

The hematopoietic stem cell MYB enhancer is essential and recurrently amplified during T-cell leukemogenesis

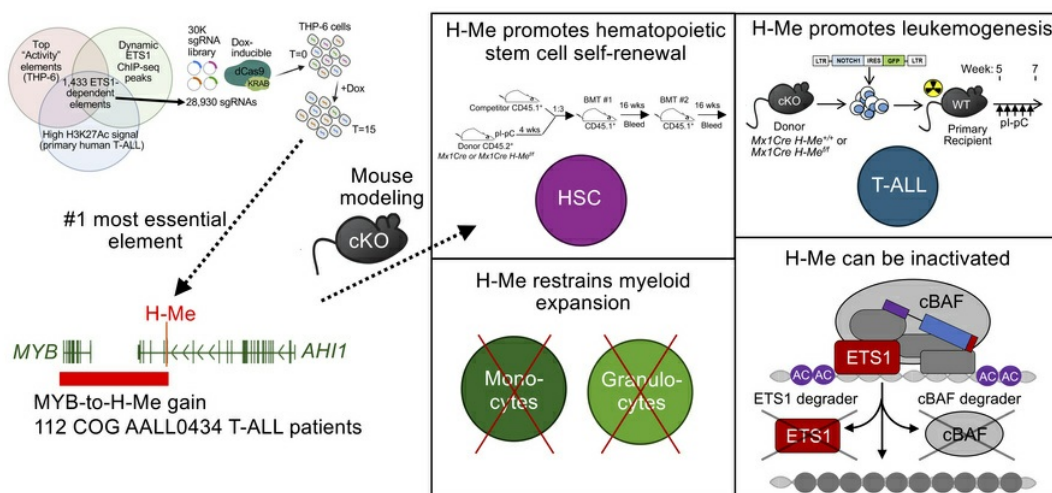
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Graphical abstract

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Title

The hematopoietic stem cell MYB enhancer is essential and recurrently amplified during T-cell leukemogenesis

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Abstract

There is an urgent need to find targeted agents for T-cell acute lymphoblastic leukemia (T-ALL). *NOTCH1* is the most frequently mutated oncogene in T-ALL, but clinical trials showed that pan-Notch inhibitors caused dose-limiting toxicities. Thus, we shifted our focus to ETS1, which is one of the transcription factors that most frequently co-bind Notch-occupied regulatory elements in the T-ALL context. To identify the most essential enhancers, we performed a genome-wide CRISPR interference screen of the strongest ETS1-dependent regulatory elements. The #1-ranked element is located in an intron of *AHL1* that interacts with the MYB promoter and is amplified with *MYB* in ~8.5% of T-ALL patients. Using mouse models, we showed that this enhancer promotes self-renewal of hematopoietic stem cells and T-cell leukemogenesis, maintains early T-cell precursors, and restrains myeloid expansion with aging. We named this enhancer the hematopoietic stem cell MYB enhancer (H-Me). The H-Me shows limited activity and function in committed T-cell progenitors but is accessed during leukemogenesis. In one T-ALL context, ETS1 binds the ETS motif in the H-Me to recruit cBAF to promote chromatin accessibility and activation. ETS1 or cBAF degraders impaired H-Me function. Thus, we identified a targetable stem cell element that is co-opted for T-cell transformation.

Introduction

The discovery of Notch-activated tumors, including ~60% of cases of T-ALL, spurred excitement to clinically test pan-Notch inhibitors, such as gamma-secretase inhibitors (GSIs), for the treatment of human cancers (1-3). Unfortunately, early clinical studies reported excessive toxicities with continuous GSI dosing (4-6). GSI toxicities result from abrogation of Notch signals crucial for normal homeostasis, particularly in the intestine (7-9). Proteomic, transcriptional genomic, and biochemical studies show that the core Notch/RBPJ complex can interact with context-dependent transcriptional regulators that co-bind its response elements (10-21). Other regulators also bind near Notch at these elements (15, 22-25). If T-lineage regulators are hijacked to drive Notch-activated T-ALL, then inhibiting them might oppose oncogenic Notch signals while circumventing the toxicities of systemic Notch inhibition. Among these factors, we prioritize ETS1 (26-28). At Notch-bound regulatory elements, we and others showed that ETS is the #1 or #2 ranked non-RBPJ motif in T-ALL in both frequency and statistical significance (21, 29). Our groups also showed that ETS1 overlaps strikingly high% (76-82%) of Notch/RBPJ-occupied elements (21, 30), more than any other transcription factor tested. Further, an ETS1 inhibitor is predicted to be safer than GSI given that postnatal *ETS1* expression is highest in the thymus and other lymphoid sites relative to other tissues (31) (Figure S1A). In contrast, Notch receptor expression shows low tissue specificity (Figure S1B-E). Accordingly, we previously showed that Ets1 deprivation in mice suppresses Notch-induced target genes, thymopoiesis and leukemogenesis like GSI but had no significant effects on overall health (21).

Because of above reasons, we shifted our focus from Notch to ETS1. Here, we sought to identify the ETS1-dependent network of essential regulatory elements through a genome-wide CRISPR interference (CRISPRi) screen. Our study identified a long-range Notch and ETS1-bound MYB enhancer as the top-ranked essential element; established the important physiological role of this

element in hematopoietic stem cells and early T-cell progenitors (ETPs); determined the essential oncogenic role of this element for T-cell leukemogenesis; showed the dependence of this element on ETS1 for chromatin accessibility and activity in one T-ALL context; and highlighted SWI/SNF and ETS1 degradation but not Notch inhibition as approaches that might target this element among multiple other effects.

Results

Genome-wide CRISPR-interference essentiality screen of ETS1-dependent regulatory elements nominates the +140kb MYB enhancer as the most essential element

To assess downstream mechanisms of ETS1, we aimed to characterize the ETS1 “essential regulome” through a high throughput DOX-inducible CRISPRi essentiality screen. CRISPRi is the preferred screening methodology to identify essential regulatory elements (32). We first used our previously published H3K27Ac ChIP-seq and ATAC-seq datasets in THP-6 cells (21) and an activity model (33) to predict highly active T-ALL regulatory elements (Figure 1A). Next, we intersected these elements with “dynamic” ETS1 peaks, which we defined as ETS1 peaks that give decreased H3K27Ac and ETS1 ChIP-seq signal upon *ETS1* knockdown in THP-6 cells (21). Lastly, we intersected these elements with strong H3K27Ac ChIP-seq peaks in primary T-ALL samples (Blueprint) to identify 1,433 candidate ETS1-dependent regulatory elements. We then transduced THP-6 cells with a custom ~30K sgRNA library targeting these elements (mean 20 sgRNAs/kb). MAGeCK analysis on paired T0 and T15 datasets (N=3) identified 10 essential elements that were as negatively selected as 39 positive control promoters of pan-essential genes (Figure 1B). The #2 and #3-ranked elements were the previously reported Notch-dependent *MYC* enhancer (“N-Me”) (34, 35) and the *BCL11B* enhancer (“ThymoD”) respectively (36, 37), thus validating our screen. WGS analysis of THP-6 cells revealed a structural variant involving ThymoD and the *NKX2-5* oncogene (Figure S2A), similar to previous reports (38-40), that led to *NKX2-5* overexpression (Figure S2B). Further WGS and STR analysis showed that THP-6 cells and CEM-CCRF cells are genetically divergent with a common origin. The #1-ranked element was an enhancer that lies +140kb from the *MYB* TSS and within Intron 23 of *AHI1*. *MYB* is a highly expressed oncogene across all T-ALL subgroups (41). This element was previously identified as a highly conserved enhancer (“MYB-enh-3”) in erythroid leukemia cells that shows correlation between *MYB* expression and chromatin accessibility in human blood cells (42). This

enhancer is an ETS1-dependent super-enhancer and makes long-range contacts with *MYB* in T-ALL cells (Figure S3A-B) (21).

