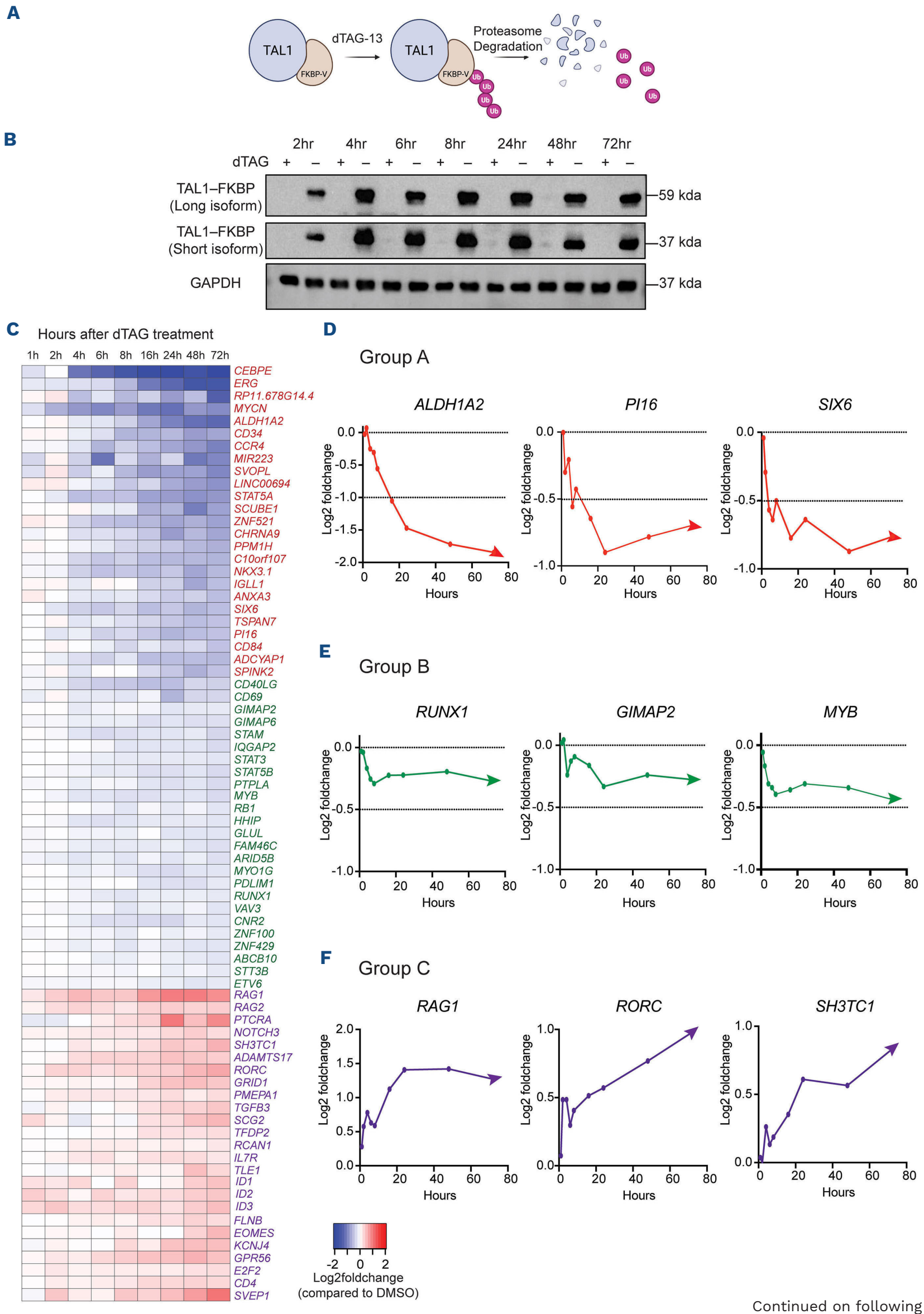

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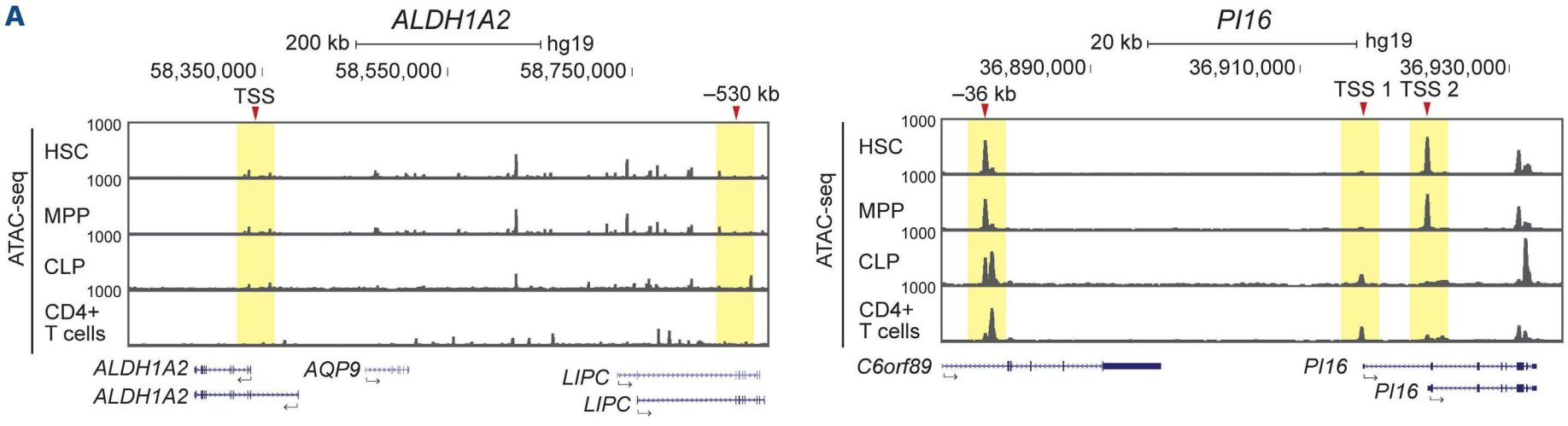
Figure 1. TAL1 differentially regulates gene expression in human T-cell acute lymphoblastic leukemia cells. (A) Illustration of the degradation tag (dTAG) system established for the TAL1 protein in the Jurkat cell line. The addition of the dTAG-13 molecule allows the dimerization of the TAL1-FKBP12^{F36V} chimeric protein to the CRBN E3 ubiquitin ligase complex to promote ubiquitination and subsequent degradation by the proteasome. (B) TAL1-FKBP12^{F36V} Jurkat cells treated with 1 μM dTAG-13 at different time points (2, 4, 6, 8, 24, 48, and 72 hours). Whole-cell lysates were collected and subjected to immunoblot analysis using antibodies specific to HA-tag and GAPDH (the loading control). A protein mobility shift of 15 kDa was observed for both long and short TAL1 isoforms after FKBP12^{F36V} domain addition. (C) Heatmap representing the mRNA expression levels of 75 representative genes identified by RNA sequencing that were significantly down- or upregulated by dTAG-13 treatment compared to dimethyl sulfoxide (DMSO)-treated cells at each time point. Genes were further stratified into group A (red), group B (green) and group C (purple) genes, which showed distinct mRNA kinetics. Group A genes included significantly downregulated genes with decreased gene expression changes over time; a log2-fold change ≤-0.7 and *P<0.05* at 72 hours were the threshold values. Red line graphs of specific group A genes (e.g., *ALDH1A2*, *PI16* and *SIX6*) are displayed on the right (D). Group B genes were downregulated with gene expression changes plateauing over time; a -0.7< log2-fold change <-0.3 and *P*<0.05 and *P*≤0.05 at 72 hours were the threshold values. Green line graphs of specific group B genes (e.g., *RUNX1*, *GIMAP2* and *MYB*) are displayed on the right (E). Group C genes included significantly upregulated genes; a log2-fold change >0.3 and *P<0.05* at 72 hours were the threshold values. Purple line graphs of specific group C genes (e.g., *RAG1*, *RORC* and *SH3TC1*) are displayed on the right (F).



