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MafB is a novel enhancer of oncogenic Notch signaling in T-cell acute lymphoblastic leukemia

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Abstract

Although activating NOTCH1 mutations in human T-cell acute lymphoblastic leukemia (T-ALL) are common, these naturally occurring Notch1 mutant alleles fail to efficiently induce leukemia in murine models. We performed a gain-of-function screen to identify gene products that enhanced signaling of leukemia-associated NOTCH1 mutants. Both MAFB and ETS2 emerged as candidates that individually enhanced Notch1 signaling, and when co-expressed, they synergistically increased signaling, comparable to levels induced by core components of the Notch transcriptional complex. In murine T-ALL models, MafB enhanced leukemogenesis of the naturally occurring Notch1 mutants, by decreasing disease latency and/or increasing penetrance. Suppressing MafB in mouse and human T-ALL cells reduced expression of Notch1 target genes, including Myc and Hes1, and sustained MafB knockdown impaired T-ALL cell growth in a competitive setting. MafB directly binds ETS2 and also interacts with PCAF and P300, highlighting its importance in recruiting co-activators that enhance Notch1 signaling. Together,

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Author Contributions: KVP designed and performed most of the experiments, interpreted all of the results, wrote the manuscript and assembled the figures. LX performed in vivo mouse experiments and bone marrow transplantations. LS performed the experiments and generated the data for Fig. 6. JP performed the H3K27Ac experiments for Fig. 3. KP generated data used in Fig. 6. YO and WB performed the array shown in Table S2. CL performed experiments shown in Fig S4 B–C. GBW analyzed the histology and tumor infiltration in leukemic transplants. RM and NM provided the ETS1 knockout bone marrow and helped design the experiments shown in Fig. S5. YL and JPPM analyzed the human T-ALL datasets shown in Fig. 2S. SCB helped design experiments and edited the manuscript. RBF helped design the Chip-Seq and analyzed the results for the experiment in Fig. 3. SC helped design the Notch gain-of-function screen, provided the cDNA library and assembled and analyzed the data shown in Fig. 1. WSP designed the experiments in this study, he interpreted all the data and helped write the manuscript.

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these data present a novel mechanism for enhancing the oncogenic potential of weak Notch1 mutants in leukemia and introduce the MAFB-ETS2 transcriptional axis as a potential therapeutic target in T-ALL.

INTRODUCTION

T-cell Acute Lymphoblastic Leukemia (T-ALL) is an aggressive malignancy of immature T-cell blasts that occurs in both children and adults (1). Although current treatments are relatively successful, especially in children (2), ~20% of patients and those with relapsed disease fail to respond to therapy (1). Activating mutations in *NOTCH1* are frequent, occurring in >60% of T-ALLs (3).

The intercellular Notch receptor-ligand interaction activates a conserved signaling pathway that regulates multiple cellular functions (4). Physiologic Notch signaling occurs when a Notch receptor interacts with a ligand of the Jagged or Delta-like family in a neighboring cell. This initiates a series of metalloprotease- and gamma secretase-mediated cleavages that release the intracellular Notch (ICN) domain from the plasma membrane. The ICN then translocates to the nucleus where it forms the Notch transcriptional complex (NTC) with the DNA binding protein RBPJ and a member of the Mastermind-like (MAML) family. This complex activates transcription of Notch target genes (5). The NTC is short-lived and is tagged for degradation by proteins including the ubiquitin ligase Fbxw7, which recognizes PEST sequences residing in the ICN C-terminus (6).

Activating *NOTCH1* mutations in T-ALL occur in the extracellular Notch1 regulatory region (NRR) and/or the C-terminal PEST domain (7). NRR mutations lead to ligand-independent signaling, whereas PEST mutations limit ICN degradation. Thus, these mutations either increase nuclear Notch1 and/or inhibit NTC turnover, which ultimately leads to dysregulated (increased) Notch1-mediated transcription. Importantly, the enhanced Notch1 signaling resulting from either of these mutations can be inhibited by γ -secretase inhibitors (GSIs), which prevent release of the ICN from the plasma membrane (3).

Expressing ICN1 in murine T-ALL models rapidly induces leukemia in all mice; however, this form of Notch1 rarely occurs in human T-ALL (3). In contrast, expressing mutated *NOTCH1* alleles commonly associated with human T-ALL results in T-ALL with a much longer latency that is incompletely penetrant (8). These findings suggest that events which synergize with the weak Notch1 mutants and/or increase their signaling strength likely contribute to T-ALL. In support of this idea, co-expressing weak Notch1 alleles with mutations found in human patients, such as oncogenic Ras, decreases T-ALL latency and increases penetrance (8).

To identify potentially oncogenic hits that directly modify Notch1 signaling, we performed a gain-of-function cDNA screen to discover molecules that enhance the ability of weak oncogenic *NOTCH1* mutants. Among the hits were the transcription factors MAFB and ETS2. MAFB belongs to the Maf family of AP1 transcription factors, all of which contain a basic leucine zipper (bZIP) domain that binds two long palindromic sequences referred to as “MARE” sequences (9). MAFB also contains an N-terminal transcriptional activation

domain (TAD), which optimally induces Maf target gene transcription by recruiting other transcriptional regulators (10, 11). MAFB serves many roles in embryonic development, including hematopoiesis, where it exerts important functions in macrophage differentiation. In cancer, translocations of *MAFB* and its closely related homologue, *MAF*, are frequent in multiple myeloma (MM) (12). ETS2 belongs to a large and nearly ubiquitously expressed family of transcription factors that function in a wide variety of developmental roles (13). In hematopoiesis, ETS2, like ETS1, with whom it shares close homology, is important for cortical thymocyte proliferation and survival (14–16). ETS transcription factors are also dysregulated in multiple cancers, including Ewing's sarcoma (17) and prostate cancer (18).

