Combinatorial ETS1-Dependent Control of Oncogenic NOTCH1 Enhancers in T-cell Leukemia

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ABSTRACT

Notch activation is highly prevalent among cancers, in particular T-cell acute lymphoblastic leukemia (T-ALL). However, the use of pan-Notch inhibitors to treat cancers has been hampered by adverse effects, particularly intestinal toxicities. To circumvent this barrier in T-ALL, we aimed to inhibit ETS1, a developmentally important T-cell transcription factor previously shown to cobind Notch response elements. Using complementary genetic approaches in mouse models, we show that ablation of Ets1 leads to strong Notch-mediated suppressive effects on T-cell development and leukemogenesis but milder intestinal effects than pan-Notch inhibitors. Mechanistically, genome-wide chromatin profiling studies demonstrate that Ets1 inactivation impairs recruitment of multiple Notch-associated factors and Notch-dependent activation of transcriptional elements controlling major Notch-driven oncogenic effector pathways. These results uncover previously unrecognized hierarchical heterogeneity of Notch-controlled genes and point to Ets1-mediated enucleation of Notch-Rbpj transcriptional complexes as a target for developing specific anti-Notch therapies in T-ALL that circumvent the barriers of pan-Notch inhibition.

SIGNIFICANCE: Notch signaling controls developmentally important and tissue-specific activities, raising barriers for developing anti-Notch therapies. Pivoting away from pan-Notch inhibitors, we show antileukemic but less toxic effects of targeting ETS1, a T-cell NOTCH1 cofactor. These results demonstrate the feasibility of context-dependent suppression of NOTCH1 programs for the treatment of T-ALL.

INTRODUCTION

The discovery of Notch-activated tumors, including approximately 60% of cases of T-cell acute lymphoblastic leukemia (T-ALL), spurred much interest and excitement to clinically test the efficacy and safety of pan-Notch inhibitors, such as gamma-secretase inhibitors (GSI), for the treatment of human cancers (1, 2). Notch receptors (Notch1–4) are activated by ligands in normal cells or additionally by activating mutations in cancer via cleavage by the gamma-secretase complex, which releases IntraCellular Notch (ICN). ICN translocates to the nucleus where it must interact with the DNA-binding Rbpj cofactor to induce transcription. Hence, small-molecule GSIs inhibit activation of Notch1–4 in normal and cancer cells. Unfortunately, early clinical trials in patients with cancer reported dose-limiting toxicities with continuous dosing of GSI (3–5). GSI toxicities result from abrogation of Notch signals crucial for normal homeostasis, particularly of the intestine (6–8), prompting the search for more specific ways to block Notch-induced oncogenic pathways. One idea to meet this challenge stems from Drosophila studies showing that cobinding of transcription factor partners at Notch response elements is necessary to generate cell-type–specific, Notch-driven gene expression programs (9).

In vertebrates, Notch1 directly induces MYC expression in T-cell progenitors and T-ALL cells through the T-cell–specific Notch-MYC enhancer (N-ME; refs. 10, 11), which is selectively active and competent for Notch1-driven transcriptional activation through the pioneering activity of the chromatin remodeler GATA3 (12). Here, we hypothesized that Notch1-collaborating transcription factors are required to drive gene expression programs that promote Notch-induced T-ALL but less so to maintain tissue homeostasis. Inhibiting these factors could effectively oppose Notch signals in cancer while circumventing the toxicities of systemic pan-Notch inhibition with GSIs.

Ets1, the founding member of the Ets family of transcription factors, has biological functions and in vivo DNA-binding specificity that are distinct from other Ets proteins (13, 14). Ets1 has been proposed as a Notch1 coregulator as Ets1...
binding at chromatin associates with Notch1 and Runx1 sites (15, 16), and like Notch1, Ets1 is required for T-cell development (17, 18). Consistently, Ets motifs are highly enriched in T-ALL-associated enhancers (19–22). These correlative observations are suggestive of a functional and clinically relevant interaction between Ets1 and Notch1 in early T-cell development and leukemogenesis. However, this concept should be formally tested, as other transcription factors involved in lymphoid development have shown to be dispensable for ALL leukemogenesis and in some cases, to actually work as tumor suppressors (23–27). Moreover, given that the scope of Ets1 expression, being primarily expressed in lymphoid cells (28), is more tissue-restricted than Notch during the postnatal period brings the possibility that a systemic Ets1 inhibitor could be less toxic than a GSI pan-Notch inhibitor. In this context, we proposed and experimentally tested the role of Ets1 as a Notch1-collaborating transcription factor in T-ALL. Our results formally establish a direct mechanistic role for Ets1 in the control of Notch1-dependent oncogenic programs in T-ALL and highlight Ets1 as a potential therapeutic target in this disease.

RESULTS

Ets1 Is Important for Notch-Dependent Steps of Early T-cell Development

A “Notch1-collaborating” transcription factor in T-ALL would be predicted to have overlapping functions with Notch1 in the normal thymic precursors from which T-ALL originates. Murine T-cell development proceeds in the thymus through a series of stages from the early T-lineage progenitor (ETP), through the double-negative (DN) stages (DN2–DN4) to the immature single-positive (ISP) and CD4+CD8+ double-positive (DP) stages, and then to the single-positive (SP) CD4+ or CD8+ stages. Notch1 is essential for two major stages of early T-cell development: ETP specification and the DN-to-DP transition (29). During thymocyte development, Ets1 and Notch target genes are concurrently expressed (Supplementary Fig. S1A). Ets1 is also expressed in the corresponding stages in human thymocytes (Supplementary Fig. S1B), which are susceptible to transformation to T-ALL (30). Thus, like Notch1, Ets1 is expressed in T-cell precursors that can transform into T-ALL.

The role of Ets1 during T-cell development was previously studied in germline Ets1-mutant mouse models. However, these early studies were hampered either because Ets1 function was partly intact (31) or because complete Ets1 deficiency was lethal to neonatal mice (32), which required studying Ets1-deficient stages in human thymocytes (Supplementary Fig. S1B), Ets1 is also expressed in the corresponding stages in human thymocytes (Supplementary Fig. S1B), which are susceptible to transformation to T-ALL (30). Thus, like Notch1, Ets1 is expressed in T-cell precursors that can transform into T-ALL.

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To delete Ets1 in hematopoietic cells, we crossed Ets1Δ/Δ mice with VarCre mice generating VarCre Ets1Δ/Δ mice (Ets1Δ/Δ). Ets1Δ/Δ mice showed >95% excision of the Ets1 allele (Supplementary Fig. S1C) and undetectable Ets1 protein in the spleen (Supplementary Fig. S1D). Similar to Notch-deficient mice, Ets1Δ/Δ mice showed normal numbers of bone marrow LinSca1hiKIt+ progenitors (LSK; Supplementary Fig. S1E and S1F) and lymphoid-primed multipotent progenitors (LMP/MPP4; Supplementary Fig. S1G and S1H), which are the major extrathymic precursors of ETPs. In contrast, Ets1Δ/Δ mice showed profound depletion in total thymocyte number (~21-fold), which was more severe than seen in VarCre Notch1Δ/Δ mice (Fig. 1A and B). Like VarCre Notch1Δ/Δ mice (33), Ets1Δ/Δ mice showed defects in ETP specification (Fig. 1C and D) and loss of subsequent DN stages (Fig. 1C and E–K). These results support that, like Notch1, Ets1 is important for ETP specification in early T-cell development.

Notch1-deficient mice (34) show a defect in the DN-to-DP transition due to impaired TCRβ rearrangement and pre-TCR signaling (34). In contrast, Ets1Δ/Δ DN3 cells showed successful TCRβ rearrangement (Fig. 1C). Nevertheless, Ets1Δ/Δ cells were hampered in the DN-to-DP transition (Fig. 1L–N) and in their progression to the DN3b stage of development (Fig. 1O). This data is in agreement with defective pre-TCR signaling, as previously suggested in germline Ets1-null thymocytes (32). Consistent with the loss of the early T-cell subsets, subsequent T-cell stages in the thymus (Fig. 1L and P–S) and spleen (Supplementary Fig. S1M and S1N) were suppressed in Ets1Δ/Δ mice. Thus, Ets1 has overlapping roles with Notch1 in promoting pre-TCR signaling and the DN-to-DP transition.