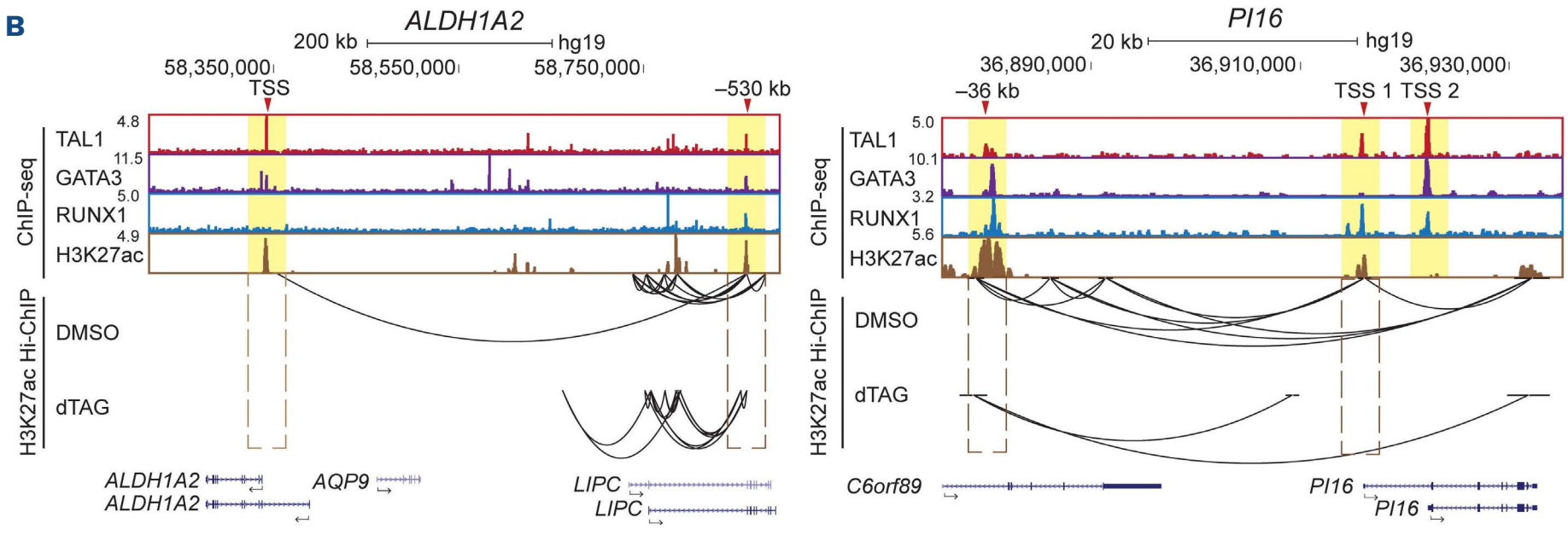


Figure 2. TAL1 induces the expression of a set of genes that are normally silenced in T cells. (A) ATAC-sequencing tracks of hematopoietic stem cells (HSC), multipotent progenitors (MPP), common lymphoid progenitor (CLP) and CD4$^+$ T cells, showing magnification of the *ALDH1A2* and *PI16* loci. The ATAC-sequencing dataset was previously reported.[33] (B) Chromatin immunoprecipitation (ChIP) sequencing tracks of the *TAL1*, *GATA3*, *RUNX1* transcription factors and activating histone mark *H3K27ac* (top) in the Jurkat cell line; the *ALDH1A2* (left) and *PI16* (right) loci are magnified. The ChIP-sequencing dataset was previously reported.[12] *H3K27ac* Hi-ChIP was performed after dimethyl sulfoxide (DMSO) treatment and dTAG treatment for 24 hours to evaluate changes in chromatin loop formation (bottom). For (A) and (B), yellow highlighting indicates the genomic region of interest: where the TAL1 transcription factor binds in baseline Jurkat cell line. For the *ALDH1A2* locus, the transcription start site (TSS) of the shorter isoform and the -530 kb putative enhancer region are highlighted. For the *PI16* locus, the TSS of both isoforms and the -36 kb putative enhancer regions are highlighted.

and CD4$^+$ T cells. The same elements were co-occupied by TAL1, GATA3 and RUNX1 and associated with an active histone mark (H3K27ac) in T-ALL cells (Figure 2B; *Online Supplementary Figure S2B*). Moreover, these genes were not expressed in normal T cells based on the Gene Expression Commons database[29] (*Online Supplementary Figure S2C*), indicating that their expression in T cells was aberrant. On the other hand, the chromatin at some regulatory elements of group A gene, such as *CD34*, *ZNF521* and *MYCN*, was open, and these genes were expressed in T-ALL cells and normal HSC but not in lymphoid progenitor or T-cell compartments (*Online Supplementary Figure S2D, E*). This result suggests that these genes were physiological targets of TAL1 in HSC but were ectopically expressed in T cells when TAL1 was abnormally overexpressed. Correspondingly, many group A genes were expressed only in TAL1-positive T-ALL cell lines, not in TAL1-negative cases (*Online Supplementary Figure S2F*).

In order to further analyze the effect of TAL1 on chromatin-chromatin interactions, we performed a H3K27ac HiChIP analysis before and after TAL1 degradation. In fact, the loss of active chromatin interactions at putative enhancers (bound by TAL1) of gene promoters was observed at the *ALDH1A2*, *PI16* and *SIX6* loci (yellow, Figure 2B; *Online Supplementary Figure S2B*). Validation of the *SIX6* locus via the 4C-sequencing also showed the loss of chromatin–chromatin interactions (*Online Supplementary Figure S2G*). Together, these results indicate that the expression of many group A genes was predominantly regulated by TAL1 via direct enhancer binding and chromatin looping. Their expression is an oncogenic signature of TAL1 expression.