To determine the relevance of the findings of our screen, we investigated the function of MAFB and ETS2 in multiple assays relevant to T-ALL pathogenesis. We found that while both MAFB and ETS2 individually enhanced the signal strength of the Notch1 gain-of-function alleles; their co-expression resulted in synergistic activity that was comparable to the core NTC. Furthermore, MAFB alone increased the penetrance and decreased the latency of T-ALL induced in mice with weak Notch1 gain-of-function mutants; whereas inhibiting MAFB in both human and murine T-ALL cells inhibited their growth and decreased expression of a subset of Notch target genes. We found that MafB interacts with ETS2, and in doing so, cooperates with the NTC to augment Notch1-mediated transcription. We thus propose that MAFB functions to amplify the signaling output of naturally occurring Notch1 mutants, a finding consistent with its expression in a high percentage of human T-ALLs. Not only do these data identify new proteins that enhance Notch1 function in T-ALL, but they also identify MAFB as a potential new therapeutic target in T-ALL.

RESULTS

cDNA screen for enhanced Notch signaling

Our Notch1 gain-of-function screen relies on three genetic components transfected into U2OS cells: 1) a sensitive, synthetic Notch signaling reporter (TP1) containing 12 iterated RBPJ-binding sites driving luciferase expression in a pSP72 backbone, 2) a CMV-vector (pcDNA3) expressing a NOTCH1 mutant, and 3) the cDNA library, containing 18,000 open reading frames in the Sport6 CMV vector, individually pre-plated in 384-well plates (Fig. 1A). U2OS cells were utilized due to their high transfectability and low basal Notch signaling. We assayed several Notch mutants previously identified in primary patient samples. We chose NOTCH1 L1601P-DeltaPest (LP P) (8), which contains both HD and PEST mutations, because it consistently yielded a luciferase signal above background and produced a significant Z' -score of 0.602 above background (Fig. S1A). In contrast, the weaker NOTCH1 L1601P mutant (8), which lacks a PEST mutation, only marginally increased background Notch signaling in U2OS cells. Both mutants were considerably weaker transcriptional activators than ICN1 (Fig. S1A).

The top 30 candidate genes identified in the screen, ranked by relative fold-induction of reporter activity, are shown in Fig. 1B and the complete list is provided in Table S1. As expected, the positive control MAML1, a critical NTC component, produced the greatest enhancement. Additional evidence that the screen was specific was the identification of

ATP2A3, which encodes SERCA3, and ERO1L, both of which are known to function in the Notch signaling pathway (19, 20).

MAFB induces dose-dependent enhancement of Notch signaling

We independently validated the ability of several top candidate hits to activate the TP1 reporter (Fig. 1C). Verified hits comprised several transcription factors including MAFB and ETS2. Of the confirmed candidates that we assayed, only MAFB showed a significant dose-dependent enhancement of Notch reporter activity in combination with LP P (Fig. 1D). This was not a general effect of MAFB on transcriptional reporters as MAFB had minimal effects on an NF- κ B reporter (Fig. S1B). These findings led us to focus on MAFB. While MAFB was not expressed in U2OS cells, it was expressed in murine and human T-ALL cell lines and primagrafts (Figs. S1D–E). Furthermore, analysis of two independent patient data sets (21, 22) identified a range of MafB expression in primary human T-ALL samples (Figures S1F–G).

MAFB and ETS2 combine to enhance Notch signaling

ETS2 was also identified as an enhancer in our screen (Fig. 1B), and its closely related homologue, ETS1, is known to interact with MAFB (23). We used Notch-reporter assays to test the potential crosstalk between MAFB and ETS2. The two genes were transfected into U2OS cells along with LP P. Not only did MAFB and ETS2 synergistically increase reporter activity but the signal was comparable to that induced by the MAML1 positive control (Fig. 1E). Although MAFB and ETS2 synergized in this reporter assay, MAFB appeared to be the limiting component. A 2-fold decrease in the amount of MAFB decreased the reporter signal by ~50%, whereas a 4-fold drop in ETS2 DNA concentration resulted in ~25% decrease in Notch1 reporter activity (Fig. S1C). These results support a robust, two-component enhancement of the LP P mutant, with MAFB playing the major role. Consistent with MafB being the limiting component, both Ets1 and Ets2 are highly expressed in post β -selection thymocytes; whereas MafB expression is much lower in these populations (<http://www.immgen.org/databrowser/index.html>, (24)).

MAFB increases penetrance and decreases latency of Notch-induced T-ALL

To assess the effect of MAFB *in vivo*, we assayed its ability to influence the leukemogenic activity of LP P in a retroviral transduction/bone marrow transplant (BMT) model (25) (Fig. 2A). Notch LP P was expressed from a MSCV retroviral vector (MigR1) co-expressing GFP (26); whereas MAFB retroviral particles co-expressed tNGFR (8), thus, providing the ability to distinguish between them.