In the case of Notch1-deficient mice, Ets1-deficient animals did not show any general defects in B-cell numbers (Supplementary Fig. S1L and S1M). Moreover, we observed a mild myeloproliferative phenotype in Ets1-deficient mice (Supplementary Fig. S1N–S1P) that was reminiscent of that observed in Notch1/Notch2-deficient and Notch signaling-defective Nicastrin-deficient mice.

Ets1 Is Important for Notch-Induced T-ALL Maintenance

Next, we wondered whether the Ets1 dependence of T-cell precursors would be conserved after they transform to leukemia. To test this possibility, we used a well-established murine model of Notch-induced T-ALL (35, 36). We transduced bone marrow stem and progenitor cells of Rosa26CreERT2, Rosa26CreERT2 Ets1Δ/+ or Rosa26CreERT2 Ets1Δ/Δ mice with an activated Notch1 allele (AE/Notch1; refs. 37, 38). We transplanted these cells into recipient mice to generate primary tumors (Fig. 2A; Supplementary Fig. S2A). To test the effect of Ets1 deletion on T-ALL maintenance, we transferred primary tumors into secondary recipients, which were then injected with tamoxifen to induce Ets1 deletion. In this setting, homozygous deletion of Ets1 alleles caused approximately 32-fold loss of circulating leukemic cells and prolonged median survival by >100% compared with vehicle treatment controls (Fig. 2B–D). In contrast, heterozygous Ets1 deletion had no effect on tumor progression and survival. We confirmed the survival benefit of homozygous Ets1 deletion compared with control/heterozygous deletion with additional independent Rosa26CreERT2 (Supplementary Fig. S2B), Rosa26CreERT2 Ets1Δ/+ (Supplementary Fig. S2C), and Rosa26CreERT2 Ets1Δ/Δ (Supplementary Fig. S2D) tumors. In these experiments, Cre activation induced mild or negligible effects in survival of control Ets1Δ/+ or Ets1Δ/Δ T-ALL mice. In contrast, tamoxifen treatment conferred a marked and
Figure 1. Ets1 is important for Notch-dependent steps of early T-cell development. A, Representative images of thymuses of VavCre control and Ets1ΔΔ mice. B, Absolute numbers of total thymocytes in VavCre control, Notch1ΔΔ, and Ets1ΔΔ mice. Representative flow cytometric profiles of DN subsets (C) and absolute numbers of ETP (D), DN2a (E), DN2b (F), DN3a (G), DN3b (H), DN3 iTCRβ+ (I), DN3 iTCRβ− (J), and DN4 (K) subsets in VavCre control and Ets1ΔΔ mice. Representative flow cytometry profiles of CD4/CD8 thymic subsets (L). %DN (M), %DP (N), DN3b/DN3a ratio (O), and absolute numbers of ISP (P), DP (Q), CD4 SP (R), and CD8 SP (S) thymic subsets in VavCre control and Ets1ΔΔ mice. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Two-sided two-sample t tests of nontransformed data were used for M–O. CD4 SP, CD4+TCRβ−; CD8 SP, CD8+TCRβ+. DN2a, LineageCD44+CD25−cKit+; DN2b, LineageCD44+CD25−cKit−; DN3, LineageCD44 CD25−; DN3a, LineageCD44 CD25+; DN3b, LineageCD44 CD25−FSC−CD27−; DN4, LineageCD44 CD25+; DP, CD4+CD8−; ETP, LineageCD44+CD25−cKit+; ISP, CD8+TCRβ−.
Figure 2. Ets1 is important for the maintenance and initiation of murine Notch-induced T-ALL. A–E, Experimental strategy to study maintenance of murine ΔE/Notch1-induced T-ALL (A). Tam, 25 mg/kg tamoxifen. Representative flow cytometric plots (B) and peripheral blood GFP+ T-ALL cell counts (C) at 2.5 weeks posttransplant, and survival curves (D) of mice transplanted with Rosa26CreER

ΔE/Notch1-induced T-ALL mouse model that were generated using the same experimental strategy (A) but were harvested from secondary recipients at 48 hours after injection with 300 mg/kg tamoxifen. J–K, Experimental strategy to study initiation of murine ΔE/Notch1-induced T-ALL (J). K, Survival curves of mice transplanted with Ets1fl/fl, Ets1fl/−, or Ets1−/− bone marrow progenitors transduced with ΔE/Notch1 (ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
significant survival benefit of approximately 125% compared with vehicle treatment in the Ets1Δ/Δ-T-ALL group (Fig. 2E).

The robust growth of Ets1Δ/Δ tumors suggested that T-ALLs can thrive with only a single Ets1 allele. Accordingly, the deletion efficiency of secondary splenic Ets1Δ/Δ tumors from morbid mice was only approximately 50%, which matched the approximately 50% deletion efficiency of Ets1Δ/Δ tumors (Supplementary Fig. S2E). To investigate this further, we transplanted secondary Ets1Δ/Δ tumors into tertiary recipients. The deletion efficiency of tertiary tumors extracted from terminally diseased mice was also approximately 50% (Supplementary Fig. S2F). Collectively, in these experiments and our other experiences breeding floxed Ets1 mice, we did not observe preferential Cre-mediated deletion of the maternal or paternal Ets1 allele. These results suggest that partial recombination resulting in deletion of only one Ets1 allele is sufficient to support leukemia cell growth and drive genetic escape.

To further test the requirement of Ets1 in Notch-induced T-ALL maintenance, we acutely deleted Ets1 in T-ALLs generated with a second conditional Ets1-deficient mouse model (39) and a different Rosa26CreERT2 strain with high deletion efficiency (40). To avoid confusion with the previous model, we have labeled these mice as “Ets1f/f-alt” and “Rosa26CreERT2-alt,” respectively. We deleted Ets1 when GFP+ Rosa26CreERT2-alt Ets1f/f-alt T-ALL blasts comprised 50% to 60% of circulating white blood cells in secondary recipients using a dose of tamoxifen that does not induce toxicity in Rosa26CreERT2-alt control tumors (10, 41) and then harvested splenic tumors 48 hours later. Compared with controls, deletion of Ets1 in Ets1f/f-alt tumors reduced spleen size (Fig. 2F and G) and increased apoptosis in T-ALL blasts (Fig. 2H) with modest effects on cell cycle (Fig. 2I). In all, complementary experiments using two independently derived genetic mouse models show that Ets1 is important for maintenance of Notch-induced T-ALL.

Ets1 Is Important for Initiation of Notch-Induced T-ALL

To determine the effect of Ets1 deletion on initiation of Notch-induced T-ALL, we used a previously described “Ets1f/f” mouse, which produces a hypomorphic Ets1 protein lacking the N-terminal PNT domain (31). These mice show a weak Ets1 loss-of-function phenotype with an approximately 2.8-fold loss of thymocytes (42). We transduced bone marrow stem and progenitor cells from Ets1+/+, Ets1f/f, or Ets1f/f mice with ΔE/Notch1 and transplanted these into isogenic recipient mice (Fig. 2J). Most mice injected with Ets1+/+ cells died of leukemia 90 days after transplant. In contrast, 6% and 0% of mice transplanted with Ets1f/f and Ets1f/f cells, respectively, died of T-ALL (Fig. 2K). These data demonstrate a strict requirement of Ets1 function for in vivo initiation of Notch-induced T-ALL.