The TAL1 complex alters chromatin accessibility of group A genes in T-cell acute lymphoblastic leukemia cells

Because group A genes were normally silenced but were induced after TAL1 expression, we hypothesized that TAL1 may affect chromatin opening. Thus, we overexpressed *TAL1* and/or its binding partner, *LMO1*, in a TAL1-negative cell line (HPB-ALL) under a doxycycline (Dox)-inducible system (Figure 3A). The HPB-ALL cell line was chosen because it expresses other regulatory partners (*E2A*, *HEB*, *GATA3* and *RUNX1*). We also confirmed that many group A genes were not expressed in HPB-ALL cells at the baseline (*Online Supplementary Table S7*).

Notably, the RNA-sequencing analysis after overexpression of either TAL1 or LMO1 alone showed the upregulation of only 24 or four genes based on the same cutoff values, while the combined overexpression of these two factors upregulated a total of 2,046 genes (Figure 3B). This result supports the fact that TAL1 works in complex with LMO1 and GATA3 in T-ALL cells. Among upregulated genes, we shortlisted 20 genes that were downregulated by dTAG TAL1 deletion in Jurkat cells; this list included many groups A and B genes, such as *ALDH1A2*, *PI16*, *GIMAP*, *RUNX1*, *MYB* and *ZNF521* (*Online Supplementary Table S8*).

Importantly, we observed an increase in chromatin accessibility at the TAL1-bound regions of the *ALDH1A2*, *PI16*, and *SIX6* loci in two independent overexpression clones (yellow, Figure 3C; *Online Supplementary Figure S3A*). Similarly, the chromatin of the regulatory elements at other group A gene loci, including *CD34*, *ZNF521* and *MYCN*, was opened after TAL1 and LMO1 were overexpressed (*Online Supplementary Figure S3B*, left). The increase in chromatin accessibility was accompanied by a significant increase in the mRNA expression of *ALDH1A2*, *PI16* and *ZNF521* (Figure 3D; *Online Supplemental Figure S3B*, right). These data indicate that the TAL1 complex opened the closed chromatin. Notably, when the TAL1 complex bound only the enhancer but not the promoter (e.g., *SIX6*, *CD34* and *MYCN* loci), the chromatin of the promoter was still closed, and thus, no increase in mRNA expression was observed even though the enhancer chromatin had been opened (*Online Supplementary Figure S3B*, right). This result indicates that both enhancer and promoter regions need to be accessible to be actively transcribed.

TAL1 promotes the expression of a set of genes that are highly expressed in different T-cell acute lymphoblastic leukemia subgroups

Next, we focused on group B genes (e.g., *GIMAP* family genes, *RUNX1*, and *MYB*) whose expression is partially dependent on TAL1. In contrast to that of the group A genes, the ATAC-sequencing profile revealed that group B gene locus chromatin was open in normal HSC and in T cells, regardless of whether TAL1 was expressed (Figure 4A, B; *Online Supplementary Figure S4A, B*). Moreover, these genes were expressed in normal hematopoietic cells (*Online Supplementary Figure S4C*), although some of them were expressed at low levels in T cells in the double-negative (DN) or double-positive (DP) stage. These genes were expressed in TAL1-positive and -negative cases, including *TLX1/3*- or *HOXA*-positive subgroups (*Online Supplementary Figure S4D*). Consistently, many of the group B gene loci were also accessible, and these genes were expressed in the TAL1-negative HPB-ALL cell line (Figure 4C; *Online Supplementary Figure S4E*). These results indicated that group B genes represent the general T-ALL gene expression signature, although their expression is positively controlled by TAL1.

Notably, the genetic regions of group B genes are composed of densely-connected chromatin-chromatin interactions, in contrast to group A genes, as indicated in the same Hi-ChIP dataset (Figure 4B; *Online Supplementary Figure S4B*). This difference was not due to the distance or gene number within the same domain (e.g., see the *ALDH1A2* and *RUNX1* loci; they were both scaled to 200 kb). Additionally, at *GIMAP* and *RUNX1* gene loci, high level of active histone

mark (H3K27ac) forming super-enhancers were observed, indicating that chromatin at these gene loci was widely open and epigenetically activated. Importantly, the rate of chromatin looping was only slightly reduced after TAL1 depletion and was not completely lost. Together, although TAL1 bound to and increased the expression of group B genes, the expression of group B genes can still be maintained, in contrast to group A genes.

TAL1 and NOTCH1 coordinately regulate *GIMAP* genes

This result prompted us to hypothesize that other transcription factors, in addition TAL1, that are expressed in