The +140kb element is a hematopoietic stem cell (HSC) MYB enhancer (H-Me) that appears relatively quiescent in the developmental stages from which T-ALL initiates.

Publicly available ATAC-seq data in sorted murine cells show that accessibility of the +140kb element is high in long term hematopoietic stem cells (LT-HSCs) but quickly begins to fall in short-term HSCs and reduces to baseline at the DN4 stage (Figure 1C). Thus, we presumptively named this element "H-Me" for hematopoietic stem cell MYB enhancer (H-Me). In human cells, the chromatin is accessible in hematopoietic progenitor cells through DN3/DN4 cells and then goes down to baseline at the ISP stage (Figure 1D). However, despite being accessible, H3K27ac profiling shows that enhancer activity drops considerably at the DN3/DN4 stage (Figure 1E). Histone profiling of human DP thymocytes suggests that the H-Me does not acquire H3K27me3 upon further differentiation (Figure 1F). These data suggest that compared to stem cells, the H-Me is less accessible or active in immature T cells (DN3-to-ISPs) but is "primed" for reactivation. Thus, T-ALL cells do not necessarily use active enhancers native to their developmental stage during oncogenesis.

The H-Me restricts HSC numbers and maintains ETPs, but has limited function after T-cell commitment

To investigate the function of the H-Me, we generated H-Me conditional knockout mice (*H-Me^{fl/fl}*). These mice contain loxP sites flanking the H-Me element, a 369-bp nucleosome-free region homologous to the human H-Me (Figure 2A). We crossed these mice with *Mx1Cre* transgenic mice (48) to generate *Mx1Cre H-Me^{fl/fl}* mice and littermate *Mx1Cre controls*. To delete the H-Me in HSCs, we injected 4-6-week-old mice with pl-pC. At 6 weeks, we observed efficient H-Me deletion (Figure S4A) and ~61% reduction in *Myb* expression in sorted HSCs (Figure 2B). In

contrast, we saw no statistically significant effects on *Ahi1* expression or in hematopoietic stem and progenitor cells (LSK cells, Figure S4B). H-Me deletion led to a ~2.1-fold increase in long-term HSC numbers (Figure 2C-D).

In contrast to HSCs, no effects were observed in LSKs (Figure 2E), MPP/ST-HSCs (Figure 2F), or lymphoid-primed multipotent progenitor cells (LMPP, Figure 2G). In the thymus, we observed a ~5.5-fold reduction in early T-cell precursors (ETPs, Figure 2H-I); no effects on DN2a (Figure 2J) and DN2b (Figure 2K) cells; ~2-fold reduction in T-committed DN3 (Figure 2L) and DN4 (Figure 2M) cells; and no effect on more differentiated thymocytes (Figure S4C-G) or total thymocytes (Figure S4H). H-Me deletion did not affect numbers of bone marrow myeloid/erythroid progenitors; total bone marrow cells; peripheral myeloid and lymphoid cells; or peripheral blood counts (Figure S4I-W). These data suggest that the H-Me has important roles in restricting HSC population growth and in maintaining ETPs. However, there are relatively modest effects, if any, on other cell types.

Next, we crossed *H-Me^{ff}* mice with *Il7rCre* transgenic mice (49), which is lymphoid-specific, to generate *Il7rCre H-Me^{ff}* mice and *Il7rCre* littermate controls. We observed efficient deletion of the H-Me in the thymus (Figure S5A). *Myb* expression decreased ~50% in ETPs but was restored in subsequent stages (Figure 2N). *Ahi1* expression was not affected (Figure S5B). Consistent with the effects on *Myb* expression, H-Me deletion reduced ETPs by ~3.4-fold (Figure S5C-D) but had no statistically significant effect on subsequent stages (Figure S5E-O). These data suggest that the H-Me has ETP stage-restricted effects in maintaining cell number and *Myb* expression during thymopoiesis.

Under stress conditions, the H-Me is important for long-term HSC self-renewal but not for primary engraftment

We wondered whether the increased numbers of phenotypically defined HSCs in *Mx1Cre H-Me^{Δ/Δ}* mice might herald eventual depletion of LT-HSC capacity as a consequence of non-self-renewing divisions. To test this possibility, we performed serial competitive bone marrow transplant (BMT, Figure 3A). We observed modest, if any, change in primary reconstitution of peripheral blood compartments after the first BMT (Figure 3B-E). However, after the second BMT, H-Me deletion gave significant loss in reconstitution of peripheral blood compartments (Figure 3F-I). Consistently, we observed no effect of H-Me deletion on reconstitution of the HSC or MPP compartments after the first BMT (Figure S6A-C) but saw significantly impaired HSC reconstitution after the second BMT (Figure 3J-K). Total MPP was not affected (Figure 3L). Similarly, in the thymus, *H-Me* deletion modestly reduced reconstitution after the first BMT (Figure S6D), but strongly impaired reconstitution after the second transplant (Figure S6E). These data suggest that under stress conditions, the H-Me is important for long-term HSC self-renewal but has relatively modest, if any, effects on primary engraftment of stem cell and thymopoietic compartments.

The floxed H-Me is a hypomorphic enhancer

The introduction of a LoxP sites might affect expression of the three genes in the TAD (*Hbs1l*, *Myb*, and *Ahi1*). To address this, we measured *Hbs1l*, *Myb*, and *Ahi1* expression in HSCs and ETPs from 5-8-week-old wildtype control and *H-Me^{fl/fl}* mice. *H-Me^{fl/fl}* HSCs showed reduced *Myb* expression compared to controls (Figure S7A). *Ahi1* and *Hbs1l* were modestly or not significantly affected (Figure S7B-C). *H-Me^{fl/fl}* ETPs showed reduced *Myb* expression compared to controls (Figure S7D). *Ahi1* and *Hbs1l* were not significantly affected (Figure S7E-F). We also observed a non-significant increase in HSCs (Figure S7G-H) and a decrease in ETPs (Figure S7I-J) in *H-Me^{fl/fl}* mice. The effect sizes of the floxed H-Me on *Myb* expression and HSC/ETP numbers were smaller than the effect sizes of H-Me deletion (Figure 2B, 2D, 2I, S5B, S5D). Taken together, these data suggest that the floxed H-Me is a hypomorphic *Myb* enhancer. These data show a

limitation of the mouse model and raise the importance of transplantation experiments for ruling out cell non-autonomous effects.