ETS1 Is Important for Propagation of Human T-ALL Cells

ETS1 was one of the most highly and consistently expressed Ets transcription factor genes in primary human T-ALL samples (Fig. 3A) and patient-derived xenografts (PDX; Supplementary Fig. S3A). In a clinically annotated cohort of pediatric T-ALL, high ETS1 expression was associated with hyperleukocytosis (white blood cell count >100K; Supplementary Fig. S3B), but not survival (Supplementary Fig. S3C). To test the functional importance of ETS1 in human T-ALL, we transduced ETS1 shRNAs into T-ALL cell lines. In-depth analysis of ETS1 knockdown in HPB-ALL cells showed effective suppression of ETS1 protein and mRNA levels (Supplementary Fig. S3D and S3E), reduced proliferation (Supplementary Fig. S3F), and delayed G1–S transition (Supplementary Fig. S3G). Similarly, ETS1 knockdown reduced proliferation of six of eight additional cell lines tested (CEM, THP-6, DND-41, SUP-T1, MOLT4, and DU.528; Fig. 3B–E; Supplementary Fig. S3H–S3M).

To test the antitumor effects of ETS1 inactivation in non-immortalized human T-ALL cells in vivo, we took advantage of the success of shRNA protocols in knocking down gene expression in PDX cells. In these experiments, we transduced PDX3 cells, which expressed high levels of ETS1 and NOTCH1 (Fig. 3F) with shETS1/YFP and transplanted them into immunodeficient NSG mice (Fig. 3G). Knockdown cells were viable, as they could expand for several more days in vitro (Fig. 3H and I). However, ETS1 inactivation markedly blunted disease progression, reducing circulating T-ALL blasts by 44-fold in 8 weeks posttransplant (Fig. 3J and K), and improved survival (Fig. 3L). Moreover, PDX cells that were recovered from terminally diseased mice showed reduced levels of YFP (Fig. 3M) and restored ETS1 expression (Fig. 3N), consistent with positive selection of cells that escaped ETS1 knockdown. Taken together, these results demonstrate a strong and highly prevalent ETS1-dependency in human T-ALL.

ETS1 Deficiency Is Less Toxic than the Pan-Notch Inhibitor GSI

Next, we explored the effects of Ets1 inactivation on overall health and, in particular, the intestine, which is the major organ affected by the toxicity of GSI in humans and mice (8, 43). We induced ubiquitous Ets1 deletion in Rosa26CreERT2 Ets1Δ/Δ mice via tamoxifen injection. In these experiments, Ets1Δ/ΔRosa26CreERT2 mice showed 70% to 90% Ets1 deletion (Fig. 4A) and undetectable Ets1 protein in the intestine (Fig. 4B). Ets1Δ/ΔRosa26CreERT2 mice showed no significant differences in weight (Supplementary Fig. S4A) and survival compared with controls (Supplementary Fig. S4B). To characterize the effects of Ets1 deletion, we analyzed Ets1Δ/ΔRosa26CreERT2 mice after tamoxifen injection. At 24 days, Ets1-deleted mice showed unremarkable blood counts other than the expected lymphopenia (Supplementary Fig. S4C). Histologic analysis of Ets1-deficient spleens showed preserved white pulp cellularity and decreased myeloid component of the red pulp compared with control spleens (Supplementary Fig. S4D). Analysis of the intestinal tissues of Ets1Δ/ΔRosa26CreERT2 mice showed morphologic changes that were similar but less pronounced than those observed in mice after pan-Notch inactivation with GSI (Fig. 4C–F). Specifically, Ets1Δ/ΔRosa26CreERT2 mice showed modest reductions in duodenal villi length (Fig. 4G), a trend toward increased duodenal crypt depth (Fig. 4H), and modest ileal goblet cell metaplasia (Fig. 4I). Similar but more pronounced changes were observed in GSI-treated
Figure 3. ETS1 is important for propagation of human T-ALL cells. A, Heatmap of Ets family member mRNA in primary human T-ALL samples (GSE13159). B, Western blot analysis of ETS1 in CEM cells transduced with shETS1. Growth curves of CEM (C), THP-6 (D), and LOUCY (E) T-ALL cells transduced with shETS1. GSI, 200 nmol/L DBZ. Fold expansion = cell count divided by cell count on day 0. F, Western blot analysis of ETS1, cleaved NOTCH1 (ICN1), and ZMIZ1 in PDX samples. G-N, Experimental strategy testing maintenance of PDX cells transduced with shETS1-3/YFP after transplantation into NSG mice (G). Flow cytometric plots (H) and growth curves (I) of PDX cells transduced on day 0, sorted on day 2, and cocultured with OP9-DLL4 cells. N = 3 transduced bioreplicates per group. Representative flow cytometric plots (J) and peripheral blood T-ALL counts (K) at 8 weeks posttransplant; survival curves (L); and % YFP (M) and ETS1 transcripts normalized to respective controls (N) of splenic tumors harvested from terminally diseased mice (99.3% CD45-positive T-ALL content) compared with cells prior to injection into NSG mice (ns, not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001).
mice (Fig. 4J–L). Furthermore, we generated mice with intestinal-specific deletion using the VillinCreERT2 transgene. Ets1ΔVillinCreERT2 mice showed 80% to 90% Ets1 deletion in intestinal crypt cells (Supplementary Fig. S4E). In contrast to Notch1ΔVillinCreERT2 mice (7), we did not observe any changes in villus morphology, goblet cell abundance, or weight (Supplementary Fig. S4F–S4K). These data suggest that systemic Ets1 inhibition only partially recapitulates the phenotype of systemic Notch inhibition with GSIs and induces limited toxicity.

ETS1 Promotes NOTCH1-Driven Oncogenic Pathways

We next sought to understand the underlying mechanism of the Ets1 requirement for Notch1-induced T-ALL initiation and maintenance. Toward this goal, we performed RNA sequencing (RNA-seq) in THP-6 T-ALL cells following ETS1 knockdown and NOTCH1 inhibition using GSI. These analyses revealed broadly overlapping signatures between ETS1 and NOTCH1 deprivation. About 33% of ETS1-regulated genes...
Figure 4. (Continued) Quantitation of villus length (G and J), crypt depth (H and K), and fraction of PAS+ area (I and L) from Rosa26CreER<sup>12</sup> control and Ets1<sup>−/−</sup>Rosa26CreER<sup>12</sup> mice after the third dose of tamoxifen (G–I) or 5 days after 5 consecutive days of injection with DMSO/GSI (J-L; ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Duo., duodenum.

Figure 5. ETS1 promotes Notch-driven oncogenic pathways in T-ALL. A, Venn diagram showing ETS1 and NOTCH1 target genes in THP-6 cells. ETS1 target genes = P < 0.01 and fold change (FC) > 1.2 for the comparison of shControl versus both shETS1 (3/2). NOTCH1 target genes = P < 0.01 and FC > 1.2 for the DMSO versus GSI comparison. Gene set enrichment analysis (GSEA) using the MSigDB HALLMARK_MYC_V2 list for the shControl versus shETS1-3/2 (B) and DMSO versus GSI (C) comparisons. D, Violin plot of RNA-seq Log<sub>2</sub>FC data showing the shControl versus shETS1-3 comparison and selected NOTCH1 (yellow) and non-NOTCH1 (blue) target genes. GSEA using list of shared NOTCH1 target genes in T-ALL (45) for the shUTR versus shETS1-4 comparison in HPB-ALL cells (E), and heatmaps of the top 28 downregulated genes (red box in E) in HPB-ALL cells merged with the corresponding genes for the shControl versus shETS1-3/2 comparison and the DMSO versus GSI comparison in THP-6 cells (F). P values represent Mantel-Haenszel chi<sup>2</sup> tests of association for the indicated comparisons between HPB-ALL cells and THP-6 cells. G, Violin plot of ETS1 ChIP-seq Log<sub>2</sub>FC in THP-6 cells showing the shControl versus shETS1-3 comparison and target genes in D that are located within the same TAD (GSM967126; ref. 65) as the ETS1 peaks. H, Violin plots showing the ETS1 ChIP-seq Log<sub>2</sub>FC in THP-6 cells of all ETS1 peaks and dynamic ETS1 peaks. Number of peaks of each type are indicated. Dynamic ETS1 peaks = union intervals with Log<sub>2</sub>FC > 0 and FDR < 0.1 for both shControl versus shETS1-3/2 comparisons. Red line = median. I, Violin plots showing the H3K27ac ChIP-seq Log<sub>2</sub>FC at all ETS1 peaks, dynamic ETS1 peaks, and ETS1 peaks that cobind RBPJ or ZMIZ1 peaks that decrease (FDR < 0.1) with both shETS1 (3/2) ETS1 and H3K27ac ChIP-seq tracks in THP-6 cells transduced with shETS1-3 at the TADs encompassing the MYC (J) and MYB (K) genomic loci. "ETS1-dependent enhancers = sites of dynamic ETS1 peaks where H3K27ac decreases with FDR < 0.1." *a and "b" are biological replicates. CUTL1 ChIP-seq (GSE51800; ref. 15), H3K27ac Hi-ChIP (GSM3967135; ref. 65), and Hi-C TAD (GSM3967126; ref. 65) datasets are shown. NES, normalized enrichment scale, with 4 hours after GSI washout.