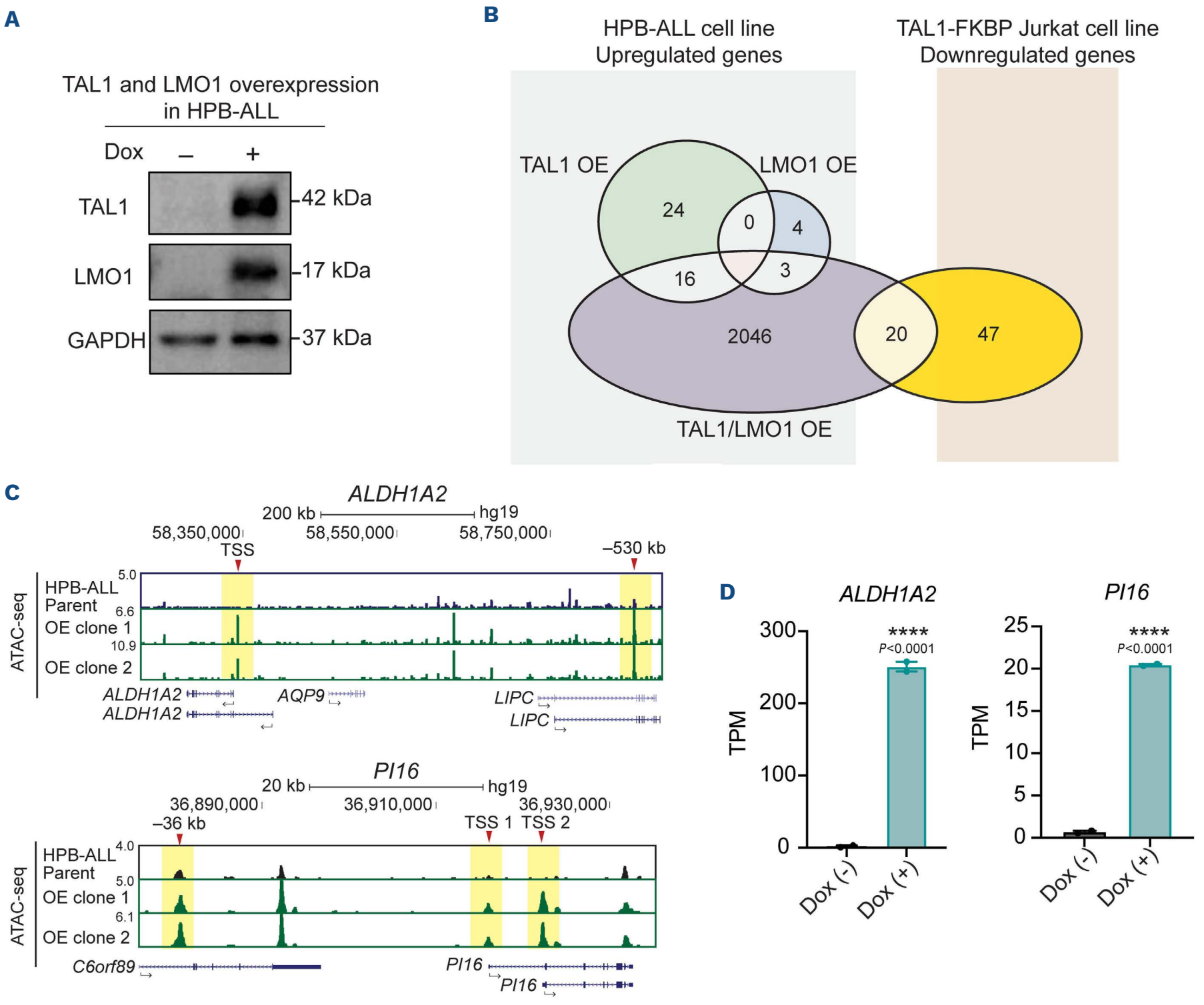


Figure 3. The TAL1 complex alters chromatin accessibility in T-cell acute lymphoblastic leukemia cells. (A) HPB-ALL cells were stably transduced with cDNA of the TAL1 and LMO1 coding regions with a doxycycline (Dox)-inducible system. Whole-cell lysates were collected after 48 hours of Dox treatment and subjected to immunoblot analysis using antibodies specific to TAL1, LMO1 and GAPDH (the loading control). The blots are representative of N=3. (B) Venn diagram analysis showing significantly upregulated (log2-fold change ≥0.7, *P<0.05*) genes between HPB-ALL cells after TAL1 was overexpressed (TAL1 OE, green circle), LMO1 was overexpressed (LMO1 OE, blue circle), and TAL1/LMO1 were overexpressed in combination (TAL1/LMO1 OE, purple circle) after 48 hours of Dox induction and significantly downregulated (log2-fold change ≤-0.3, *P<0.05*) genes in TAL1-FKBP12^{F36V} Jurkat cells after 48 hours of 1 μM dTAG treatment. (C) ATAC-sequencing tracks of the *ALDH1A2* (top) and *PI16* (bottom) loci in the basal-state HPB-ALL cell line (black) and TAL1/LMO1 overexpression in the HPB-ALL cells, displayed as 2 independent clones (green). Yellow highlighting indicates the genomic region of interest: where the TAL1 transcription factor binds in the baseline Jurkat cell line. For the *ALDH1A2* locus, the TSS of the shorter isoform and the -530 kb enhancer region are highlighted. For the *PI16* locus, the TSS of both isoforms and the -36 kb putative enhancer regions are highlighted. (D) *ALDH1A2* and *PI16* mRNA expression measured by RNA sequencing in Dox (-) and Dox (+) TAL1-/LMO1-overexpressing HPB-ALL cells. ****P<0.0001 by Student's *t* test compared to Dox (-) cells. TPM: transcripts per million.