The H-Me limits myeloid expansion and HSC loss with aging

To understand possible toxicities of systemic H-Me inhibition, we compared H3K27ac signals in a variety of tissues in ENCODE (Figure S8A). H3K27ac signals at the H-Me were relatively strong in T-ALL cell lines and primary tumors, moderate in erythroid/B-cell derived cells and undetectable in non-blood cells. Based on these profiles, we predicted that systemic H-Me inactivation would be tolerable and avoid the toxicities of Notch inhibition. To test this, we bred *H-Me^{ff}* mice with *Rosa26CreER^{T2}* mice. The progeny spontaneously recombined the floxed H-Me and were intercrossed to generate germline *H-Me^{-/-}* mice. Next, we observed H-Me-deficient and littermate control mice from birth to 12 months of age. Initially, these mice showed no differences in weight up to 6 months of age. Afterwards, H-Me-deficient mice showed 6-10% reductions in weight that were stable over several months (Figure S8B). Importantly, overall survival was not affected (Figure S8C). Since our serial transplant studies revealed the importance of the H-Me for long-term HSC self-renewal, we wondered whether H-Me inactivation might cause long-term hematopoietic toxicity. At 8-months of age, peripheral blood analysis showed no differences (Figure 4A-E). However, at 12-months of age, WBC, neutrophil, and monocyte counts were increased 1.3-fold, 1.3-fold, and 2.1-fold respectively while platelet counts were mildly decreased (Figure 4F-J). In the BM, long-term HSCs were depleted 7.2-fold while MPP/ST-HSCs were depleted 2.8-fold (Figure 4K-O). In the thymus, ETPs were severely depleted 9.1-fold (Figure 4P-Q) while more differentiated populations and total thymocytes decreased by 1.9-8.6-fold (Figure 4R-U) and 2.2-fold (Figure 4V) respectively. These data suggest that the H-Me is important for maintaining HSCs and thymopoiesis and restraining myeloid cell expansion with aging.

Effects of H-Me deletion on HSPCs at steady state

To examine the cell cycle status of *H-Me*^{-/-} HSPCs as compared to wildtype at steady state, 10-13-week-old mice were injected with EdU followed by a 3-day maintenance period with EdU-containing water. We chose the 10-13-week-old age range in order to reliably analyze adult LT-HSCs at steady state. No difference was observed between groups with respect to EdU uptake in the LSK or LT-HSC populations (Figure S9A-B). Further, Ki67 staining showed no differences in LSK or LT-HSC cells in the G0, G1, or S-G2-M phases of the cell cycle (Figure S9C-D). These data suggest that cell cycle dynamics were unchanged in adult *H-Me*^{-/-} HSPCs as compared to *H-Me*^{+/+} at steady state.

To determine if H-Me deficiency leads to changes in the progenitor cell populations at steady state, we enumerated HSCs and MPPs (50). This staining protocol fractionates MPPs into MPP1 (CD229⁻CD244⁻; with balanced lymphoid and myeloid potential, MPP2 (CD229⁺CD244⁻), MPP3 (CD229⁺CD244⁺; a myeloid-biased subpopulation), and a poorly characterized MPP other population (Figure S10A) (51). At 10-13 weeks of age, there were no differences in the overall cellularity of the bone marrow (Figure S10B). However, there was a non-significant 1.9-fold increase in HSCs (Figure S10C), which aligns with the HSC expansion seen at an earlier time point (Figure 2D). We did not observe any differences between control and H-Me knockout mice in the total MPP, LSK, and individual MPP populations (Figure S10D-H). However, we observed a trend towards increased cell number and relative expansion of the myeloid-biased MPP3 population compared to other MPPs (Figure S10H-I). These data are consistent with the eventual myeloid expansion seen in aged H-Me-deficient mice (Figure 4F-H). These data suggest that at steady state, the HSC expansion seen acutely after H-Me deletion has weakened and there are hints of the myeloid bias seen later in life.

Effects of H-Me deletion on intestinal morphology

Given the weight loss of H-Me-deficient mice over time and given the importance of *Myb* and *Ets1* for intestinal homeostasis (21, 52-56), we compared intestinal tissue in the above 10-13-week-old H-Me deficient mice compared to *H-Me*^{+/+} control mice. Overall morphology of the intestinal tissue was not changed as visualized by H&E staining (Figure S11A) or PAS/AB staining of mucous cells (Figure S11B).

The H-Me is important for murine Notch-activated T-ALL leukemogenesis

Since the H-Me seemed less accessible, active, and functional in early T-committed thymocytes compared to more primitive ancestors, we wondered whether H-Me activity is increased during transformation to drive T-ALL initiation. To test this possibility, we used a well-established murine model of Notch-induced T-ALL (57, 58). We transduced bone marrow stem and progenitor cells from *H-Me*^{-/-} mice with an activated *Notch1* allele ($\Delta E/Notch1$) (59, 60) and transplanted these cells into recipient mice to generate T-ALL (Figure 5A). H-Me-deleted mice generated ~38-fold fewer peripheral T-ALL blasts (Figure 5B-C) and showed significantly prolonged survival (Figure 5D) relative to littermate control mice.

To further test whether the H-Me is important for T-ALL leukemogenesis, we transduced bone marrow stem and progenitor cells from *Mx1Cre H-Me*^{fl/fl} mice with $\Delta E/Notch1$, transplanted these cells into recipient mice, and injected pl-pC at Week 5 (Figure 5E). H-Me deletion induced a ~105-fold reduction in peripheral T-ALL blasts (Figure 5F-G) and significantly prolonged survival (Figure 5H) relative to *Mx1Cre* littermate control mice. Further, H-Me deletion significantly impaired leukemogenesis in a second Notch-activated T-ALL mouse model (*Lmo2-tg*) that is induced by an *LMO2* transgene in which activating *Notch1* mutations are acquired (Figure 5I-J) (61). These data suggest that the H-Me is important for murine Notch-activated T-ALL leukemogenesis.

The H-Me is frequently co-amplified with *MYB* in human T-ALL patients

Previous studies showed that the *MYB-AHI1* region is a frequent target of tandem duplications in ~8%-40% of pediatric T-ALL patient samples (62-66). Thus, we wondered whether there is positive selection for including the H-Me in these duplications in human T-ALL patients. Consistently, WGS analysis in 1,309 T-ALL samples from the Children's Oncology Group clinical trial AALL0434 (40) showed that 8.8% of patients had *MYB* amplifications, of which 97% involved the H-Me (Figure 6A). The genomic segment extending from the *MYB* gene to the H-Me appears to be a minimally amplified region. Further, ~50% of cell lines were found to carry *MYB* duplications, such as CEM cells (63). Consistently, WGS analysis showed that THP-6 cells contain *MYB*/H-ME duplications and is thus a clinically relevant model (Figure 6B). WGS confirmed duplication in CEM cells but did not detect it in other T-ALL cell lines used in this study (see below) (Figure 6C). These data support the importance of the H-Me in human T-ALL patients and solves the puzzling mystery of why *MYB* duplications nearly always contain part of the *AHI1* gene.