were also controlled by NOTCH1 (Fig. 5A). Conversely, about 22% of NOTCH1-controlled genes were also regulated by ETS1. Of the 467 ETS1-NOTCH1 coregulated genes, 290 (62%) were regulated in the same direction. Interestingly, pathway analyses showed Myc target genes as the most prominent hallmark signature enriched in ETS1-induced and NOTCH1-induced genes (ETS1 NES = −5.81, FDR < 0.0001; NOTCH1 NES = −6.15, FDR < 0.0001; Fig. 5B and C; Supplementary Table S1). Consistently, pathway analyses showed that the list of genes containing at least one Myc binding motif (CACGTG) and the C6 oncogenic signature of Myc-induced genes were among the topmost enriched in ETS1-induced and NOTCH1-induced genes (FDR < 0.0001; Supplementary Table S1; Supplementary Fig. S5A and S5B). In addition, pathway analyses showed that the C6 oncogenic signature of mTOR-induced genes and the mTOR-related Rapamycin_Response and Leucine_Deprivation gene sets were highly enriched in ETS1-induced and NOTCH1-induced genes (FDR < 0.01; Supplementary Table S1; Supplementary Fig. S5A and S5B). These results reinforce the well-established roles for Myc and mTOR as major oncogenic effector pathways in Notch1-induced T-ALL (2). To test the importance of MYC and mTOR as downstream effectors of the ETS1-driven oncogenic/dependency programs, we expressed Myc or myr-AKT in THP-6 cells (Supplementary Fig. S5C) and CEM cells (Supplementary Fig. S5D) using previously described constructs (44). Enforced expression of Myc or myr-AKT was sufficient for partial rescue from the antiproliferative effects of ETS1 deprivation. These data support the prominent role of MYC and mTOR signaling in ETS1 programs convergent with NOTCH1 in the control of T-ALL proliferation. Moreover, it did not escape our attention that several known direct NOTCH1 target genes were downregulated by ETS1 knockdown, including MYC, NOTCH3, HES1, DTX1, SHQ1, NOTCH1, and IIL7R (Q < 0.05; Fig. 5D; Supplementary Fig. S5E–S5G; Supplementary Table S2).

Similarly, ETS1-regulated genes in HPB-ALL cells (Supplementary Table S3) were significantly enriched in a core set of NOTCH1-regulated genes shared among seven T-ALL cell lines (ref. 45; P < 0.001; Fig. 5E). Moreover, corner gene analysis showed markedly concordant effects of ETS1.
deprivation between THP-6 and HPB-ALL (P = 1E-190; Supplementary Fig. S5i and S5j; Supplementary Table S2). Of the top 28 NOTCH1-induced genes, most downregulated by shETS1-4 in THP-6 ALL-cells, 23 were also downregulated by shETS1-3 in THP-6 cells (P = 9.0E-14; Fig. S5j; Supplementary Table S2), including well-established NOTCH1-induced target genes like MYC, DTX1, HES1, and NOTCH3 (Fig. 5d and F; Supplementary Fig. S5h). In all, the results show that ETS1 and NOTCH1 coinduce shared target genes and oncogenic pathways across multiple T-ALL cell lines.

***Dynamic ETS1 Binding Increases H3K27ac at GATA-Associated Response Elements***

We next asked, what are the direct effects of ETS1 on enhancer and transcriptional activation? To answer this question, we performed ETS1 chromatin immunoprecipitation sequencing (ChIP-seq) in THP-6 cells following ETS1 knockdown. We defined “dynamic ETS1 peaks” as those with differential ChIP-seq read count FDR < 0.1 for two independent ETS1 shRNA knockdowns compared with controls. Using these parameters, we identified 3,697 dynamic ETS1 peaks (Fig. 5g; Supplementary Fig. S5k) in which ETS1 knockdown significantly reduced ETS1 occupancies compared with all ETS1 binding sites (Fig. 5h). Only approximately 15% of ETS1 peaks met criteria as dynamic ETS1 peaks. Moreover, transcription factor motif analysis showed that 70% of dynamic ETS1 peaks contained the HOMER Ets1 motif compared with only 35% of nondynamic ETS1 peaks, indicating that dynamic ETS1 peaks could signify a class of high-confidence ETS1 binding sites. Next, we identified dynamic ETS1 peaks located within the same topologically associating domain (TAD) of 1,739 genes differentially expressed after ETS1 knockdown (DEG; Q < 0.05 for both shETS1) and designated these loci as “high-confidence direct ETS1 target genes” (Fig. 5g; Supplementary Fig. S5k; Supplementary Table S4). Among these, 1,011 genes showed chromatin looping linking promoter regulatory sequences with a distal dynamic ETS1 peak based on H3K27ac Hi-ChIP (Supplementary Table S4). Of these genes, 430 (43%) were also NOTCH1 target genes at Q < 0.05. These data suggest that ETS1 directly co-regulates a large fraction of the Notch transcriptional program.

To evaluate the functional role of ETS1 in enhancer regulation, we performed H3K27ac ChIP-seq in THP-6 cells in basal conditions and following ETS1 knockdown. In these experiments, ETS1 deprivation reduced H3K27ac read counts at dynamic ETS1 peaks compared to all ETS1 peaks (Fig. 5i). Mean Log₂FC differences were -0.479 and -0.412 for shETS1-3 and shETS1-2 compared with controls, respectively, in line with the known interaction of ETS1 with P300/ CBP acetyl transferases. However, only approximately 51% of dynamic ETS1 peaks were associated with differential H3K27ac read counts. To better understand the regulatory logic by which ETS1 facilitates activation of response elements, we performed de novo HOMER motif analysis of dynamic ETS1 peaks associated with differential H3K27ac read counts following ETS1 knockdown (Supplementary Fig. S5l). These analyses recovered an Ets family motif as the top hit and a sequence closely matching GATA family motifs as the second most frequent element associated with dynamic ETS1 peaks linked to H3K27ac regulation compared with all other dynamic ETS1 peaks (26% vs. 16%; P = 1E-8). On the basis of these findings, we propose that ETS1 and GATA3 cooperate in promoting enhancer activation.

To better understand the impact of ETS1 control of oncogenic programs in T-ALL, we examined the effect of ETS1 deprivation on enhancers located in the same TADs containing MYC (Fig. 5j) and MYB (Fig. 5k), two major T-ALL oncogenes. H3K27ac Hi-ChIP data identified the N-ME (purple box, Fig. 5j) as the only enhancer associated with the MYC promoter in CUTLL1 cells at FDR < 1E-10. In contrast, multiple enhancers were associated with the MYB promoter (Fig. 5k). Two MYB enhancers showed dynamic NOTCH1/RBPJ binding associated with differential H3K27ac signals (Fig. 5k). In this context, ETS1 deprivation reduced ETS1 and H3K27ac read counts at the N-ME and multiple MYB-associated enhancers, including the Notch-bound MYB distal regulatory sites. Importantly, we observed convergent and largely overlapping ETS1 and H3K27ac peaks associated with MYC and MYB in THP-6 and CUTLL1 cells, supporting a common regulatory logic across T-ALL tumors. Overall, 72% of ETS1 peaks identified in THP-6 cells were detected in CUTLL1 cells (Supplementary Fig. S5m). Altogether, these results indicate a prominent role for ETS1 binding in the activation of enhancers driving oncogene expression in T-ALL.