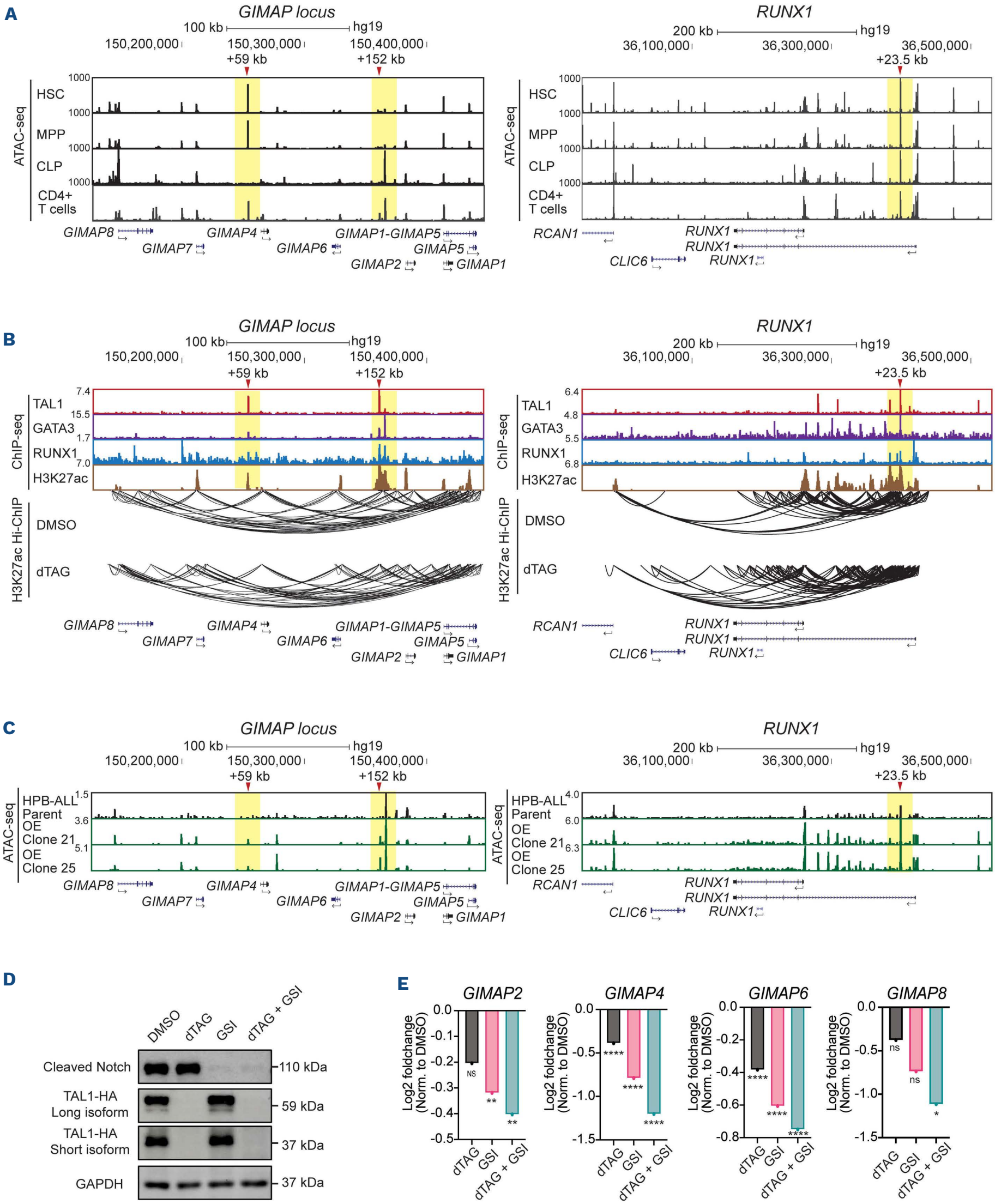


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Figure 4. TAL1 promotes the expression of a set of genes that are highly expressed in different T-cell acute lymphoblastic leukemia cell subgroups. (A) ATAC-sequencing tracks of hematopoietic stem cells (HSC), multipotent progenitors (MPP), common lymphoid progenitors (CLP) and CD4$^+$ T cells; the *GIMAP* family and *RUNX1* loci are magnified. The ATAC-sequencing dataset was previously reported.[33] (B) Chromatin immunoprecipitation (ChIP)-sequencing tracks of the *TAL1*, *GATA3*, and *RUNX1* transcription factors and activating histone mark *H3K27ac* (top) in the Jurkat cell line; the *GIMAP* family (left) and *RUNX1* (right) loci are magnified. The ChIP-sequencing dataset was previously reported.[12] *H3K27ac* Hi-ChIP was performed after dimethyl sulfoxide (DMSO) treatment and dTAG treatment to evaluate changes in active chromatin loop formation (bottom). (C) ATAC-sequencing tracks of the basal-state HPB-ALL cells (black) and TAL1/LMO1 overexpression in the HPB-ALL cells, displayed as 2 independent clones (green). For (A), (B) and (C), yellow highlighting indicates the genomic region of interest: where the TAL1 transcription factor binds in the baseline Jurkat cell line. For the *GIMAP* family locus, the +59 kb and +152 kb enhancer regions (with respect to *GIMAP7*) are highlighted. For the *RUNX1* locus, the +23.5 kb enhancer region is highlighted. (D) TAL1-FKBP12^{F36V} Jurkat cells were treated with either DMSO, dTAG, GSI or dTAG + GSI for 24 hours. Whole-cell lysates were collected and subjected to immunoblot analysis using antibodies specific for cleaved notch, HA-tag and GAPDH (the loading control). The blots are representative of N=3. (E) The mRNA expression of several GIMAP family genes (*GIMAP2*, *GIMAP4*, *GIMAP6*, and *GIMAP8*) was measured by RNA sequencing after dTAG, GSI or dTAG + GSI treatment in biological duplicates. Error bars: the mean ± standard error of the mean; NS: not significant; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 by Student's two-tailed *t* test compared to control cells.

T-ALL cells, may regulate group B gene expression. In this regard, the *GIMAP* gene locus had been demonstrated to be activated by NOTCH1 in T-ALL cells.[16,30] Hence, we focused on this locus as a representative example.

In order to analyze the contribution of each factor, we inhibited NOTCH1 activity using a γ-secretase inhibitor (GSI) with or without TAL1 depletion. We confirmed the status of TAL1 expression and the activated form of NOTCH1 (Figure 4D). In this setting, we found that inhibition of either TAL1 (by dTAG-13) or NOTCH1 (by GSI) resulted in the partial downregulation of multiple *GIMAP* genes while combination treatment further downregulated their expression (Figure 4E). This result was independently validated by qRT-PCR (*Online Supplementary Figure S4F*). Correspondingly, we performed a 4C-sequencing analysis for different treatment conditions, and the results demonstrated that inhibition of either TAL1 or NOTCH1 alone was not sufficient to reduce chromatin–chromatin interactions, while dual inhibition resulted in a marked reduction (*Online Supplementary Figure S4G*). Hence, in this instance, both TAL1 and NOTCH1 independently contributed to the expression of *GIMAP* genes and did not require each other. This mechanism is ideal for sustaining the activation status after the loss of one factor and to prevent the complete loss of targets that are required for T-ALL cells. Of note, the majority of group B genes, including *RUNX1*, *MYB* and *ARID5B*, were not downregulated by either GSI alone or in combination treatment with dTAG, suggesting that NOTCH1 was not the only transcription factor that sustained the expression of group B genes and that other factors may be involved.

Transcriptional activity of GATA3 and RUNX1 depends on TAL1

TAL1 regulates downstream targets in conjunction with its regulatory partners, RUNX1 and GATA3, which forms the CRC.[12] Independent validation via shRNA knockdown of *GATA3* or *RUNX1* resulted in the downregulation of many groups A and B genes (Figure 5A). This result indicates that GATA3 and RUNX1 also positively regulate the same target genes in the presence of TAL1, thus showing coordination among three factors. However, it remains unclear whether their functions are redundant or whether their activities depend on TAL1.

In order to address these possibilities, we performed a cut-and-run assay with GATA3 and RUNX1 proteins before and after TAL1 depletion. Interestingly, GATA3 and RUNX1 still bound to many of the group A and B gene loci (Figure 5B), even if TAL1 was depleted and thus target mRNA expression was reduced. The binding of GATA3 to several gene loci was validated by ChIP-qPCR (*Online Supplementary Figure S5A*). This suggests that DNA bindings of GATA3 or RUNX1 alone is not sufficient to induce the transcription of TAL1 targets. In contrast, knockdown of GATA3 or RUNX1 in the presence of TAL1 resulted in downregulation of the same targets (Figure 5A). Hence, the transcriptional activity of GATA3 and RUNX1 depends on the presence of TAL1.

TAL1 induces lethality via the inhibition of the NOTCH1 pathway in TAL1-negative/TLX3-positive T-cell acute lymphoblastic leukemia cells

Previous studies have shown that TAL1 is usually not expressed together with other Type A abnormality (i.e., TLX1, TLX3, and HOXA), showing an exclusive relationship.[7,17,19] This outcome suggests either functional redundancy or potential synthetic lethality when these genes are expressed together. In order to gain an understanding of the mechanism, we utilized HPB-ALL cells, which belong to the TLX3 subgroup and performed functional and gene expression analyses after overexpressing TAL1 and/or LMO1, as described above (Figure 3A). Interestingly, overexpression of either TAL1 or LMO1 did not affect cell proliferation, but their combined overexpression significantly reduced growth rates (Figure 6A). The same result was confirmed in two other TAL1-negative cell lines, DND-41 and KOPT-K1 (*Online Supplementary Figure S6A*). DND-41 has been verified to expresses TLX3. KOPT-K1 has a high LMO2 baseline expression and thus overexpression of TAL1 alone could produce a significant arrest in cell proliferation. A cell cycle analysis using propidium iodide staining revealed profound G1-phase arrest (Figure 6B), while Annexin-V staining con-