The H-Me is important for human T-ALL maintenance

Within the topologically associating domain (TAD) that contains *MYB*, ATAC-seq of T-ALL cell lines and primary samples showed that the H-Me ranked amongst the strongest nucleosome-free signal, stronger than previously identified *MYB* enhancers in T-ALL (67-69) (Enhancers “A” and “B” in Figure 7A). To test the importance of the H-Me, we transduced H-Me sgRNAs and dCas9-KRAB into T-ALL cell lines that represent genomically defined subtypes (40) expressing a range of *MYB* levels in *ETS1*-enriched T-ALL (Figure S12). We selected several *NOTCH1*-mutated cell lines -- THP-6 (NKX2-5), Jurkat (TAL1 (67)), MOLT14 (STAG2/LMO2 (70)), CEM (NKX2-5 (39), related to THP-6), SUP-T1 (TAL1-negative NOTCH1 t(7;9) otherwise not classified (71, 72)) and MOLT4 (TAL1 (73)) -- as well as one *NOTCH1*-wildtype cell line HSB2 (TAL1 (74)). The H-Me sgRNAs repressed expression of *MYB* by 1.3-fold to 5.5-fold (Figure 7B-D, S13A-D) and impaired

cell growth by 1.6-fold to 113-fold (Figure 7E-G, S13E-H)). MOLT4 cells appeared to be MYB-independent as MYB promoter guides suppressed *MYB* expression but not growth. To rule out non-MYB effects of the H-Me, we ectopically expressed *Myb* and then transduced sgH-Me. Enforced *Myb* expression rescued growth of H-Me repressed cells (Figure S13I-J). To test the importance of the H-Me in vivo we injected NSG mice with doxycycline-inducible dCas9-KRAB-expressing CEM cells transduced with sgRNA and then treated the mice with doxycycline. At 4 weeks, sgH-Me repressed peripheral blood blast count by 5-fold (Figure 7H-I) and significantly extended survival (Figure 7J). These effects were just as strong as repressing a pan-essential gene. Taken together, these data show that the H-Me has limited function in normal T-lineage committed progenitors but rises in importance for malignant transformation, induction of *MYB*, and maintenance of T-ALL cells without compensation by other *MYB* enhancers.

The ETS motif in the H-Me is primarily bound by ETS1 and is important for H-Me activity in one T-ALL context

To better understand how ETS1 regulates the H-Me, we first generated THP-6 and CEM (related to THP-6) cell lines in which ETS1 was fused to FKBP^{F36V}. Adding dTAG^V-1 robustly degraded ETS1 protein, resulting in suppression of *MYB* protein and reduction of *MYB* RNA by ~4.1-fold in THP-6 cells (Figure 8A-B) and ~2.3-fold in CEM cells (Figure S14A-B). Next, we performed HOMER analysis of the H-Me across several species, which revealed strong conservation of several transcription factor motifs including a single ETS motif (Figure S15). Human-to-mouse conservation at the H-Me was high at 93% compared to just 12-15% in introns overall (75-77). To test whether ETS1 bound this motif, we performed an assay, termed "reverse ChIP", which was originally developed by the Pimanda lab (78). In this assay, nuclear extracts are incubated with a biotinylated 254bp H-Me DNA fragment containing wildtype ETS motif (AGAGGAAGTG) or mutated ETS motif (AGAGAAAATG, full sequence in Figure S16A). The mutant DNA fragment pulled down less ETS1 in THP-6, CEM, and Jurkat T-ALL cells compared to wildtype control

(Figure 8C-D, S16B-E). Next, we performed mass spectrometry of "reverse ChIP" pulldowns. As a pulldown assay, this assay could in theory detect closely related ETS factors. However, ETS1 was the only protein that was differentially pulled down by wildtype compared to mutated DNA fragment by two statistical methods (Figure 8E). As a complementary approach, we attempted to generate a mutant THP-6 ETS1-FKBP^{F36V} clone with homozygous deleted ETS binding sites. We observed strong selection against creating this clone but eventually created one clone with homozygous partial mutations (Figure 8F). ChIP showed undetectable ETS1 occupancy at the mutant H-Me relative to wildtype control (Figure 8G). These data suggest that ETS1 binds the endogenous ETS site in the H-Me.

Next, we wondered whether the ETS site in the H-Me is functional. To test this, we measured *MYB* mRNA in wildtype and ETS-mutant cells with or without ETS1 degradation using dTAG. Consistently, *MYB* expression was ~60% reduced in mutant cells compared to wildtype control (Figure 8H). Further, ETS1 degradation reduced *MYB* expression in wildtype control but had no effect in the mutant clone. The lack of *MYB*-responsiveness to ETS1 degradation is not due to clone-specific damage to ETS1-FKBP^{F36V} functionality as dTAG treatment reduced the ETS1 target genes *LYL1* and *HHEX* (21) (Figure S16F-G). As an orthogonal approach, we developed an H-Me luciferase reporter assay, which showed that the H-Me was active in T-ALL cells but not in U2OS or 293T cells (Figure S16H). Mutating the ETS motif (Figure S16I) or *ETS1* knockdown (Figure S16J) impaired H-Me reporter activity. Although Notch/RBPJ bound the H-Me with gamma-secretase dependence (Figure S3A) (21), Notch inhibition with GSI did not impair H-Me activity (Figure S16K). These data are consistent with multiple public RNA-seq datasets showing that Notch does not generally regulate *MYB* (Figure S16L) (30, 84, 85). Thus, Notch appears to defer to ETS1 to induce *MYB* directly through the ETS site in the H-Me in one T-ALL context.

The SWI/SNF complex is a top-ranked candidate ETS1 cofactor at the H-Me

Since enhancers are difficult to target, we sought to identify ETS1-bound cofactors at the H-Me. Initially, we examined our H-Me "reverse ChIP" mass spectrometry data and identified 71 transcriptional regulators that differentially bound wildtype H-Me bait compared to bead controls (Figure S17A-B), including several DNA-binding sequence-specific transcription factors (Figure S17C). Next, we transduced CEM cells with Flag-ETS1 and performed anti-Flag co-immunoprecipitation followed by mass spectrometry. We identified 60 transcriptional regulators that differentially bound Flag-ETS1 compared to vector control (Figure S17D-E), including known partners RUNX1 and its cofactor CBFB (86) (Figure S17F). Finally, we intersected the H-Me-interacting proteins with the ETS1-interacting proteins to identify 13 proteins common to both groups and ranked them by strength of binding to ETS1 (Figure 8I). The top two proteins were RUNX1 and CBFB. The third ranked protein was SMARCC1, which is a subunit of SWI/SNF complexes (Figure S17C, blue dot). In addition to SMARCC1, 6 other SWI/SNF subunits differentially bound ETS1 (Figure S17D, yellow cluster; Figure S17F, blue dots). Co-IP confirmed the interaction between Flag-ETS1 and endogenous SWI/SNF subunits SMARCC1 and SMARCB1 (Figure 8J). Reciprocal co-IP confirmed the interaction between endogenous SMARCC1 and ETS1 (Figure 8K). These data suggest that SWI/SNF complexes might be recruited by ETS1 to activate the H-Me.