***ETS1 Interacts with NOTCH1 and Cooccupies Most ICN1/RBPJ Binding Sites***

The convergent transcriptional effects of ETS1 and NOTCH1 in T-ALL led us to ask the question, does ETS1 interact closely with NOTCH1? To answer this question, we first tested whether ETS1 could physically bind the Notch transcriptional complex. Accordingly, communoprecipitation experiments in THP-6 and HPB-ALL cells showed interactions between endogenous ETS1 and activated intracellular NOTCH1 proteins (Fig. 6a; Supplementary Fig. S6a). In
ETS1-Dependent Control of Notch Transcriptional Programs

De novo motif analysis of 65 RBPJ peaks that cobind dynamic ETS1 peaks and change with FDR<0.1 for shCtrl vs. shETS1

<table>
<thead>
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Known motif analysis (N = 65) (FDR<0.1) vs. shETS1

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<td>35%</td>
</tr>
<tr>
<td>1E-02</td>
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Notch-MYCe (N-ME) Notch-MYBe Notch-HES1p Notch-NOTCH3e Notch-DTX1e

FDR<0.1 for shCtrl vs. shETS1-2b shETS1-2a shETS1-3b shETS1-3a

Log2 FC ICN1 tags

-6 -4 -2 0 2 4 6

ETS1-Dependent Control of Notch Transcriptional Programs

A

B

C

D

E

F

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addition, we verified interactions between ETS1 and ZMIZ1, a context-dependent direct transcriptional cofactor of Notch1 (33, 46). Next, we performed ICN1, RBPJ, and ZMIZ1 ChIP-seq in control and shETS1-transduced THP-6 cells. These analyses identified 971 sites where ETS1, ZMIZ1, ICN1, and RBPJ peaks overlapped (Fig. 6B). Remarkably, 94% of overlapping ICN1 and RBPJ peaks contained an overlapping ZMIZ1 peak. Moreover, 35% and 28% of ETS1 peaks were cobound by RBPJ and ZMIZ1, respectively. In contrast, 77%, 73%, and 85% of ICN1 peaks were cobound by ETS1, RBPJ, and ZMIZ1, respectively. Furthermore, analysis of the 24,552 THP-6 ETS1 binding sites with ETS1, NOTCH1, RBPJ, and H3K27ac peaks in CUTLL1 and JURKAT cells revealed largely overlapping signals (Supplementary Fig. S6B and S6C). These results support that ETS1 binds NOTCH1 and cooccupies Notch complex binding sites.

**ETS1 Facilitates Recruitment of Notch Complex Members and H3K27ac Deposition at Response Elements Enriched for GATA Factor Binding Motifs**

Because ETS1 and NOTCH1 cobind chromatin and physically interact, we next asked, does ETS1 facilitate the recruitment of Notch complex members to chromatin? To answer this question, we investigated whether ETS1 deprivation could reduce RBPJ occupancy at sites where RBPJ peaks overlap with dynamic ETS1 peaks. Accordingly, ETS1 deprivation reduced read counts of RBPJ peaks that overlapped with dynamic ETS1 peaks compared with all RBPJ peaks (Fig. 6C, top). Mean RBPJ ChIP-seq Log2FC differences were −0.517 and −0.436 by shETS1-1 and shETS1-2 compared with controls, respectively. Similarly, ETS1 deprivation reduced read counts of ICN1 and ZMIZ1 peaks that overlapped with dynamic ETS1 peaks compared with all ICN1 and ZMIZ1 peaks, with mean Log2FC differences of −0.895 and −0.665 for ICN1 (Fig. 6D) and −0.921 and −0.780 for ZMIZ1 (Fig. 6E) by shETS1-3 and shETS1-2 compared with controls, respectively. Consistently, metagene plots showed that ETS1 deprivation reduced average read counts of RBPJ and ICN1 peaks that overlapped with dynamic ETS1 peaks (Supplementary Fig. S6E) compared with all RBPJ and ICN1 binding sites (Supplementary Fig. S6D). We conclude from these observations that ETS1 can facilitate the recruitment of Notch complex members to a subset of the regulatory sites controlling the transcription of Notch targets in T-ALL.

To better understand the mechanisms by which ETS1 facilitates recruitment of RBPJ to chromatin, we first defined “dynamic RBPJ peaks” as sites with differential RBPJ read counts following ETS1 knockdown (FDR < 0.1). Next, we performed motif analysis on dynamic ETS1 peaks that overlapped with dynamic RBPJ peaks. Similar to what we found for dynamic ETS1 peaks associated with differential H3K27ac signals (Supplementary Fig. S5L), a GATA family motif was the top-ranked non-ETS motif present in these locations. Interestingly, this GATA family motif was more closely associated with dynamic ETS1 peaks that overlapped with dynamic RBPJ peaks than dynamic ETS1 peaks that overlapped with nondynamic RBPJ peaks (43% vs. 17%; P = 1E-3; Fig. 6C, bottom). Similar associations with GATA3 were found for dynamic ICN1 peaks (P = 1E-10; Supplementary Fig. S6F) and dynamic ZMIZ1 peaks (P = 1E-10; Supplementary Fig. S6G). These data suggest that ETS1 cooperates with GATA3 to facilitate the recruitment of Notch factors to transcriptional control sites.

Because the Notch complex is known to facilitate H3K27ac deposition, we wondered whether ETS1 deprivation would weaken H3K27ac signals at dynamic ETS1 sites where RBPJ binding was reduced. In support of this hypothesis, ETS1 knockdown significantly reduced H3K27ac read counts at dynamic ETS1 peaks that overlap with dynamic RBPJ peaks compared with all dynamic ETS1 sites (Fig. 5I). H3K27ac ChIP-seq mean Log2FC differences were −0.292 and −0.152 for shETS1-3 and shETS1-2 compared with controls, respectively. Similar results were found for dynamic ZMIZ1 peaks. We next considered the possibility that ETS1 recruits Notch to response elements that regulate shared NOTCH1/ETS1 target genes (Fig. 5D and F; Supplementary Fig. S5I). To test this possibility, we examined ETS1, RBPJ, and H3K27ac ChIP-seq tracks at Notch-bound response elements of MYC (N-ME), MYB (Notch-MYBe), HES1 (Notch-HES1p), NOTCH3 (Notch-NOTCH3e), and DTX1 (Notch-DTX1e; Fig. 6F). ETS1 knockdown in THP-6 cells reduced RBPJ and H3K27ac read counts at the Notch-MYBe, Notch-HES1p, Notch-NOTCH3e, and Notch-DTX1e enhancers, but not N-ME. In contrast, ETS1 knockdown significantly reduced RBPJ signals at the N-ME in CEM cells (Supplementary Fig. S6H and S6I). These results further support that ETS1 might cooperate with GATA3 to facilitate recruitment of Notch to activate chromatin.