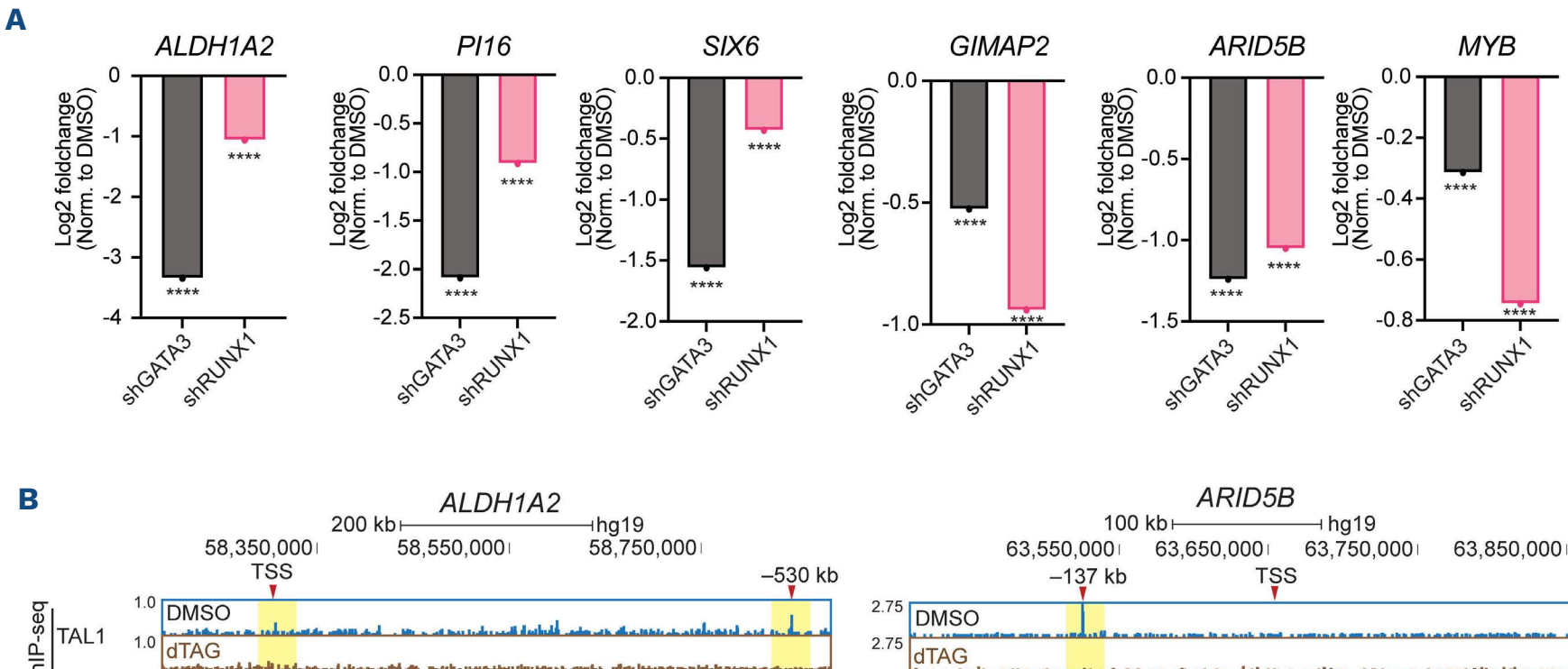
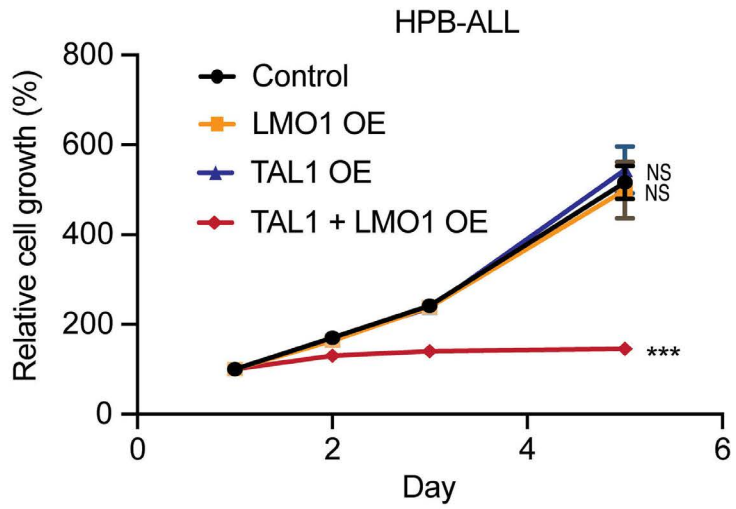
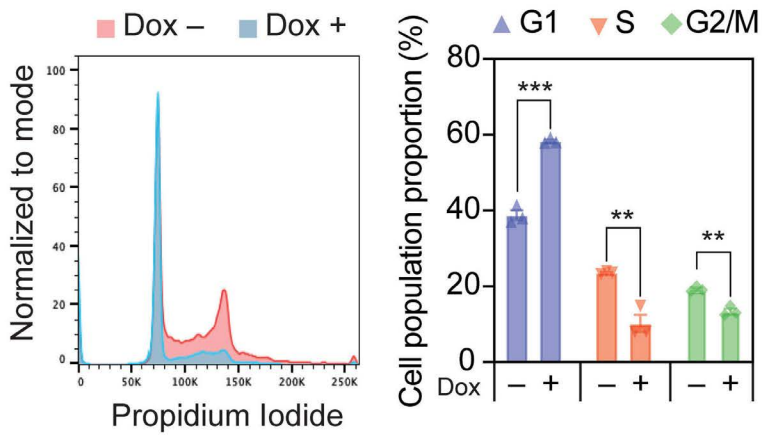


Figure 5. Transcriptional activity of GATA3 and RUNX1 depends on TAL1. (A) mRNA expression of *ALDH1A2*, *PI16*, *SIX6*, *GIMAP2*, *ARID5B* and *MYB* as measured by RNA sequencing after 72 hours of small hairpin RNA (shRNA) genetic knockdown of *GATA3* (black bar) and *RUNX1* (pink bar) in biological duplicates. ****P<0.001 by Student's two-tailed t test compared to control cells. (B) Chromatin immunoprecipitation (ChIP)-sequencing and cut-and-run tracks for *ALDH1A2*, *PI16*, *MYCN*, *ARID5B*, *MYB* and *RUNX1* loci. A ChIP-sequencing assay was performed for TAL1-FKBP12^{F36V} Jurkat cells treated with dimethyl sulfoxide (DMSO) or dTAG for 24 hours, using antibodies specific to TAL1. A cut-and-run assay was performed for TAL1-FKBP12^{F36V} Jurkat cells treated with DMSO or dTAG for 24 hours, using antibodies specific to GATA3 and RUNX1. Yellow highlighting indicates the genomic region of interest: where the TAL1 transcription factor binds in the baseline Jurkat cell line. TAL1 binding was abolished after 1 μM dTAG treatment.