Reconstituted mice were monitored at various times post-BMT. At 4 weeks post-BMT, CD4⁺CD8⁺ T cells accumulated in the peripheral blood (PB) of ICN and LP P+ MAFB mice (Fig. 2B). This population also expressed the GFP⁺ (ICN or LP P) or GFP⁺NGFR⁺ (LP P+ MAFB⁺) surrogate markers indicating that they originated from the transduced progenitors. Consistent with previous data, mice reconstituted with ICN1 succumbed to T-ALL with a 100% penetrance within 80d post-BMT, with a median survival time of 58d; whereas mice receiving LP P alone developed T-ALL with a penetrance of 36% and latency greater than 100d (8, 25, 27) (Fig. 2C). None of the mice receiving MAFB alone succumbed

to leukemia. In contrast, 16/16 mice reconstituted with LP P/MAFB succumbed to T-ALL with a median survival of 72 days. Consistent with the Kaplan-Meier plots, the WBC of the LP P+MAFB mice was significantly higher than the LP P-only transduced mice (Fig. 2D), as were the spleen weights and percentage of infiltrating tumor cells in tissues compared to the LP P-only mice (Fig. S2). Together, these data show that MAFB strongly enhances the ability of weakly activating Notch1 mutants to induce T-ALL.

Suppressing MafB inhibits Notch signaling and T-ALL cell growth

To understand the function of MafB in T-ALL, *MafB* was suppressed in T6E cells with fluorophore-conjugated siRNAs. T6E cells were chosen as they express high levels of *MafB* (Fig. S1D). At 48h post-treatment, >90% of T6E cells expressed the fluorophore (Fig. S3A), and both siRNAs reduced *MafB* expression by ~50% (Fig. 3A). There was a concomitant decrease in the direct Notch1 targets *Hes1*, *Myc*, *Notch3*, and *Deltex1* as well as the Myc target *CAD* (Fig. 3B). In contrast, expression of *Hey1* did not change, neither did that of the *GAPDH* negative control (Figs. 3B, S3B).

To persistently inhibit MafB, T6E cells were transduced with retroviral vectors expressing MafB shRNAs. Both shRNAs suppressed *MafB* as well as *Hes1* and *Myc* expression (Fig. S3C–E). As NTCs recruit p300 to activate transcription (28, 29), we performed ChIP-PCR for this protein on T6E cells treated with *MafB* shRNAs to determine p300 occupancy at the 5' promoter region of *Hes1* and at the 3' Notch-dependent *Myc* enhancer (NDME), both of which are known to recruit p300 in a Notch-dependent manner (30). When compared to scrambled shRNA treated cells, loss of MafB significantly reduced p300 interaction at both the *Hes1* promoter and the *NDME* (Fig. 3C).

To gain further insights into the effect of MafB on Notch1-dependent transcription, we performed chromatin immunoprecipitation sequencing (ChIP-seq) for the histone acetylation mark H3K27Ac in T6E cells treated with scrambled shRNAs or *MafB* shRNAs. We chose H3K27Ac as changes in Notch occupancy produce dynamic alterations in H3K27ac levels at both enhancers and promoters of Notch1-dependent genes in T-ALL (31). To identify Notch1-dependent genes in T6E cells, we performed a GSI washout experiment (32) to compare expressed genes in the Notch “off” to “on” states (31, 32). Using stringent criteria (see methods), we identified 59 Notch1-positively regulated transcripts (Table S2). We then analyzed the effect of MafB knockdown on the putative enhancers, marked by H3K27Ac of these 59 Notch positively regulated transcripts. Suppression of MafB decreased H3K27Ac by at least 1.4-fold in 37/59 (62%) Notch1 responsive genes (Fig. 3D and Table S2). These included the well-known direct Notch targets *Dtx1*, *Myc*, *Notch3*, *NRarp*, *Notch1* and *IL2RA* (Fig. 3E and fig. S3F; Table S2). Although *Hes1* expression significantly decreased in the GSI washout condition, the decrease in H3K27Ac abundance did not meet our 1.4-fold threshold, as it decreased by only 1.2-fold; nevertheless, there was a decrease in H3K27Ac load at the site 5' of *Hes1* transcription start site (Fig S3F). Furthermore, shMafB is not a global inhibitor of transcription, given that many genes, such as *Trib1*, which is not a Notch1 target, showed no change in H3K27Ac (Fig. S3F). Together, these data suggest that inhibiting MafB decreases Notch-mediated transcription at a significant number of direct Notch target genes (Binomial p-value = 0.06).

To test the fitness of T-ALL cells treated with MafB-shRNAs, we performed an *in vitro* competition assay comparing the shMafB transduced T6E cells to cells receiving the scrambled-shRNA control (shScrmB) (Fig. 3F). Unlike cells transduced with scrambled control vectors, the shMafB-transduced cells were outcompeted by the shScrmB cells (Figs. 3G). These findings suggest that inhibiting MafB represses T-ALL cell growth by inhibiting expression of crucial Notch1 targets, such as *Myc*, *Dtx1* and *Hes1*.

Increased MAFB expression promotes growth and subverts the effects of GSI in T-ALL cells

To better understand the effect of increased MAFB expression in T-ALL cells, human-MAFB was retrovirally expressed in T6E cells. Due to the poor quality of commercial anti-MafB antibodies, we appended 5' HA or FLAG molecular tags to the MAFB coding sequence. We selected cell lines with different amounts of MAFB overexpression; HA-MAFB was slightly higher while FLAG-MAFB was much higher than endogenous murine MafB protein (Fig. 4A). In both cell lines, the expression of the Notch1 target genes *Hes1* and *Myc* was significantly increased (Figs. 4B–C), and this was accompanied by increased cell proliferation (Fig. S4A).