ETS1 recruits the cBAF complex to remodel chromatin at the H-Me in one T-ALL context

To test whether ETS1 recruits SWI/SNF to the H-Me, we degraded ETS1 in THP-6 cells and performed ChIP for SMARCC1 at the H-Me. Consistently, ETS1 degradation reduced SMARCC1 occupancy by ~2.5-fold in THP-6 cells (Figure S18A) and ~1.6-fold in CEM cells (related to THP-6, Figure S18B). ETS1 degradation reduced H3K27ac by ~3.2-fold at the H-Me (Figure S18C). ETS1 degradation reduced ARID1A signals (specific for cBAF) by ~2.3-fold but had no effect on PBRM1 occupancy (specific for PBAF) at the H-Me (Figure 8L). Next, we performed ATAC-seq. ETS1 degradation strongly reduced H-Me chromatin accessibility by ~4.9-fold at FDR<6.8E-15

(Figure 8M). Next, we wondered whether the ETS motif in the H-Me is required for SWI/SNF function. To test this, we measured *MYB* mRNA in the ETS-mutant cells (Figure 8F) after SWI/SNF degradation with AU-15330. Consistently, SWI/SNF degradation reduced *MYB* expression in ETS-mutant cells by only ~20% in contrast to ~80% in control cells (Figure 8N). The lack of *MYB*-responsiveness to SWI/SNF degradation was not due to clone-specific damage as AU-15330 treatment strongly repressed the ETS1 target genes *LYL1* and *HHEX* (21) in this clone (Figure S18D-E). These data suggest that ETS1 recruits the cBAF complex to the H-Me to promote chromatin accessibility and *MYB* induction in one T-ALL context.

cBAF inhibitors inactivate the H-Me and downregulate *MYB* in two T-ALL contexts

The above data raised the possibility that proteolysis-targeting chimera (PROTAC) degraders of SMARCA2/4, such as ACBI1 (87) and AU-15330 (88), might be effective in targeting the H-Me. To test this, we generated dose-response curves with these compounds on CEM, Jurkat, and THP-6 cells. All three cell lines showed <100nM sensitivity to AU-15330, which classifies them as “sensitive” (Figure S19A) (88). Jurkat and THP-6 cells, but not CEM cells, were also sensitive to ACBI1 (Figure S19B). To test whether SMARCA2/4 degradation inactivates the H-Me, we performed H3K27ac qChIP. AU-15330 treatment reduced H3K27ac signals by ~11.1-fold in THP-6 cells (Figure S19C) and ~2.1-fold in CEM cells (Figure S19D). Consistently, AU-15330 suppressed *MYB* transcripts by ~1.3-4-fold (Figure S19E-G) and *MYB* protein levels by ~2.3-9.7-fold (Figure 8O, S20A). ACBI1 failed to degrade SMARCA2/4 protein levels in CEM cells (Figure S20B), which is consistent with its weak growth inhibitory effects and lack of effect on *MYB* transcripts (Figure S20C) and protein (Figure S20B) in these cells. In contrast, ACBI1 suppressed *MYB* transcripts by ~1.4 and ~2-fold and *MYB* protein by ~7 and ~25-fold in Jurkat and THP-6 cells respectively. These data suggest that SMARCA2/4 degradation might be an effective strategy for inhibiting H-Me activity and *MYB* expression, among multiple other effects (89), in two T-ALL contexts.

Discussion

While many T-ALL-associated transcriptional regulators have been identified, there remains limited understanding of the non-coding elements at which these factors assemble and coordinate in hierarchies to promote oncogenesis. Despite important work in this area (22, 67, 68, 90-92), there are knowledge gaps, particularly in identifying the most important oncogenic enhancers and finding ways to safely eject transcription factors bound to these elements as potential therapeutic strategies. To help close these knowledge gaps, we performed an unbiased essentiality screen of regulatory elements that bind ETS1 and are dependent on ETS1 for H3K27ac modification in the Notch-activated THP-6 cell line. Our screen showed that the most essential ETS1-dependent element was the H-Me, a Notch-independent superenhancer that induces *MYB*. In T-ALL, supraphysiological MYB activity can be induced through translocations, tandem duplications, and genetic variants conferring increased protein stability; however, most T-ALL tumors (~83-95%) have no known *MYB* genetic lesion (62-68, 93). Thus, native elements like the H-Me are likely important drivers of MYB expression in most cases, including tandem duplications since they nearly always amplify the H-Me. Our screen also showed that the second most essential element was the Notch-dependent MYC enhancer (N-Me) (34, 35).

Although the H-Me and the N-Me share similarities as developmental enhancers that are co-opted to promote T-ALL oncogenesis, they confer different stage-specific dependences. The marked T-cell developmental defects in *LckCre Myc^{Δ/Δ}* mice phenocopy the marked defects in N-Me deficient mice (34). In contrast, the marked T-cell developmental defects in *LckCre Myb^{Δ/Δ}* mice (94) are not seen in H-Me deficient mice. These phenotypic differences might be explained by the disparate chromatin configurations of these enhancers in DN3/pre-T cells when *LckCre* initially becomes active (95). While N-Me accessibility peaks at DN3/DN4 (22), H-Me accessibility peaks

developmentally earlier in primitive HSCs. While the N-Me shows high H3K27ac signals in DN3/DN4 cells, the H-Me shows low H3K27ac signals (Figure 1E). Hence, it is not surprising that N-Me deletion caused marked T-cell developmental defects whereas H-Me deletion did not. We and others previously showed that DN3, DN4, or ISP cells are highly enriched for leukemia-initiating cells, depending on the T-ALL mouse model (43-45) and similar to human T-ALL (47). Thus, the N-Me appears to be fully accessible to immature T cells as they transform to T-ALL. In contrast, it is not apparent how developing T-ALL cells can easily access the oncogenic potential of the H-Me. These cells presumably restructure the H-Me to a more open configuration or aberrantly sustain the stem cell configuration during T-cell differentiation in order to hijack this stem cell element to drive oncogenesis. Emerging evidence suggests that developmental T-ALL enhancers can be separated into thymocyte-active elements (e.g. *Myc* and *Pten*) and stem cell-active elements (e.g. *Myb* and *Mycn*) (34, 35, 96, 97).

Myb deletion and H-Me deletion show similarities and differences. Conditional *Myb* deletion using *Mx1Cre* led to dramatic reductions in HSCs, MPPs, myeloid progenitors and differentiated blood populations (98). In contrast, conditional H-Me deletion using *Mx1Cre* acutely led to an increase in HSCs and no statistically significant effects on MPPs, myeloid progenitors, or differentiated blood populations. Later, between intermediate and aged time points, the H-Me-deficient HSC expansion wanes and then reverses to HSC depletion with evolving relative and/or absolute myeloid expansion, particularly monocytosis. The H-Me-deficient HSC and monocyte phenotypes are similar to what has been reported comparing 2-month-old and 12-month-old *Myb*^{+/-} mice (99). Induction of *Myb* haploinsufficiency leads to time-dependent accumulation of myeloid proliferation disease by 22 months of age (99). Germline *Myb* deletion is embryonic lethal due to severe anemia (100) while germline H-Me-deleted mice have normal survival with normal blood counts and weights up until 6-8 months. A single competitive bone marrow transplant of conditional *Myb*-deleted or *Myb*^{+/-} bone marrow revealed impaired HSC self-renewal or reconstitution (98, 99).