A



B



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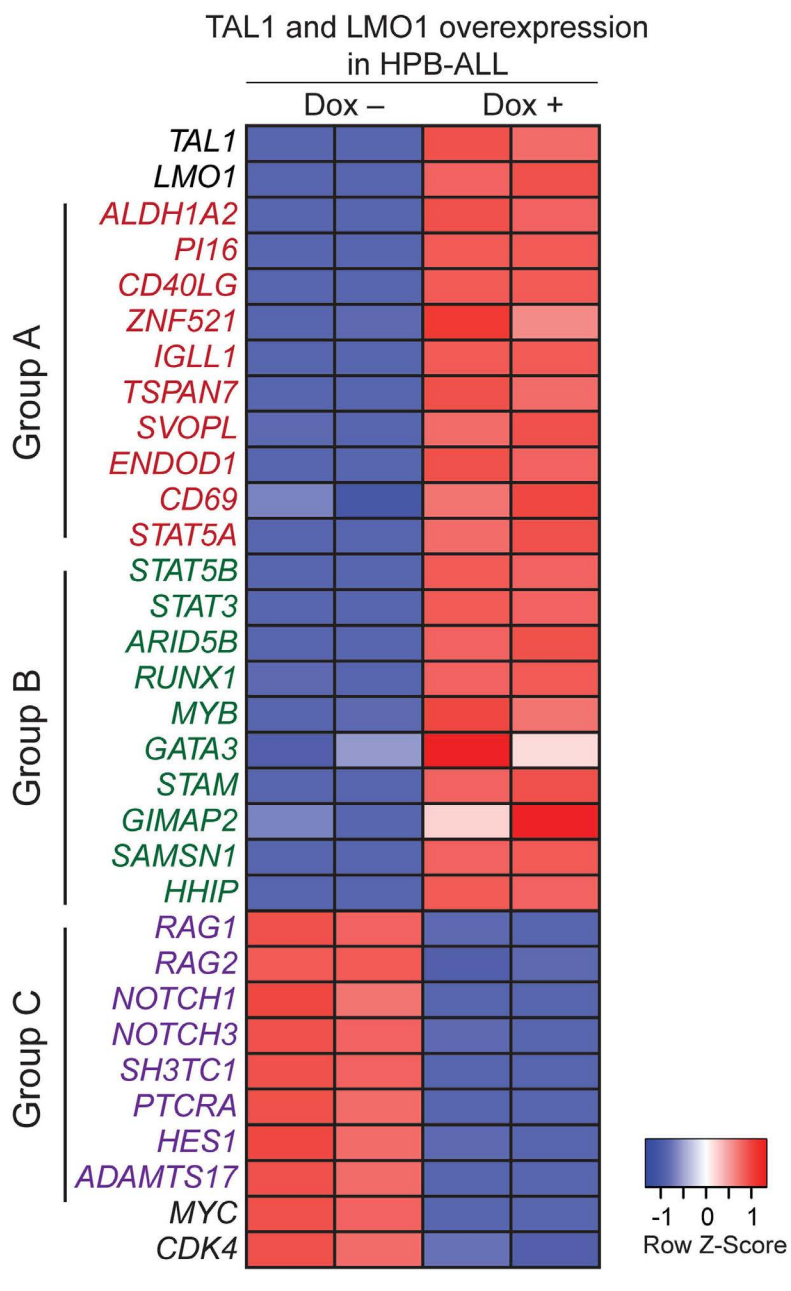

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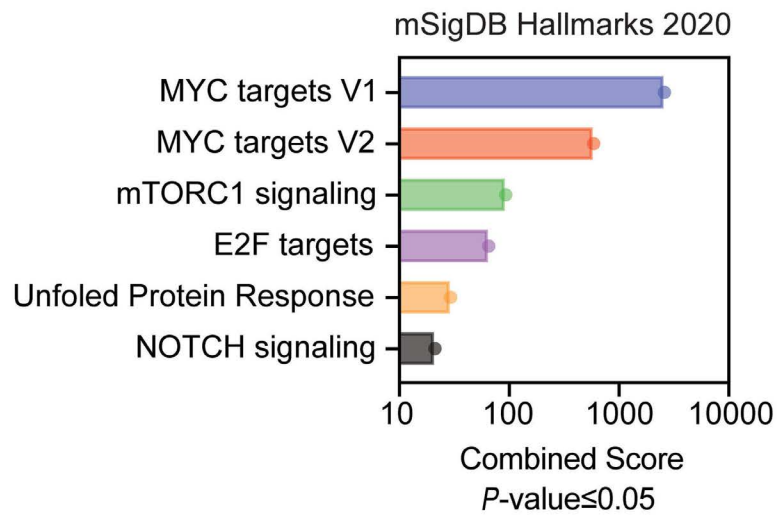

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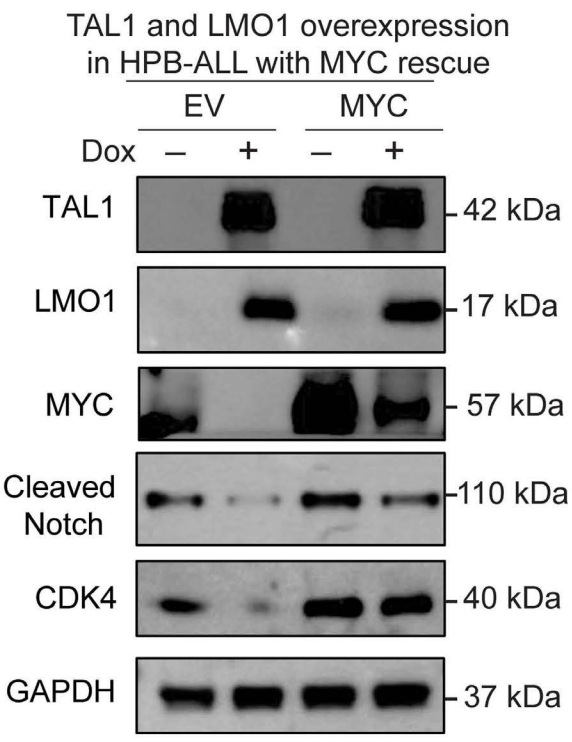

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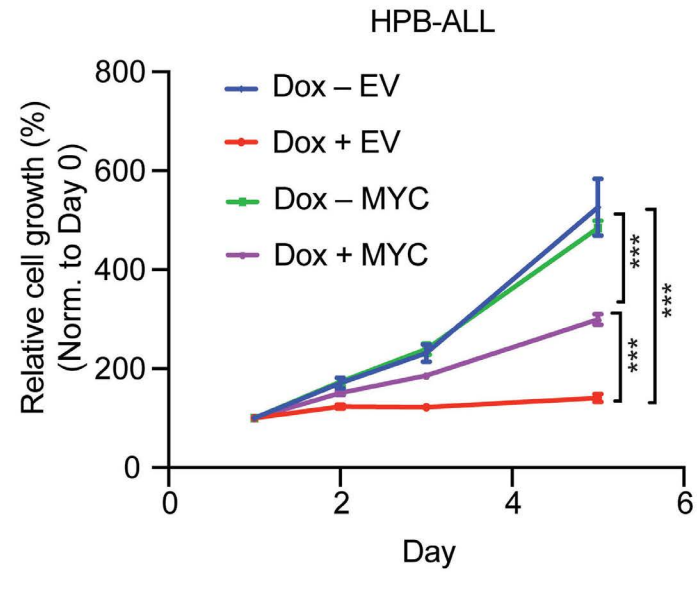