**Ets1 Deprivation Sensitizes Leukemic Cells to Notch Inhibition**

Several strategies have been proposed to maximize the therapeutic activity of anti-Notch therapies, including the combination of a GSI with agents targeting critical effectors downstream of Notch (43, 47, 48). Because our data show that ETS1 promotes MYC and mTOR (Supplementary Fig. S5A; Supplementary Table S1), two major oncogenic pathways downstream of NOTCH1 (48, 49), we wondered whether suppressing Ets1 signals might confer increased sensitivity to low doses of GSI. To test this possibility, we knocked down ETS1 in human T-ALL cell lines and treated them with a partially inhibitory dose of GSI (0.2 μmol/L; Supplementary Fig. S7A). As expected, partial Notch inhibition had only modest effects on the growth of GSI-sensitive cell lines (Supplementary Fig. S3H–S3J). ETS1 deprivation sensitized five of six cell lines to partial Notch inhibition, including all three GSI-resistant lines (CEM, THP-6, and JURKAT; Fig. 3C and D; Supplementary Fig. S5I, S3J, and S3L). Consistently, ETS1 knockdown and NOTCH1 deprivation cooperatively reduced MYC expression in THP-6 cells (Fig. 7A). Moreover, Ets1 deletion and Notch inhibition with a GSI in Notch-induced Rosa26CreERT2 Ets1iTRA murine T-ALL cells cooperatively downregulated Myb and Dtx1 expression (Fig. 7B–D). In addition, and of therapeutic relevance, in vivo deletion of Ets1 by tamoxifen treatment in this model enhanced the antileukemic effects of intermittent GSI dosing, resulting in reduced peripheral blood T-ALL blasts and increased survival compared with controls (Fig. 7E–H; Supplementary Fig. S7B). In all, these results support a therapeutic role for Ets1 inhibition in combination with anti-Notch1 therapies for the treatment of T-ALL.
Figure 7. Ets1 deprivation sensitizes Notch-activated T-ALLs to the pan-Notch inhibitor GSI. A, Relative MYC expression in THP-6 cells transduced with shETS1 for 4 days and treated with GSI (DBZ, 1 μmol/L) 1 day prior to harvest. Expression of Ets1 (B), Myb (C), and Dtx1 (D) in murine Rosa26CreER^{T2} Ets1^{f/f} cells (#643; GFP-positive T-ALL cell content 99.5%) treated with 12 nmol/L OHT to induce Ets1 deletion for 42 hours ± low-dose GSI for 18 hours (DBZ, 0.1 μmol/L). E–H, Experimental strategy to study effects of combinatorial effects of Ets1/Notch deprivation on in vivo maintenance of murine ΔE/Notch1-induced T-ALL (#182; E). GSI, 10 μmol/kg DBZ; Tam, 25 mg/kg tamoxifen. Representative flow cytometric plots (F) and peripheral blood GFP^+ T-ALL cell counts (G) at 26 days posttransplant, and survival curves (H) (ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
DISCUSSION

During T-ALL transformation, Notch1 can become supraphysiologically activated or “hijacked,”” leading to widespread, intense expression of cleaved intracellular Notch1 (50). Current models support that Notch in turn hijacks its normal cellular partners including the co-binding transcription factors that it normally relies upon to promote T-cell development. In this context, such “Notch-collaborating” transcription factors might comprise new vulnerabilities in Notch-dependent cancers like T-ALL. These concepts are clinically relevant as continuous pan-Notch inhibition with GSI is poorly tolerated (43).

Here, we present evidence that Ets1 is a Notch-collaborating transcription factor and a potential therapeutic target in T-ALL. Using mouse models, we show that Ets1 inactivation resembled the Notch1 inhibition phenotype by impairing ETP specification and the DN-to-DP transition, which are the two major Notch-dependent steps during early T-cell development. Ets1 inactivation also impaired leukemic proliferation and Notch-induced gene expression in complementary mouse and human models of Notch-activated T-ALL. Accordingly, ChIP-seq analyses of T-ALL cells showed that approximately 80% of ICN1/RBPJ sites were co-occupied by ETS1. Compared with Notch inhibition, the effects of Ets1 inactivation were strong in T-cell precursors or T-ALL cells but relatively weak in the intestine. Thus, Ets1 might have more substantial Notch-collaborating functions in the context of T-cell development and leukemogenesis than in the context of intestine stem cell differentiation.

It is worth noting that outside the T-cell compartment Ets1 plays a role in the maturation of other hematopoietic cells, such as B cells (31, 39, 42, 51–59). Thus, even though the effects of Ets1 on thymopoiesis are cell autonomous (17, 18) and B cells are dispensable for early T-cell development, it cannot be ruled out that some effect of Ets1 inactivation in non–T-cell hematopoietic populations could influence the thymic phenotypes described here.

Functional analysis of ETS1 binding to chromatin sites in T-ALL revealed that only a minority of ETS1 peaks were suppressed upon ETS1 knockdown (dynamic ETS1 peaks). This finding is reminiscent of an earlier study showing that fewer than 10% of NOTCH1 peaks in CUTLL1 cells were dynamic when switching between Notch-on and Notch-off states (15). Like dynamic ETS1 peaks, dynamic NOTCH1 peaks were associated with differential H3K27ac read counts (15). For ETS1, our motif analyses suggest that dynamic peaks might signify a class of high-confidence binding sites that are highly sensitive to ETS1 dose reduction. In contrast to the high percentage of ICN1/RBPJ peaks that are co-occupied by ETS1, a much smaller percentage of ETS1 peaks were co-occupied by ICN1/RBPJ. Consistently, we identified several ETS1 target genes that, to our knowledge, have not been linked to dynamic NOTCH1 peaks or implicated as direct NOTCH1 target genes in publicly available T-ALL gene expression screens. Many of these genes, such as GATA3, LYL1, PTPN11, LM02, LCK, and HHX5, have prominent roles in early T-cell development and leukemia transformation.

ETS1 loss often reduced H3K27ac read counts without affecting ICN1/RBPJ binding. However, we observed a small subgroup of ETS1 sites containing GATA motifs where ETS1 knockdown reduced ICN1/RBPJ binding and strongly reduced H3K27ac tags. Of note, this is a discrete feature suggestive of a strong functional interaction, as GATA motifs were uncommonly associated with ETS1 peaks (~15% of total). Combined with our observations of protein–protein interactions between ETS1 and NOTCH1 in two T-ALL cell lines, these data suggest the possibility of context-dependent transcriptional complexes that stabilize NOTCH1 complex interactions with chromatin (Supplementary Fig. S8). GATA3 is the predominant GATA family member expressed in T-ALL cells and is the only GATA family member expressed in the cell line we analyzed by ChIP-seq. It is possible that GATA3 might act as a scaffold linking ETS1 to the NOTCH1 complex. GATA3 pioneering factor activity can facilitate nucleosome eviction at the N-ME to promote transcription factor binding (12). Thus, GATA3-driven chromatin remodeling might help other proteins “connect” ETS1 to the Notch complex. Accordingly, our motif analysis suggests that transcription factors other than GATA3 are associated with dynamic changes of Notch factors and H3K27ac upon ETS1 knockdown. Moreover, because GATA3 is an ETS1-induced gene, ETS1 might indirectly promote Notch complex recruitment and H3K27ac deposition through GATA3 induction.

Therapeutically, our study demonstrates for the first time that it is possible to disengage activated Notch from its chromatin and gene expression functions in cancer cells without directly targeting the formation of the NOTCH1–RBPJ–MAML1 ternary complex. A corollary of these results is that combination strategies with anti-ETS1 and anti-Notch agents could synergize to enhance the antileukemic effects of Notch suppression. Finally, our study supports a model in which Notch-collaborating partner transcription factors like Ets1 create a favorable chromatin context for Notch1 to activate a subset of response elements. This evolutionary conserved principle already present in Drosophila (9) can be relevant to human disease as we show that context dependence through Ets1 could be exploited to oppose parts of the Notch downstream pathway in cancer with less toxicity than pan-Notch inhibitors. Moreover, given emerging data supporting a role for ETS1 in tumors such as breast, ovarian, and colon cancer (60) that are Notch dependent (1), our findings raise the possibility that ETS1 could cooperate with Notch to drive transformation in a diverse number of human cancers.