We tested whether increased MAFB expression could compensate for decreased Notch1 signaling in T-ALL cells treated with GSIs. To determine the appropriate dose of GSI treatment for T6E cells, we titrated the concentration of GSI and determined that the range from 0.1 μM to 0.01 μM reduced *Hes1* expression by ~50% (Fig. S4B). We next performed a GSI washout experiment (33) with 0.1 μM GSI for 24 hours. As expected, GSI treatment downregulated the Notch targets *Hes1* and *Myc*, which were upregulated following GSI washout (Fig. 4D–E, **black bars**). However, the effects of both the GSI treatment and the washout were blunted in T6E cells expressing HA-MAFB (Figs. 4D–E, **grey bars**). We next assayed cell growth under GSI treatment. As anticipated, MigR1 cells treated with DMSO continued to proliferate while those treated with GSI experienced growth arrest as early as 48 h after treatment. On the other hand, the MAFB overexpressing cells continued to expand (Fig. 4F). These findings support a role for MAFB to enhance Notch1 function, and suggest that T-ALL tumors expressing high amounts of MAFB may be more resistant to GSI-based therapies.

MAFB enhances Notch signaling by interacting with ETS

MAFB and ETS2 were initially detected by our luciferase Notch reporter screen, thus we tested whether MAFB directly binds to the *Rbpj/Notch1* sites in the TP1 reporter. For this, we employed oligo-immunoprecipitation (OIP) assays, where a biotinylated oligonucleotide containing the consensus *Rbpj/Notch1* (CSL) binding sequence (CGTGGGAA) found in the TP1 reporter was added to the lysate of U2OS cells transfected with vector control, *MAFB*, *ETS2*, or *MAFB* and *ETS2* (Fig. 5A). Of particular relevance, we identified the core DNA sequence for *ETS* factor binding (C/A)GGAA(G/A) (34) interspaced between the *Rbpj* binding sequences of the TP1 reporter (underlined in Fig. 5A). The OIP assay revealed ETS2 binding to the TP1 oligo; however, MAFB bound only in the presence of exogenous ETS2, which is only marginally expressed in U2OS cells (Fig. 5B). This same dependency of MAFB on ETS factors was confirmed for ETS1 (Fig. S5A).

These data also suggest that lack of Ets factors will limit MafB-dependent enhancement of Notch signaling and delay T-ALL onset in vivo. We tested this using the BMT model described in Fig. 2, however for these studies, we transplanted BM from ETS1^{-/-} donor hematopoietic progenitors (35) that were transduced with ICN1 or LP P+MAFB. Although Ets1-deficiency had minimal effects on T-ALL onset in cells transduced with ICN1, loss of a single Ets factor significantly decreased T-ALL penetrance in recipient mice, with median survival increasing from 72d in WT BM donors transduced with LP P+MAFB to 160d in ETS1^{-/-} BM donors transduced with LP P+MAFB (Fig. S5B). These results suggest that the amount of Ets may influence disease latency, even in the presence of MafB.

MAFB interacts with ETS2 through its bZIP domain and functions to recruit transcriptional co-activators

To determine whether MAFB directly binds ETS factors in T-ALL cells, we transduced T6E cells with epitope-tagged MAFB, and performed IPs to identify binding partners (Fig. 5C). MAFB strongly bound both ETS1 and ETS2, which are highly homologous (16). Previous studies of the MAFB homologue, MAFA, showed that it forms complexes with important transcriptional co-activators with histone acetyltransferase activity, including the PCAF complex (36) and p300 (37), both of which are recruited to NTCs where they activate transcription (38, 39). To test whether MAFB interacts with PCAF and P300, we performed IPs in T6E cells expressing FLAG-tagged MafB. Our results indicate that MafB interacts with both PCAF and P300 in T-ALL cells (Fig. 5D), suggesting a mechanism through which it enhances Notch signaling.

We next set out to determine how MAFB interacts with ETS2. We used site-directed mutagenesis to remove the basic leucine zipper domain (bZIP) domain from MAFB (9, 11, 23) (Fig. 5C). Using T6E cell lysates, we first verified the deletion and the size of the mutant (Fig. 5C, **right panel**,). We then performed IP experiments (Fig. 5E), which showed that deleting the MAFB bZIP domain markedly diminished the MAFB-ETS2 interaction. MAFB bZIP also markedly decreased p300 binding, however, the bZIP domain was not required for the PCAF interaction (Fig. 5E). Previous ChIP-seq analysis showed that ETS binding sites are enriched within 250bp of Notch1/RBPJ binding sites, thus placing ETS factors in the immediate vicinity of the NTC (40). We tested if MAFB can interact with components of the NTC, specifically the cleaved intracellular Notch1, as detected by the Val1744 antibody, and RBPj. We found that MAFB co-immunoprecipitated in T6E cells with cleaved Notch1 (Fig. 5F) and RBPj (Fig. 5G), and that this interaction with the NTC was lost when the MAFB bZIP domain was deleted (Fig. 5G). These findings further highlight the ability of MAFB:ETS complexes to cooperate with the NTC to activate transcription.

To determine the effect of the MAFB bZIP mutant on Notch transcriptional activity, we tested its effect in T6E cells using the TP1 reporter. The MAFB bZIP mutant decreased reporter activity by over 60% (Fig. 5H). When ETS2 was co-transfected in the assay with the MAFB bZIP mutant, the overall Notch activity was increased, but the loss of the bZIP domain did not significantly enhance Notch signaling more than when ETS2 or MAFB were added alone, thus suggesting that MAFB-mediated enhancement of Notch transcription occurs through its bZIP domain. We next assayed the MAFB deletion mutant in vivo using

the BMT model. As before, MAFB greatly decreased the latency and increased the penetrance of T-ALL development. In contrast, the MAFB bZIP mutant dramatically limited the ability of MAFB to enhance LP P leukemogenicity (Fig. 5I). Together, these data show that the bZIP domain of MAFB is required for MAFB to enhance LP P activity both *in vitro* and *in vivo*.