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Figure 6. TAL1 induces lethality via the inhibition of the NOTCH1 pathway in TAL1-negative/TLX3-positive T-cell acute lymphoblastic leukemia cells. (A) Relative growth (%) of HPB-ALL cells overexpressing TAL1, LMO1 or TAL/LMO1 measured 1, 2, 3 and 5 days after doxycycline (Dox) treatment using a Cell TiterGlo luminescence assay kit; N=3. Error bars: the mean ± standard error of the mean (SEM); NS: not significant; ***P<0.001 by Student's two-tailed t test compared to Dox (-) control cells. (B) Cell cycle analysis using propidium iodide performed with Dox (-) *versus* Dox (+) TAL1-/LMO1-overexpressing HPB-ALL cells. Representative flow cytometry plot (left) and cell population proportions (%) at the corresponding cell cycle stages (right); N=3. Error bars: the mean ± SEM; **P<0.01, ***P<0.001 by Student's two-tailed t test compared to Dox (-) cells. (C) Heatmaps representing the mRNA expression of 10 representative significantly upregulated group A and B genes and 10 representative significantly downregulated group C genes after TAL1/LMO1 are overexpressed, as measured by RNA sequencing. The heatmap data are based on biological duplicates. (D) Gene Ontology analysis (mSigDB Hallmarks 2020) performed with significantly downregulated genes after TAL1/LMO1 was overexpressed in HPB-ALL cells. Significantly enriched pathways ($P<0.05$) are displayed in a bar chart in descending order of the combined scores. (E) HPB-ALL cells after Dox-induced TAL1/LMO1 overexpression were stably transduced with either an empty vector (EV) or the cDNA carrying the *MYC* gene. Whole-cell lysates were collected 48 hours after Dox treatment to induce TAL1/LMO1 overexpression and then subjected to immunoblot analysis using antibodies specific for TAL1, LMO1, MYC, cleaved NOTCH, CDK4 and GAPDH (loading control). The blots are representative of N=3. (F) Relative growth (%) of HPB-ALL cells after Dox-induced TAL1/LMO1 overexpression and transduction with either an EV or *MYC* plasmid. Cell growth was measured 1, 2, 3 and 5 days after Dox induction using a CellTiter Glo luminescence assay kit; ***P<0.001 by Student's two-tailed t test compared to Dox (-) cells.

firmed that the cell proliferation was not reduced due to the induction of apoptosis (*Online Supplementary Figure S6B*). These results indicated that TAL1 exhibits lethality in the context of TAL1-negative/TLX3-positive T-ALL when overexpressed with LMO1/LMO2.

Next, we analyzed RNA-sequencing data to identify the gene expression changes underlying this phenotypic change. As described earlier, many group A and B genes (e.g., *ALDH1A2* and *GIMAP*) were significantly upregulated after TAL1 and LMO1 were overexpressed, while group C gene expression (e.g., RAG1 and RAG2) was downregulated (Figures 3B and 6C; *Online Supplementary Table S9*). These results indicate that these genes were regulated by TAL1 in TLX3-positive T-ALL, regardless of whether the cells underwent proliferation arrest.

Importantly, we observed that many genes involved in the NOTCH pathway, such as *NOTCH1*, *NOTCH3*, *PTCRA*, *HES1* and *SH3TC1*, was significantly downregulated after TAL1/LMO1 were overexpressed (Figure 6C, D; *Online Supplementary Table S9*). The expression of direct targets of NOTCH1, including *MYC* and *CDK4*, which promote cell proliferation, was also significantly downregulated. This result was validated in DND-41 and KOPT-K1 cells (Figure 6E; *Online Supplementary Figure S6C*). Additionally, stable overexpression of MYC partially restored the cell proliferation rate as well as the CDK4 expression level in HPB-ALL cells after TAL1 overexpression (Figure 6E, F). Cleaved NOTCH1 protein was also slightly upregulated by MYC overexpression due to transcriptional upregulation of NOTCH1 mRNA (*Online Supplementary Figure S6D*). These results indicated that the growth-suppressing effect induced by TAL1/LMO1 overexpression was due to the downregulation of NOTCH1-MYC pathway while they can induce their own targets (i.e., group A and B genes). These effects were marked contrast to that in TAL1-positive T-ALL cells, in which TAL1 collaborated with NOTCH1. Moreover, forced expression of TAL1 and LMO1 affected the expression of differentiation markers in HPB-ALL cells, as demonstrated by downregulation of CD4, CD3 and CD1 and upregulation of CD8a (*Online Supplementary Figure S6E*). Although the resulted immunophenotype does not represent a typical differentiation stage of T cells, our data indicates that TAL1 overexpression affects cell differentiation status.

Discussion

Since the discovery of TAL1 from chromosomal translocation in T-ALL cells,[31] its roles in T-cell leukemogenesis have been extensively studied. Early studies with mouse models demonstrated that TAL1 exerted oncogenic effects via the inhibition of E-proteins,[8,9] as confirmed with our study on group C genes, which included many E-protein targets. This mechanism primarily results in differentiation arrest of developing thymocytes, which may eventually lead to the acquisition of additional abnormalities such as activating mutations of *NOTCH1*. However, several questions remain unanswered.

In this study, we reviewed the transcription targets driven by TAL1 using a protein degradation system, revisiting known mechanisms and highlighting novel mechanisms. First, we found that two groups of genes are positively regulated by TAL1, and each group is characterized by unique features. Group A genes are highly dependent on the activity of TAL1, and many of them are not expressed in normal T cells or TAL1-negative T-ALL cells, thus representing the oncogenic signature of TAL1. Importantly, closed chromatin at many of Group A loci were opened by overexpression of TAL1 and LMO1. Previously, Ferrando's group implicated GATA3 as a pioneer factor in T-ALL.[32] Thus, one possible hypothesis is that when GATA3 forms a complex with TAL1 via LMO1, it is recruited to target gene loci and changes the chromatin status to ectopically induces gene expression.

In contrast, group B genes represent a more general gene expression signature of T-ALL and are not solely dependent on TAL1. For example, the *GIMAP* gene locus shows densely-connected chromatin-chromatin interactions that are also regulated by NOTCH1. Inhibition of TAL1 or NOTCH1

alone did not fully block the expression of group B genes, but the combined inhibition of these two factors strongly downregulated their expression. TAL1 and NOTCH1 equally contributed to the expression of *GIMAP* genes. Hence, group B genes demonstrate the collaborative roles of TAL1 with other transcription factors, which is likely required to sustain the gene expression program in the maintenance or progression of T-ALL cells.

Our study also suggested a functional relationship among CRC members. When TAL1 was rapidly depleted, binding of GATA3 and RUNX1 was mostly unchanged at target gene loci, while knockdown of each of them led to the downregulation of target genes. These results indicate that although CRC members share target genes and positively regulate them, GATA3 and RUNX1 activities may depend on TAL1, and thus cannot replace TAL1. Thus, TAL1 plays a dominant role among CRC members, which is different from the relationship between TAL1 and NOTCH1.

Another long-standing question about T-ALL is why the expression of Type A abnormalities (e.g., TAL1 and TLX3) is exclusively observed (see introduction for the definition). Our study demonstrated that the induction of TAL1 and its downstream targets is potentially lethal in the presence of TLX3. TAL1 overexpression in TLX3-positive T-ALL cells induced group A genes but inhibited NOTCH1-MYC pathway, although TAL1 collaborated with NOTCH1 in TAL1-positive T-ALL cells. These results also suggest that TLX3-positive cells are highly dependent on the NOTCH1/MYC pathway. Altogether, our study indicates that the roles of TAL1 and NOTCH1 depend on the cellular context.

Disclosures

No conflicts of interest to disclose.

Contributions

JZLO and LW performed the experiments. TKT conducted the bioinformatics analyses. SHT and TS supervised the study. JZLO and TS wrote the manuscript.

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Data-sharing statement

All sequencing dataset have been deposited in the Gene Expression Omnibus database under the accession number GSE225941.

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