However, conditional H-Me-deficient mice required serial competitive bone marrow transplants to reveal impaired HSC self-renewal or reconstitution. *Myb* deletion using *LckCre* led to ~10-fold loss in thymus cellularity, particularly at the DP thymocyte stage (94). In contrast, conditional H-Me deletion using *Mx1Cre* or *I17rCre* caused stage-restricted ETP loss during thymopoiesis. Taken together, H-Me deletion has milder and/or stage-restricted effects compared to *Myb* deletion. While these data highlight an example of theoretical safety advantage of enhancer targeting over gene targeting, they also show that enhancer targeting would still have important ramifications, particularly in old age.

We are mindful that the H-Me is a single enhancer within a large transcriptional hub (Figure S3A). Thus, it is possible that deletion of the H-Me or insertion of the LoxP sites might affect other *Myb* regulatory elements in the TAD. Insertion of the LoxP sites also creates a hypomorphic H-Me enhancer. This is an important limitation of our floxed mouse model although the conclusions of this manuscript are based on comparisons between *H-Me*^{+/+} and *H-Me*^{Δ/Δ} mice as well as orthogonal models, such as germline H-Me knockout mice and human T-ALL cell lines.

The literature is replete with examples of Notch signaling being central to T-ALL oncogenesis (3). However, we here highlight a contrasting example of a top oncogenic element where Notch is not dominant, instead deferring to ETS1. The reason for why NOTCH1 binds the H-Me is unclear. It is possible that NOTCH1 induced H-Me activity early during leukemogenesis, but subsequent compensatory Notch-independent signals later converged on the H-Me, rendering Notch occupancy dispensable. Consistently, HSB2 cells, which lack *NOTCH1* mutations, require Notch-independent signals at the H-Me for population cell growth. Mechanistically, ETS1 appears particularly important since it binds and recruits the cBAF complex to the ETS site to promote accessibility and induce *MYB* in THP-6/CEM cells. Since open chromatin is required for transcription factor binding, the chromatin remodeling function of ETS1 likely explains our

previous observations that *ETS1* knockdown reduced Notch and Notch cofactor occupancy at a subset of *ETS1*-bound enhancers (21) (Figure S20D).

The importance of SWI/SNF and *ETS1* occupancy at the H-Me might have therapeutic implications. Consistently, SMARCA2/4 and *ETS1* degradation inactivated the H-Me, resulting in downregulation of *MYB*. The resultant growth inhibitory effects are consistent with recent reports that cBAF is important for promoting chromatin accessibility and transcriptional activity of RUNX1/CBFB, which are the top *ETS1* interaction partners in our screen (Figure 8I) (89, 101). While these findings are promising, there are some limitations. First, we caution that the details of how *ETS1* activates the H-Me and *MYB* has not been tested in all T-ALL contexts. Second, our H-Me ETS motif knockout reporter assay showed expected loss of gene activation; and while reporter assays are statistically predictive of true regulatory activity in high-throughput assays (79-83), effects of individual reporter constructs may not always recapitulate all regulatory effects in situ. While we were able to generate a THP-6 clone with homozygous H-Me mutations that led to loss of *ETS1* binding and regulatory effects on *MYB*, we were unable to derive clones with homozygous loss of the core ETS motif, possibly due to strong negative fitness effects. Third, since transcriptional regulators like *ETS1* and cBAF are not specific to *MYB* or the H-Me but regulate thousands of target genes, inhibiting them might not be safe in humans. (21, 89). Fourth, our study does not offer a unique mechanism or strategy to target a single gene out of thousands of genes or a single enhancer out of thousands of enhancers. Despite these limitations, given the safety of ubiquitous *Ets1* deletion in mice (21) and since SWI/SNF inhibitors are entering clinical trials (102), our study suggests that *ETS1* and cBAF degradation are reasonable therapeutic strategies to test in human T-ALL.

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Authorship Contributions

The order of the co-first authors C.M. and K.L. was established based on equal contribution. Conceptualization: M.Y.C., C.M., K.L., R.J.H.R., A.C.M (Monovich), P.P, D.T.T., C.G.M., M.C. Investigation: C.M., K.L., C.S., A.C.M. (McCarter), E.C., A.F.M, S.L., Q.W., A.I., K.B.R., M.J., K.C., T.K., P.E., A.W., J.N., S.K., Z.S. Visualization: M.Y.C., R.J.H.R., C.M., K.L., E.C., K.C., M.J., T.M.K., P.P. Formal Analysis: M.Y.C., C.M., K.L., C.S., E.C., Y.D., F.V.L., K.B.R., P.P. Data Curation: M.Y.C., C.M., K.L., D.S. Funding acquisition: M.Y.C. and C.G.M. Writing of the original draft: M.Y.C., C.M., K.L., E.C., K.C., M.J., L.C.S. Review and editing of the manuscript: C.M., K.L., C.S., A.C.M (Monovich), E.C., A.F.M, S.L., Q.W., A.I., A.C.M. (McCarter), D.T.T., C.G.M., P.P., M.C., Q.L., M.J., L.C.S., Y.D., D.S., F.V.L., A.I.N., R.J.H.R. Supervision: M.Y.C., A.I.N., D.S., D.T.T., C.G.M., R.J.H.R.

Conflict-of-interest

The authors have declared that no conflict of interest exists.

Methods

Sex as a biological variable

For developmental and gene expression analyses, mice of both sexes generated from heterozygous-to-heterozygous matings were used to generate data. These data were combined when no differences were noted. Since no differences were noted, sex was not considered as a biological variable for bone marrow transplantation experiments.

Mice

C57/BL6N mice between 4-8 weeks old were purchased from Taconic. 564 B6-Ly5.1/Cr mice between 4-8 weeks were purchased from Charles River Breeding Labs. Mice were backcrossed to the C57/BL6N strain at least 5 times. *Mx1Cre* mice were a gift from Qing Li (University of Michigan, Ann Arbor). *Il7rCre* mice were a gift of Hans-Reimer Rodewald (German Cancer Research Center, Heidelberg) (49). NSG mice were obtained from Jax (Strain #:005557). The conditional H-Me floxed mice were generated using Easi-CRISPR (Quadros, Genome Biology, 2017) by the University of Michigan Transgenic Animal Model Core. Briefly, ES cells were microinjected with a megamer donor containing 5' and 3' loxP sites flanked the 369bp H-Me segment (chr10:21,054,606-21,054,974; mm10), Cas9, and 5' and 3' sgRNA guides. A 47-bp rabbit beta globin splice acceptor sequence (103) was placed in the megamer donor between the 3' loxP site and Exon 20 of *Ahi1*. The *Mx1Cre H-Me^{ff}* and *Il7rCre H-Me^{ff}* mice were generated by crossing *H-Me^{ff}* mice with Mx1-Cre mice and Il7rCre mice respectively. Cre expression in *Mx1Cre H-Me^{ff}* mice that were 4-6 weeks of age was induced with pl-pC (Amersham, 40 µg i.p. every 2 days for 5 times) and analyzed at 6 weeks after the last injection (11-13 weeks of age). *H-Me^{ff}* mice of both sexes were analyzed at 5-8 weeks of age. *Il7Cre H-Me^{ff}* mice of both sexes were analyzed at 5-10 weeks of age. Germline H-Me knockout mice of

both sexes were analyzed at 10-13 weeks of age. Aged H-Me deficient mice of both sexes were analyzed at 52-54 weeks of age.