MAFB enhances Notch signaling in human T-ALL cells

To determine whether MafB served a similar role in human T-ALL we determined the levels of *MAFB* in three T-ALL cell lines (Fig. 6A). Of these, KOPT-K1 had the highest levels of *MAFB* expression. KOPT-K1 cells were treated with shRNAs designed against human *MAFB*, which decreased *MAFB* expression by over 50% (Fig. 6B). The effect of suppressing *MAFB* in human KOPT-K1 cells was similar to murine T6E cells as expression of the direct Notch target genes *HES1*, *DTX1* and *MYC* was suppressed (Fig. 6C). Likewise, KOPT-K1 cells treated with anti-*MAFB*-HU-shRNAs were at a disadvantage in cell growth assays when compared to cells treated with scrambled shRNAs (Fig. 6D).

We next tested whether ETS factors also played a similar role in human T-ALL cells. First, we confirmed that three human cell lines expressed ETS1 and ETS2 (Fig. 6E). Next, we tested whether MAFB binds ETS2 in human T-ALL cells. We transduced KOPT-K1 cells with FLAG-tagged MAFB, and performed IPs to detect interactions and found that MAFB strongly bound both ETS2 and RBPJ in KOPT-K1 cells (Fig. 6F). This suggests that a similar MAFB-ETS2 pathway enhances Notch signaling in human T-ALL.

DISCUSSION

The finding that human and murine T-ALLs with activating NOTCH1 mutations frequently require persistent Notch signaling for growth and survival led to the idea that Notch is a druggable target in this disease. Although multiple studies support this idea, one paradox is that the common NOTCH1 mutations in T-ALL patients are weak oncogenic drivers in murine T-ALL models. This raises the question of whether there are additional molecules that potentiate Notch signaling, which could identify additional therapeutic targets.

In this study, we set out to identify proteins that enhance the activity of the common Notch1 mutations in T-ALL patients. Our screen identified multiple genes, some of which were previously verified to be important in Notch signaling (19, 20). We chose to focus on MAFB and ETS2. These transcription factors have well-established roles in myeloid and lymphoid development (15, 41) respectively, however, their involvement in T-ALL is poorly understood. In reporter assays, MAFB and ETS2 demonstrated strong synergy and enhanced Notch activity to a level on par with MAML1, a core component of the NTC. In our functional studies, MAFB seems particularly important as expressing MAFB along with the weak oncogenic NOTCH1 mutant L1601P P in BM progenitors accelerated T-ALL onset and increased T-ALL penetrance. Inhibiting *MAFB*, by either siRNA or shRNA, downregulated expression of multiple direct Notch targets, including *MYC*, *DTX1* and *HES1*, in both murine and human T-ALL cells and also decreased the proliferation of murine and human T-ALL cells. In contrast, overexpressing MAFB enhanced the expression of these Notch targets and promoted T-ALL growth. Thus, we propose that the combination

of MAFB and ETS2 has the potential to amplify the activity of weak activating Notch1 mutations. Although multiple Notch1 targets are inhibited by blocking MAFB, additional analyses are needed to determine whether MAFB influences all direct Notch1 target genes. It is unlikely that withdrawal of MafB broadly represses transcription as the expression of many genes, such as *Trib1*, was not affected by MafB knockdown.

How MAFB boosts the signaling of mutant Notch receptors was not immediately obvious. Previous work characterized MafB as a differentiation factor, with roles in monocyte-to-macrophage differentiation (42). In T cells, MAFB expression is low (43), however, data from published databases, and our own analysis of cell lines, patient primagrafts, and primary tumors show that MAFB is frequently expressed in T-ALL cells and primary samples; however, there was no correlation with Notch1 mutation subtypes and there was no evidence of MAFB or ETS1/2 genetic amplification in primary T-ALLs (21, 22). Thus, we hypothesize that in these tumors, MAFB expression, driven by unknown mechanisms, potentiates both Notch signaling and its oncogenic capacity. Our findings also suggest that in other tumors with weakly activating Notch1 mutations, such as CLL, additional co-factors analogous to MAFB may be required to potentiate oncogenic Notch1 signals.

MAFB is a multi-domain transcription factor that can bind DNA directly as well as form protein-protein interactions (9, 44). In our T-ALL studies, MAFB did not appear to bind DNA independently; instead, it appears to either bind cooperatively with ETS transcription factors or to be tethered to DNA through the ETS transcription factors. *ETS* binding sites are common and in close proximity with *RBPJ* binding sites (40). Since MAFB is known to directly interact with ETS1 (23), we believe that MAFB is recruited near sites of NTC formation through a similar interaction with ETS2 or ETS1. Suppression of MafB does not affect the expression of ETS factors nor does it alter the occupancy of ETS2 at the 5' *Hes1* promoter in T-ALL cells. (Fig. S6A–C). We found that MafB interacts with both P300 and PCAF, which are important for Notch-induced transcriptional activation (38) and that loss of MafB decreases occupancy of p300 at the *Hes1* and *Myc* Notch-dependent enhancers. The bZIP domain of *MafB* is required for this activity, thus bringing PCAF and P300 and by connection, the Notch/RBPJ/MamL complex into a larger protein complex capable of high transcriptional output. This MAFB:ETS2 synergy and its ability to enhance Notch signaling is also conserved in human T-ALL cells. Recent work showed that the T-cell transcription factor Zmiz1 interacts with Notch1 and regulates expression of oncogenic Notch targets such as *Myc* (45), indicating that enhancing Notch signaling by co-factors is an important step in T-ALL onset. In our studies, suppressing MafB did not affect Zmiz1 expression (Fig. S6D). Circulating DP T-cells, which express high levels of ETS factors, are observed in the BMT model of NOTCH1 LP P induced T-ALL (8), however, these mice develop T-ALL at low frequency. From the results of our study, we predict that when MAFB is expressed at sufficient levels, it creates an environment where transcriptional co-factors are recruited at a higher density near sites of Notch transcriptional targets, thus raising Notch1 signaling output to levels that exceed the threshold for oncogenic transformation.