METHODS

Mice

C57BL/6 mice ranging from 4 weeks to 8 weeks of age were obtained from Taconic for bone marrow transplantation experiments. Rosa26CreER<sup>T2</sup> Ets<sup>Flox</sup> mice were generated by crossing Ets<sup>Flox</sup> mice (M.C. Ostrowski; submitted for publication) with Rosa26CreER<sup>T2</sup> mice (Jackson). Rosa26CreER<sup>T2-alt</sup> Ets<sup>Flox-alt</sup> mice were generated by crossing Ets<sup>Flox-alt</sup> mice (39) with Rosa26CreER<sup>T2-alt</sup> mice (40). The “alt” term was used to avoid confusion with the Jackson/Ostrowski Rosa26CreER<sup>T2</sup> Ets<sup>Flox</sup> mouse model described above. In Ets<sup>Flox</sup> and Ets<sup>Flox-alt</sup> mice, the Ets1 conditional allele was constructed by flanking exons 7 and 8 with loxP sites. Ets<sup>Flox</sup> mice, a gift from Dr. Garrett-Sinha (University at Buffalo, Buffalo, NY), were described previously (42).
In these mice, the exons 3 and 4 of Ets1 (containing the PNT domain) were deleted, thus creating a hypomorphic "p" allele (31). Notch1
t and VarCre (also known as "Var-1 Cre") mice were obtained from The Jackson Laboratory. VillinCreER
t mice were obtained from S. Robine, Institut Curie, Paris, France (61). Mice used for T-cell developmental studies were 5 to 8 weeks of age. Mice used for Rosa26-CreERT2 and VillinCreERT2 experiments were 8 to 12 weeks old. Per animal protocol, mice were sacrificed when weight dropped to 80% or less of starting weight. All mouse experiments were performed according to NIH guidelines with approved protocols from the Institutional Animal Care and Use Committees at the University of Michigan (Ann Arbor, MI) and Columbia University Medical Center (New York, NY). A summary of the mice used is provided in Supplementary Table S5.

Histologic Analysis

Paraffin sections (5 μm) were stained with periodic acid-Schiff (PAS)/Alcian Blue (AB; Newcomer Supply) to assess mucin-containing goblet cells. PAS/Al-stained images from mouse intestines were quantified for proportion of blue staining (blue stained region in μm2/total villus region in μm2) using ImageJ software (NIH, Bethesda, MD). Villi length and crypt depth were traced and analyzed using the Measure tool. All quantitative analyses of the intestinal histology were performed by an observer who was blinded to the genotypes and treatment of the mice.

Cell Lines

Jurkat cells were provided by Jon Aster (Harvard University, Boston, MA). CEM cells were CEM/SS (a subclone of CCRF-CEM), which were provided by Katherine Collins (University of Michigan, Ann Arbor, MI). THP-6, DU.528, MOLT4, DND-41, and SUP-T1 cells were provided by Andrew Weng (Terry Fox Laboratory, Vancouver, BC, Canada). THP-6 is a GSI-resistant LYL1/LMO2-type T-ALL that expresses ICN1 (33, 46). HPB-ALL cells were obtained from DSMZ. LOUCY cells were obtained from ATCC. OP9-DL4 cells were provided by J.C. Zuniga-Pﬂucker (University of Toronto, Toronto, Ontario, Canada). Six hundred forty-three cells were established from a primary Rosa26-CreERT2 Ets1
t splenic tumor. In these cells, Ets1 can be deleted in vitro via the administration of 4-hydroxystauroxifen. All human cell lines were authenticated using STR analysis prior to use (Genetica Corporation). All cell lines were cultured less than 3 months after resuscitation. All cell lines were cultured less than 3 months after resuscitation. All cell lines were cultured less than 3 months after resuscitation. All cell lines were cultured less than 3 months after resuscitation.

Cell Culture Conditions

T-ALL cell lines were grown in RPMI1640 (Invitrogen) supplemented with 10% FBS (HyClone or Gibco), 2 mmol/L L-glutamine, 2-mercaptoethanol [0.0005% (v/v), Sigma], penicillin, and streptomycin. Two hundred ninety-three T cells were maintained in DMEM (Invitrogen) with the same supplements except 2-mercaptoethanol. Cells were grown at 37°C under 5% CO2. Retroviral and lentiviral transduction of T-ALL cells and sorting or selection were performed as described previously (62, 63). DBZ (GSI) was obtained from EMD chemicals (for growth assays). Cells were analyzed via flow cytometry. Cells were analyzed via flow cytometry. Cells were analyzed via flow cytometry. Cells were analyzed via flow cytometry. Cells were analyzed via flow cytometry.
ChIP-seq Library Preparation and Sequencing

ChIP chromatin was prepared as described above from THP-6 cells in biological duplicates with shControl (SHC002, Sigma), shETS1-3 (TRCN000005591, Sigma), or shETS1-2 (TRCN00001916, Sigma), treated at 48 hours with puromycin, and harvested after an additional 48 hours. ChIP-seq libraries were prepared as described previously (64). Briefly, ChIP DNA was end-repaired (End-It, Epicentre), A-tailed (Klenow fragment 3′→5′ exo-, New England Biolabs), and ligated to barcoded Illumina adaptors (Quick T4 DNA ligase, NEB; adaptors produced by RIKEN). PCR amplification was followed by cleanup with SPRI beads (AmpureXP, Beckman Coulter). Ligation products were amplified by 14 cycles of PCR with Illumina indexing primers and Pfu Ultra II HS PCR mix (Agilent). Library size selection for 300 to 600 bp chromatin was performed using two-step SPRI bead selection (AmpureXP, Beckman Coulter). Library size was confirmed via Tapestation D1000 (Agilent). Final libraries were sequenced with 75-cycle paired end (38 bp × 2) on a Nextseq (Illumina) according to manufacturer’s protocols.

ChIP-seq Alignment, Filtering, Track Generation, Peak Calling, and Overlaps

Reads were aligned to the hg19 genome assembly using bwa-aln (bwa version 0.7.12). Data were filtered to remove PCR duplicates and reads mapping to >2 genomic sites. All peak sets were also post-filtered for known ENCODE blacklist regions (available at http://hgdownload.cse.ucsc.edu/goldenpath hg19/encodeDCC/wgEncode Mapability/wgEncodeDacMapabilityConsensusExcludable.bed.gz). Filtered bam files were marked as “pruned bam files.” Next, bigwig display files were generated with igvtools count and deepTools bamCoverage. Bed peak files were generated with HOMER findPeaks (-style-factor, -exclude factors). Scaling for all ChIP-seq tracks in the figures is equal to local paired-end fragment coverage × (1,000,000/totalCount). To determine overlaps between ETS1, NOTCH1, ZMIZ1, and RBPJ peaks, we first concatenated the two shControl bioreplicates for each transcription factor, using mergeBed in BEDTools. We then identified the total number of peaks for each transcription factor as well as the overlaps between the transcription factors using intersectBed in BEDTools. These data were then plotted in a Venn diagram using Microsoft Excel.

Identification of High-Confidence ETS1 Direct Target Genes

To identify high-confidence ETS1 direct target genes, we concatenated peaks from the two shControl ETS1 ChIP-seq biological replicates and merged overlapping ones using mergeBed in BEDTools. We performed differential binding analysis comparing shETS1-3 or shETS1-2 versus shControl using DiffBind (2.14.0; http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf). Because DiffBind can use either DESeq2 or edgeR to do normalization and differential tests, it was run twice to generate results from both methods. The DESeq2 method was used, as it identified more differentially regulated peaks than edgeR. We defined “dynamic ETS1 peaks” as ETS1 peaks that gave FDR < 0.1 for the shControl versus shETS1-3 comparison and the shControl versus shETS1-2 comparison. Next, we extracted from the RNA-seq data in THP-6 cells (above) the DEGs that were shared between the shControl versus shETS1-3 and shControl versus shETS1-2 comparisons requiring Q < 0.05 in both and the changing direction to be the same. We extracted TADs based on Hi-C data in CUTLL1 cells (GSE134761; ref. 65). For each dynamic ETS1 peak, we found the TAD(s) it intersected with and the DEGs that intersected with the same TAD(s) using intersectBed in BEDTools. TAD boundaries were first extended to cover the flanking gaps. These DEGs were designated as high-confidence direct NOTCH1 target genes as they were linked to at least one ETS1 dynamic peak within the same TAD as the DEG. Dynamic ETS1 peaks that overlapped with two adjacent TADs were assigned to both TADs. DEGs that overlapped with at least two adjacent TADs were assigned to both TADs. We also extracted H3K27ac Hi-ChIP intervals in CUTLL1 cells (GSE134761; ref. 65). We filtered the DEGs to include only those DEGs with TSS within Hi-ChIP intervals that were linked to another Hi-ChIP interval that contained at least one dynamic ETS1 peaks using R version 3.6.1 with tidyverse (1.3.0). The DEGs linked to dynamic ETS1 peaks within the same TAD and the filtered DEGs that were additionally linked to dynamic ETS1 peaks through H3K27ac Hi-ChIP are listed in separate tabs in Supplementary Table S4.