Constructs

Flag-ETS1 construct was generated as previously described¹. H-Me^{WT} and H-Me^{Mut} constructs were generated by subcloning from designed gBlocks from IDT into the pGL3-luciferase-promoter vector (Promega). The TET3G activator plasmid was generated by subcloning the TET3G from pLVX-EF1a-Tet3G (Clontech #631359) into pRRLsin.cPPTCTS.MNDU3.BXE.PGK.NGFR.WPRE (gift from Andrew Weng, British Columbia Cancer Agency, Vancouver). CRISPRi sgRNAs were cloned into the sgOPTI virus (Addgene #85681; RRID:Addgene_85681) and co-transduced with TRE-KRAB-dCas9-IRES-GFP virus (Addgene #85556; RRID:Addgene_85556) and TET3G activator virus (104). These constructs were used for all CRISPRi experiments with the exception of CEM and THP-6 cells in Figure 3I-J, which used a constitutive CRISPRi construct, pLV-hU6-sgRNA-hUbc-dCAS9-KRAB-T2a_puro (Addgene #71236; RRID:Addgene_71236).

Cell lines

CEM/SS, THP-6, SUP-T1, MOLT4, and Jurkat cells were obtained and cultured as previously published (20, 21). HSB2 and MOLT14 cells were obtained from Charles Mullighan (St. Jude Children's Research Hospital, Memphis). DOX-inducible CRISPRi cell lines were generated as previously published (104). All human cell lines were authenticated using STR analysis prior to use to match an established cell line standard (DSMZ) or an internal standard when a reference is not available (THP-6) (Labcorp). All cell lines were cultured less than 3 months after resuscitation and tested for contaminants using MycoAlert (Lonza) every 1-3 months to ensure they were free of Mycoplasma contamination.

Antibodies, sgRNAs, HDR templates and primers

These reagents are listed in Table S1.

Statistics

Unless otherwise indicated, P-values were derived from two-sided two-sample t-tests of Log2-transformed data for comparisons in experiments involving two groups and 1-way ANOVA for pairwise contrasts in experiments with more than two groups. Unless otherwise stated, horizontal lines are means and values are shown as mean $\pm$ standard deviation. Survival curves (or time to event data) was tested with log-rank tests comparing pairs of groups. A P value less than 0.05 was considered significant.

Data availability

High-throughput sequencing data, results, and statistics were deposited in the Gene Expression Omnibus database with accession number GSE263585, GSE263913, GSE263952, and GSE263977 and are publicly accessible. The publicly available NGS datasets used during the present study can be found in GEO under accession numbers: GSE225559, GSE221345, GSE151075, GSE51800, GSE29600, GSE134761, GSE94000, GSE151075, GSE29181, GSE79422, GSE117749, GSE93755, GSE138516, GSE109125, GSE22601, GSE129086, GSE110630, GSE76783, GSE90715, GSE116873, and GSE138659. H3K27ac ChIP-seq profiles of sorted thymocyte subsets were obtained from <https://viz.stjude.cloud/mullighan-lab/collection/the-genomic-basis-of-childhood-t-lineageacute-lymphoblastic-leukemia~29>.

BLUEPRINT project DP thymocyte histone profiles were obtained from EGAD00001002369. WGS datasets were obtained from phs002276.v2.p1, phs000218, phs000464. Supporting data values are in the XLS file, as described above.

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Figure legends

Figure 1. The hematopoietic stem cell MYB enhancer (H-Me) is a top-ranked essential regulatory element in T-ALL cells that appears relatively quiescent in the developmental stages from which T-ALL initiates. A) Schematic of the CRISPRi screen to identify essential ETS1-dependent enhancers in the THP-6 T-ALL cell line. B) CRISPRi essentiality screen results. MAGeCK analysis in Table S2. C) ATAC-seq profiles of the murine H-Me in long-term HSCs (LT-HSCs) through T-cell development (Immgen). D) ATAC-seq profiles of the human H-Me in sorted thymocytes subsets (GSE151075 (105)). D1 = Donor 1; D2 = Donor; HSC=CD34⁺ cord blood; ETP/DN2 = CD34⁺CD4⁻CD1⁻; DN3/DN4 = CD34⁺CD4⁻CD1⁺; ISP = CD28⁺CD4⁺CD3⁻CD8⁻; DP = CD4⁺CD8⁺; SP = single positive. E) H3K27ac ChIP-seq profiles of normal thymocyte subsets at the H-Me and the N-Me in 50kb windows (St. Jude Cloud at <https://viz.stjude.cloud/mullighan-lab/collection/the-genomic-basis-of-childhood-t-lineageacute-lymphoblastic-leukemia~29>). F) Histone chromatin profiles of DP cells of 4 donors (D1-D4; BLUEPRINT project) comparing a silenced region to the *MYB-AHI1* region.

Figure 2. The H-Me normally restricts hematopoietic stem cell (HSC) numbers and maintains early T-cell precursors (ETPs) under steady state conditions. A) Schematic of the floxed H-Me allele (f). SA=Rabbit beta-globin splice acceptor sequence (103). B) qRT-PCR of *Myb* and *Ahi1* in sorted LT-HSCs (CD150⁺CD48⁻Lineage⁻Sca-1⁺Kit⁺ (LSK)) cells from *Mx1Cre H-Me^{Δ/Δ}* (Δ/Δ) and littermate control *Mx1Cre* (+/+) mice. C-G) Representative bone marrow Lineage⁻ flow cytometry plots (C) and absolute numbers of LT-HSCs (D), LSK (E), MPP/ST-HSC (F, CD150⁺CD48⁻ LSKs), and LMPPs (G, Lineage⁻Sca-1⁺Kit^{hi}Flt3^{hi}). H-M) Representative thymus Lineage⁻ flow cytometry plots (H) and absolute numbers of ETP (I, Lineage⁻CD44⁺CD25⁻cKit^{hi}), DN2a (J, Lineage⁻CD44⁺CD25⁺cKit^{hi}), DN2b (K, Lineage⁻CD44⁺CD25⁺cKit^{lo}), DN3 (L, Lineage⁻CD44⁻CD25⁺), and DN4 (M, Lineage⁻CD44⁻CD25⁻) cells. N) *Myb* qRT-PCR in sorted DN cells from

Il7rCre H-Me^{Δ/Δ} (Δ/Δ) and littermate control *Il7rCre* (+/+) mice. Unpaired 2-tailed t-test. *P<0.05; **P<0.01; ***P<0.001.

Figure 3. The H-Me is important for long-term HSC self-renewal under stress conditions.