In summary, our findings show that the MAFB-ETS2 interaction enhances Notch1 signaling in a leukemic setting by supporting the higher expression of Notch targets. These findings suggest that the MAFB-ETS2 axis may serve as a therapeutic target in T-ALL. Our findings

also raise issues regarding the efficacy of GSI therapy in tumors with increased MAFB abundance, as our data show that cells expressing high amounts of MAFB were less sensitive to GSI treatment. Thus, strategies that decrease *MAFB* expression or formation of the MAFB:ETS2 complex may enhance the sensitivity of Notch-dependent T-ALL cells to GSI therapy.

MATERIALS AND METHODS

Cell Culture

T-ALL cell lines were cultured in RPMI1640 (Invitrogen) supplemented with 10% FBS (Hyclone), 2mM L-glutamine, 1% Non-essential amino acids (GIBCO), 1% Sodium Pyruvate (GIBCO) and 2-mercaptoethanol (0.0005% [v/v], Sigma-Aldrich), with antibiotics. U2OS cells were maintained in DMEM (Invitrogen) with 10% FBS (GIBCO) and antibiotics. Cells were grown at 37°C under 5% CO₂. Retroviral transduction, sorting, and expression analysis of T-ALL cells were performed as described (3). The GSI, Compound-E (Calbiotech 565790) was titrated on T6E cells for 12 h at concentrations ranging from 1μM to 1nM. GSI washout experiments were performed as described (32) after 24 hours of culture in 0.1μM GSI.

Constructs and Retroviruses

MigR1 (46), MigR1-NGFR, MigR1-Notch1-L1601P and MigR1-Notch1 L1601P P are described (8). Sport6-MafB (MafB coding sequence 780bp) and Sport6-ETS2 (ETS2 coding sequence 822bp) were obtained from the library screen. The 5' HA or FLAG tags added to MafB were generated by PCR. MafB deletions were generated using QuickChange II (Agilent Technologies) and primer design was based on the QuickChange Primer Design program (www.genomics.agilent.com).

Luciferase Screen and Reporter Assays

The cDNA screening strategy involved the use of three key components: 1) a pcDNA3 plasmid encoding a modestly strong *NOTCH1* gain-of-function mutant, L1601P P, driven from a CMV promoter (40 ng of cDNA/well), 2) a Notch firefly luciferase reporter (TP1) containing 12 CSL binding sites (50 ng of cDNA/well), and 3) a pre-plated cDNA library cloned into the Sport6 plasmid (40 ng of cDNA/well). A *MAML1* cDNA was the positive control for each screen plate, while empty vector and a *DTX1* cDNA were background and negative controls (40 ng of cDNA/well) respectively. DNA spotting was performed using a Matrix Platemate (Thermo, Waltham, MA, USA) for the control wells. The Matrix Wellmate (Thermo) was used to dispense the transfection mix containing the reporter and Notch1 mutant plasmids in combination with transfection reagent (Fugene6 Promega), which were added to the wells (4000 U2OS cells/well) after a 30 min incubation. Luminescence was measured 48 hours after plating using BriteLite-Plus (PerkinElmer, Waltham, MA, USA) luciferase reagent with LJI Biosystems Analyst HT96:384. Methods for screen validation are provided in the supplementary materials.

Luciferase Screen Validation

For the validation of screen candidates and independent luciferase assays, 4000 U2OS cells/well were seeded on Corning opaque 384-well plates. Fugene6 transfection mix was prepared in OptiMEM (Gibco, Grand Island, NY, USA) serum-free medium with three plasmid components: 1) 50 ng/well of Notch TP1 firefly reporter plasmid; 2) 40 ng/well of pcDNA3-L1601P P plasmid; and 3) 40 ng/well of screen component cDNAs cloned into pCMVSPORT6 (ex. MafB or ETS2). pcDNA3-MAML1 and Flag-CMV-DTX1 plasmids (40 ng/well) served as positive and negative controls, respectively. 4) 5 ng of pRL-TK *Renilla* luciferase internal control plasmid. Following 20 min at room temperature, 20 µl of the transfection reaction mix was added to the cells by multi-channel pipette. 24 wells were analyzed for each individual transfection sample set. After a 48hr incubation with the transfection mix, 35 µl/well of BriteLite-Plus luciferase reagent was added by multi-channel pipette; luminescence was measured by an LJM Biosystems Analyst HT96:384 (LJM Biosystems, Inc, Sunnyvale, CA, USA). Stop-N-Glo buffer and renilla luciferase reagent (Promega) was used to assess transfection efficiency. At least three individual repeats were performed for each experiment.