Comparative ChIP-seq Analysis

To determine differential binding of Notch complex members and differential H3K27ac signals, we created a set of union intervals by merging the bed files for the ETS1, NOTCH1, RBPJ, and ZMIZ1 peaks. Narrow windows for transcription factors were 200 bp, centered on the union interval peak. Broad windows for H3K27ac were 2,000 bp, centered on the union interval peak. For each of the union intervals, we found the intersection counts and overlap fraction from each factor’s peak sets, using annotateBed in BEDTools. We used annotatedPeaks.pl in HOMER to calculate normalized tag count distribution surrounding the interval centers. DiffBind (2.14.0) was run twice to generate results using both DESeq2 and edgeR methods. DESeq2 was used for ETS1 and H3K27ac ChIP-seq data. edgeR was used for ICN1, RBPJ, and ZMIZ1 ChIP-seq data. Binding/H3K27ac quantitation and differential binding/H3K27ac analysis were then obtained. For violin plot analyses, we defined transcription factor peaks as union intervals that intersected with at least one peak of that transcription factor in at least one control bioreplicate. Unless otherwise indicated, we defined “dynamic ETS1 peaks” as union intervals that intersected with at least one ETS1 peak in at least one control bioreplicate and that gave FDR < 0.1 for the shControl versus shETS1-3 comparison and the shControl versus shETS1-2 comparison. Likewise, we defined dynamic ICN1, RBPJ, and ZMIZ1 peaks as union intervals that intersected at least one peak of that factor in at least one control bioreplicate and gave FDR < 0.1 for the shControl versus shETS1-3 comparison and the shControl versus shETS1-2 comparison. To generate metagene plots, two interval sets were selected: (i) “All Peaks” = intervals that intersect with at least one peak of that factor in all of the shETS1 bioreplicates (shETS1-3 and shETS1-2) or intersect with at least one peak of that factor in both shControl replicates and (ii) intervals from (i) that intersect with at least one dynamic ETS1 peak. To generate metagene plots in Supplementary Fig. S6D and S6E, we used annotatedPeaks.pl in HOMER to calculate normalized tag count distribution surrounding the interval centers for each of the two groups (i and ii) above and plotted them using R. We also calculated the normalized tag count distribution for (iii) the broader H3K27ac intervals that intersected with at least one ETS1 peak in all of the shETS1 bioreplicates (shETS1-3 and shETS1-2) or intersected at least one ETS1 peak in both shControl replicates and (iv) broad intervals from (iii) that intersected with at least one dynamic ETS1 peak. Volcano plots for ETS1 ChIP-seq differential binding were generated using R Version 3.6.1 with the following packages: tidyverse (1.3.0), ggplot2 (0.8.1), extrafont (0.17), and stringr (1.4.0). De novo DNA motif enrichment analysis was performed with HOMER findMotifs, using findMotifsGenome.pl with a setting of -size 200.
The background peakset was HOMER generated and GC content normalized. Known motif analysis was performed with the HOMER motif library version 4.10.4, using two sets of peaks: (i) peaks identified as differentially bound by the given transcription factor in both the shETS1-3– and shETS1-2–treated cells (vs. shControl, FDR < 0.1) and (ii) peaks that were NOT identified as differentially bound by the given transcription factor in either the shETS1-3 or shETS1-2 comparison (vs. shControl, FDR > 0.1). To obtain P values for the enrichment of the GATA family motif in the first peakset versus the second peakset, we inputted the first peakset as the “target” peaks and the second peakset as the “background” peaks and the known motif Gata4(ZF)/Heart_Gata4_Chip-seq (GSE35151) into the HOMER find-MotifsGenome.pl command. De novo DNA motif analyses were also performed for all ETS1, ICN1, and RBPJ peaks (Supplementary Fig. S9).

Deposition of Sequences

The high-throughput sequencing and microarray data were deposited in the Gene Expression Omnibus database with accession GSE138660 for the superseries. Subseries include GSE138516 (ChIP-seq), GSE138659 (RNA-seq), and GSE138803 (microarray).

Disclosure of Potential Conflicts of Interest

A.C. McCarter reports grants from NIH (T32-GM007315, T32-GM007863, and F30-CA228228) during the conduct of the study. G. Della Gatta reports other from Regeneron Pharmaceuticals (currently an employee of Regeneron Pharmaceuticals studying the molecular basis of obesity and other metabolic syndromes) outside the submitted work. A. Melnick reports grants from NIH during the conduct of the study. J.K. Nalamolu reports grants from NIH during the conduct of the study. N. Kunnath reports grants from NIH (to institution, the University of Michigan) outside the submitted work. A. Rao reports other from Voxel Analytics, LLC (consulting) and grants from NIH, American Cancer Society (ACS), and Agilent Technologies outside the submitted work. L.C. Samuelson reports grants from NIH during the conduct of the study. M.C. Ostrowski reports grants from Medical University of South Carolina during the conduct of the study. A.A. Ferrando reports grants from NCI, NIH and Leukemia & Lymphoma Society during the conduct of the study and personal fees from Ayala Pharmaceuticals, SpringWorks Therapeutics, and Pfizer outside the submitted work. In addition, A.A. Ferrando has a patent for 20110098735 issued and with royalties paid from Pfizer, Merck; a patent for 20100003588 issued and with royalties paid from Pfizer, Merck; a patent for 20100093684 issued and with royalties paid from Pfizer; has patents for 20110118192, 8633179, and 8716233 issued; and his lab generated the CUTLL1 cell line, which is licensed to Novartis, EMD Millipore, and Applied Biological Materials. M.Y. Chiang reports grants from NIH, Rally Foundation for Childhood Cancer, Bear Necessities Pediatric Cancer Foundation Research (outside the submitted work), and Alex’s Lemonade Stand Foundation (during the conduct of the study), and Rally Young Professionals; and grants, personal fees, and non-financial support from the University of Michigan. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

A.C. McCarter: Formal analysis, funding acquisition, investigation, visualization, writing–original draft, writing–review and editing. G. Della Gatta: Formal analysis, investigation, visualization, writing–original draft, writing–review and editing. A. Melnick: Formal analysis, validation, investigation, writing–review and editing. E. Kim: Validation, visualization. C. Sha: Investigation, visualization. Q. Wang: Investigation, visualization. J.K. Nalamolu: Investigation, visualization. Y. Liu: Investigation, visualization. T.M. Keeley: Formal analysis, investigation, visualization, writing–review and editing. R. Yan: Investigation, visualization. M. Sun: Investigation. R. Kodgule: Investigation. N. Kunnath: Software, formal analysis. A. Ambesi-Impiombato: Software, formal analysis. R. Kuick: Data curation, software, formal analysis, writing–original draft, writing–review and editing. A. Rao: Supervision. R.J.H. Ryan: Conceptualization, resources, data curation, software, formal analysis, supervision, visualization, methodology, writing–original draft, writing–review and editing. B.L. Kee: Conceptualization, resources, supervision, funding acquisition, methodology, writing–original draft, writing–review and editing. L.C. Samuelson: Conceptualization, resources, supervision, funding acquisition, visualization, writing–original draft, writing–review and editing. A.A. Ferrando: Conceptualization, formal analysis, supervision, funding acquisition, visualization, methodology, writing–original draft, writing–review and editing. M.Y. Chiang: Conceptualization, formal analysis, supervision, funding acquisition, visualization, methodology, writing–original draft, writing–review and editing.

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REFERENCES


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