A) Schematic of serial competitive BMT experiment. B-I) Peripheral blood analysis of indicated subsets tracking %donor (CD45.2⁺)-derived cells after the first BMT (B-E) and the second BMT (F-I). J-L) Representative Lineage⁻ flow cytometry plots (J) and %donor-derived (CD45.2⁺) analysis of LT-HSCs (K) and MPP/ST-HSCs (L) at 16 weeks after the second BMT. Unpaired 2-tailed t-test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 4. The H-Me limits myeloid expansion and HSC loss with aging. A-J) WBC (A, F), neutrophil counts (B, G), monocyte counts (C, H), hemoglobin concentrations (D, I), and platelet counts (E, J) comparing 8-month-old (A-E) and 12-month-old (F-J) indicated mice. K-O) Representative flow cytometric plots (K) and absolute numbers of LT-HSCs (L), MPP/ST-HSCs (M), hematopoietic stem and progenitor cells (LSK, N), and total BM cells (O) in 1-year old mice as defined in Fig. 2. P-V) Representative flow cytometric plots (P) and absolute numbers of ETP (Q), DN2a (R), DN2b (S), DN3 (T), DN4 (U), and total thymocyte cells (V) as defined in Fig. 1. Unpaired 2-tailed t-test. *P<0.05; **P<0.01; ***P<0.001.

Figure 5. The H-Me is an essential regulatory element for T-ALL leukemogenesis in multiple genetically engineered mouse models.

A) Schematic showing generation of Notch-induced T-ALL by transducing $\Delta E/Notch1$ retrovirus (59, 60) into hematopoietic stem and progenitor cells from *H-Me^{-/-}* mice or littermate controls followed by transplantation. B-D) Representative flow cytometry plots (B), GFP⁺ blast counts at 4 weeks after transplant (C), and leukemia-free survival curves (D) for the experiment in (A). E) Schematic showing generation of Notch-induced T-ALL

by transducing $\Delta E/Notch1$ retrovirus into hematopoietic stem and progenitor cells from *Mx1Cre* *H-Me^{fl/fl}* mice and *Mx1Cre* littermate controls followed by transplantation and injection of pl-pC to delete the H-Me. F-H) Representative flow cytometry plots (F), GFP⁺ blast counts at 4 weeks after injection of pl-pC (G), and leukemia-free survival curves (H) for the experiment in (E). I-J) *Lmo2-tg* *H-Me^{-/-}* and littermate control *H-Me^{+/+}* mice were observed for survival (I) and development of T-ALL, which was confirmed by flow cytometry of thymic mass (#33, #98, and #61) or spleen mass (#772) (J). Unpaired 2-tailed t-test and log-rank test. ****P<0.0001.

Figure 6. The H-Me is co-amplified with MYB in nearly all human T-ALL patients with MYB amplifications. A) Amplified region in primary human T-ALL in the WGS dataset from AALL0434 (N=1,309 patients) (40). Each row is a unique patient (N=115). Bar shows the *MYB*-to-H-Me region. B-C) WGS tracks showing sequence reads in the *MYB-AHI1* region including the H-Me in THP-6 cells (B) and other T-ALL cell lines used in this study (C).

Figure 7. The H-Me is important in diverse models of human T-ALL maintenance. A) ATAC-seq profiles (green) of a panel of T-ALL cell lines and primary samples across the *MYB* TAD. ETS1 and H3K27ac tracks in THP-6 cells are shown in blue (GSE138516). MYB enhancers “A” and “B” were previously described (67-69). ATAC-seq datasets from GSE129086, GSE110630, GSE263585, GSE263977, and GSE225559. TAD datasets from GSE134761. B-G) THP-6 (B, E), Jurkat (C, F), and MOLT14 (D, G) cells were transduced with constitutive (THP-6) or dox-inducible (Jurkat, MOLT14) dCas9-KRAB and sgRNAs against the H-Me or the *MYB* promoter and then tested for expression of *MYB* (B-D) and measured for cell growth (E-G). *HBS1L* and *AHI1* are flanking genes of *MYB*. H-J) CEM cells, related to THP-6, transduced with indicated sgRNAs were injected into NSG mice and 2-5 days later treated with doxycycline in drinking water to activate dCas9-KRAB-GFP. Representative GFP/hCD45.2 flow cytometry plots of peripheral blood at 4 weeks after injection (H), GFP⁺/hCD45⁺ blast counts (I) and survival (log-rank test P values, J)

were measured. N=9 (control); N=10 (PE); N=10 (H-Me). NT=non-targeting; PE=pan-essential gene *RPL34*. 1-way ANOVA test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 8. ETS1 recruits cBAF through the ETS motif in the H-Me to activate *MYB* expression in THP-6/CEM cells. A-B) Western blot of ETS1 and MYB proteins (A) and *MYB* qRT-PCR (B) showing the effect of 500nM dTAG^V-1 in degrading ETS1 in ETS1-FKBP^{F36V} knock-in THP-6 cells. C-D) Representative Western blots (C) and quantitative Image J analyses (D) showing effect of the ETS motif mutation on ETS1 binding (p54 and p42 isoforms) in "reverse ChIP" in THP-6 cells. E) Normalized abundance plot of transcriptional regulators that were pulled down by "reverse ChIP" and identified by mass spectrometry comparing wildtype and ETS motif mutated H-Me; and analyzed with PD (Thermo Fisher Proteome Discoverer) and FragPipe (106-108). Full results are in Table S3. F-H) Sequences of homozygous partially mutated ETS sites in the 3 H-Me alleles (F), ETS1 qChIP at the H-Me (G); and *MYB* qRT-PCR (H) in a subclone of ETS1-FKBP^{F36V} knock-in THP-6 cells after CRISPR/Cas9 editing and homology directed repair (HDR). I) Venn diagram showing intersection of the H-Me and ETS1 interactomes ranked by strength of interaction with Flag-ETS1. The H-Me interactome was supplemented with transcriptional regulators that met Padj<0.1/LFC>0 criteria by Proteome Discoverer. Full results are in Table S4 and S5. J) Flag co-IP assay in vector-transduced (Ctrl) and Flag-ETS1-transduced CEM cells showing interactions with endogenous SMARCC1 and SMARCB1. K) Reciprocal co-IP assay comparing IgG and anti-SMARCC1 pulldowns in CEM cells to detect interactions with endogenous ETS1. L) ARID1A and PBRM1 qChIP using primers at the H-Me peak center or a negative control site 1.25kb downstream in ETS1-FKBP^{F36V} knock-in THP-6 cells treated with 500nM dTAG^V-1 (dTAG) to degrade ETS1. M) ATAC-seq profiles of the *MYB* topologically associating domain (TAD) in ETS1-FKBP^{F36V} knock-in THP-6 cells treated with dTAG to degrade ETS1. DeSeq2 analysis. N) *MYB* qRT-PCR in ETS1-FKBP^{F36V} knock-in THP-6 cells with mutated ETS-binding sites in the H-Me (F) treated with DMSO vs AU-15330. O) Western blot for indicated proteins in DMSO-treated

or AU-15330-treated T-ALL cells. 1-way ANOVA test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