Human T-ALL data analysis and statistical analysis

Normalized microarray expression analysis for the Haferlacht dataset was obtained from the Oncomine database and Zuurbier dataset was provided by Dr. Jules Meijerink. Each set was separately analyzed for expression of MafB, Notch1 receptor and Notch1 target genes: Hes1, Deltex and cMyc. Each sample that displayed higher than 2-fold Notch receptor and Notch target expression was parsed from the total data set. MafB expression was crossreferenced for any individual sample that showed a Notch enhancement signature. The comparative values of Notch1, Notch targets and MafB were plotted as a log2 median value for all the samples that show Notch enhancement in the Haferlacht and Zuurbier datasets. Statistical analysis was performed using Prism 6 (GraphPad). Survival curves were computed using Kaplan-Meier analysis and comparison of survival curves was performed using Mantel-Cox test provided through Prism. Predicted experimental mouse numbers for the Notch1-L1601P P and MafB experiment were powered (0.8). Z' score for Notch1 gain-of-function screen determined by the following formula: $Z' \text{ score} = 1 - [(3SD_- + 3SD_+) / (Av_+ - Av_-)]$. An unpaired t-test P value of less than 0.05 was considered to be significant in all experiments, unless noted otherwise; * p 0.05, ** p 0.005, *** p 0.0005, **** p 0.00001.

qPCR

RNA was extracted using Qiagen RNeasy Mini and Micro Kits. cDNA was synthesized from RNA with the SuperScript III kit (Invitrogen). Transcripts were amplified with SYBR green PCR reagent (Applied Biosystems), and qPCR was performed on the ABI Prism 7900HT system (Applied Biosystems). mRNA quantities, either in absolute or relative quantification, were normalized to EF1α and GAPDH, respectively. Primer3 software was used for primer design and sequences are provided in Supplemental Table 4.

siRNA and shRNA

RNA interference was carried out with small-interfering RNA duplexes designed, then screened for specific and effective knockdown of target genes. Fluorescence-conjugated duplexes (n=3) targeting *MafB* were ordered directly as ON-TARGETplus siRNA (J-) from Thermo/Dharmacon. siRNA methods are provided in Supplementary Materials. For shRNA suppression, two different *MafB*-targeting shRNAs and a scrambled shRNA were purchased from Open Biosystems (sequences are provided in Supplemental Table 4). For siRNA control transfections, nontargeting siRNA and siGlo-Green were purchased from Thermo/Dharmacon. For transfections of cells, siRNA duplexes were resuspended in siRNA buffer (Dharmacon), and added to the cell growth media for 12hrs with siImporter transfection reagent (Millipore) as per manufacturer's instructions. 48–72 hr after transfection RNA was harvested from cells with RNeasy mini kit (QIAGEN) and 500 ng of total RNA was used in qPCR analysis. For shRNA suppression, two different *MafB*-targeting shRNAs and a scrambled shRNA were purchased by Open Biosystems (sequences are provided in Supplemental Table 3, and Supplementary materials) and were subcloned into the pLMP-GFP or pLPM-NGFR vector. For viral transduction cells were centrifuged with viral supernatant and 8 µg/mL hexadimethidine bromide (Sigma) at 2500RPM for 90 min at 25°C. FACS-purified shRNA-treated cells were analyzed for growth or gene expression at the indicated times after transduction. For growth curves or competition assays, 5×10^5 sorted cells were seeded.

ChIP-seq Analysis

ChIP-Seq reads were aligned to mm10 using BWA (47) and filtered to remove PCR duplicates and multi-mapped reads. All reads post-filtered by known ENCODE blacklist regions. Peak calling was performed using MAC2 (version 2.0.9) (48) with the following parameters: --nomodel, --shiftsize=(1/2 estimated fragment length), --keep-dup=1 and FDR threshold 1×10^{-6} . ChIP-Seq display files were generated using samtools, bedtools, and ucsc utilities. Scaling for all ChIP-Seq tracks in figures is equal to local fragment coverage $\times (1,000,000 / \text{totalCount})$.

The peaks were associated to their most proximal gene as defined in Ensembl GRCm38 transcript model using bedtools and HOMER-annotatePeaks (version 4.8) (49). H3K27ac signal was compared between the GSI and GSI-washout conditions on the regions 1500 pb flanking the peak summits. Regions of 1500 kb flanking the summits of peaks were merged across GSI and GSI-washout conditions using bedtools-merge (version 2.25.0). In each library, peak-filtered H3K27ac signal was quantified and normalized to fragment per kilobase per million reads (FPKM). The logarithmic fold change of H3K27ac load on with non-zero read counts in at least one condition was calculated as $\log_2$ FPKM of the GSI versus GSI-washout condition with a pseudo-count of 1, and the regions with absolute fold change greater than or equal to $\log_2(0.5)$ were indicated in Table 2 and *MafB*-dependent.

Mice and Bone Marrow Transduction

Bone marrow transductions and transplantation into lethally irradiated recipients were performed as described (26, 50) Recipient mice used in these experiments were 6–8-week-old C57BL/6 female mice were obtained from Charles River Laboratories. In cases where

two retroviruses (ex. MigR1-L1610P P and MigR1-NGFR-MafB) were used to transduce the bone marrow progenitors, the total viral titer and multiplicity of infection was adjusted to reflect previously used T-ALL Notch-mutant virus titers (8). Mice were maintained on antibiotics in the drinking water for 2 wks after BMT and peripheral blood was drawn every 2 wks to monitor blood counts and evaluate the presence of circulating immature T cells by flow cytometry. Mice with WBC $>4.0 \times 10^6$ /mL and a body condition score of 2 were euthanized and tissues were harvested for flow cytometry and histology (H&E) analysis. All mice were housed in specific pathogen-free facilities at the University of Pennsylvania. Experiments were performed according to the guidelines from the National Institutes of Health with approved protocols from the University of Pennsylvania Animal Care and Use Committee.

Flow Cytometry

Flow cytometry was performed on BD LSRII; cell sorting was performed on BD AriaII; and FlowJo software was used for analysis. Peripheral blood (bi-monthly), spleen, thymus and bone marrow (terminal analysis) were harvested and cell suspensions were generated from mice transduced as described above. Antibodies used in staining of the tissues include: CD45.2 (104, BD) CD25 (PC61 Biolegend) CD44 (IM7 eBiosciences), CD3 (17A2 eBiosciences), CD4 (RM4-5; eBiosciences), CD8 (53-6.7; eBiosciences), CD19 (1D3; eBiosciences), CD11b (M1/70; eBiosciences), F480 (BM8; eBioscience) and Gr-1 (RB6-8C5; eBiosciences). DAPI was used for Live/ Dead determination. Transduced T6E cell lines were sorted on the basis of internal GFP fluorescence or surface staining with anti-NGFR-biotinylated antibody generated in-house from the 8737 hybridoma line.

Western Blot and Immunoprecipitation Analyses

Whole-cell lysates were prepared with RIPA buffer or FLAG-IP lysis buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 0.5% NP40, 10% Glycerol); with protease inhibitor tablets (Roche Complete). Protein concentration was determined with the Bio-Rad protein assay dye reagent (Bio-Rad). Proteins were separated using SDS-PAGE and wet-transferred to PVDF membranes. Blots were visualized with SuperSignal west pico chemiluminescence or SuperSignal west femto chemiluminescence substrate (Thermo Scientific). Antibodies used for Western blot were cleaved Notch1 (Val1744) antibody (Cell Signaling Technology, no. 2421), RBPJSUH (Cell Signaling Technology D10) MafB (Santa Cruz P20) and rabbit mAb (abcam ab66506), GAPDH (Santa Cruz FL-335), ETS1 (Santa Cruz C-20), ETS2 (Sigma E3783), PCAF (Santa Cruz E-8), P300 (Thermo RW109), HA (Covance MMS-101P), FLAG (Sigma M2) and secondary anti-mouse-HRP (Pierce) or anti-rabbit-HRP (Pierce).

IP experiments were performed using HA-probe agarose beads (Santa Cruz Biotech) or anti-FLAG Agarose beads (Sigma M1). Samples were loaded and western analysis was performed as described above. OIP experiments were performed as described (51). Oligonucleotide sequences for the OIP experiments are provided in Table S3. For each pulldown 0.5–1 mg of fresh protein lysate was used and combined with 25–30 μ L of beads for incubation overnight at 4°C with rotation. Beads were washed four times in lysis buffer with increasing concentration of NaCl 200nM (2x) and 300nM (2x). The IP experiments depicted in Figure 7 were conducted in similar fashion however the cells were lysed with

FLAG-IP lysis buffer and washed only three times, once with lysis buffer and twice with lysis buffer with higher NaCl concentration (150mM). Protein was released from washed beads by addition of 2x Laemli buffer and boiled for 10m at 95°C.

Microarray Analysis

Mouse Gene 2.0 ST Affymetrix array CEL files were imported and normalized and summarized using robust multichip average (RMA) and median-polish algorithms, respectively, using the Bioconductor “oligo” package (52). Limma (53) was used for differential gene expression on triplicate samples of GSI and GSI-washout conditions. Package *mogene20sttranscriptcluster.db* in Bioconductor was used for annotation. Genes with greater than 2-fold change and Benjamini Hochberg FDR of less than 0.1 were called as differentially expressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Screen to identify novel enhancers of Notch signaling

A. Schematic of the methodology used in the cDNA gain-of-function screen. **B.** List of candidate genes potentiating NOTCH1-L1601P- P (LP P) activity. MAML1^{Control} is the MAML1 positive control. ●, symbol indicates candidates that were independently verified in reporter assays. **C.** Luciferase induction using the Notch-responsive TP1 reporter and LP P to validate candidates identified in the screen. Data were quantified relative to the empty vector (EV) control. **D.** Luciferase assay using the TP1 reporter (40ng/well) and LP P (40ng/well) and assaying dose dependency of Notch activation by ETS2 (40, 80, 120ng/well) or MAFB (40 and 80ng/well). **E.** Luciferase TP-1 reporter assay with LP P when MAFB and ETS2 was singly or co-transfected (at 40ng each). To normalize the amount of plasmid per well, EV was added so that the total amount of DNA was 80ng/well (not including reporters). Data are each from 3 independent experiments. * p 0.05, ** p 0.005, *** p 0.0005, **** p 0.0001 by unpaired t-tests.

Figure 2. MAFB enhances T-ALL onset of a weak Notch gain-of-function allele

A. Schematic representation of experimental design for retroviral transduction of 5-FU-treated bone marrow progenitors. When only a single cDNA was expressed (MAFB -only, ICN-only or LP P-only), an equivalent dose of the retroviral vector (either MigR1 or MigR1-NGFR) was used to normalize the total retroviral titers. **B.** Peripheral blood analysis of the four cohorts of transplanted mice (ICN n=8, MAFB n=9, LP P n=14, LP P+MAFB n=15) 4 weeks after bone marrow transplant (BMT). Representative populations shown were gated for live, singlet and Lin⁻ cells. NGFR is the surrogate marker for MAFB transduction and GFP is the surrogate marker for Notch mutant transduction. **C.** Kaplan-Meier plot indicating percent T-ALL-free mice after BMT. Additional animals that succumbed to non-T-ALL conditions, such as irradiation poisoning (MAFB-only n=1, LP P-only n=1) and bone marrow failure or anemia (LP P-only n=1) were excluded from analysis. **D.** White blood cell (WBC) counts in the peripheral blood of the mice in each of the experimental cohorts. A diagnostic threshold for tumor onset of 40 million/mL WBC and circulating double-positive T-cells in the peripheral blood were used as a benchmark for T-ALL, as previously described (8, 25). *** p < 0.0005 by Mantel-Cox test (C) or unpaired t-test (D).

Figure 3. MafB-deficient T-ALL cells downregulate Notch targets and perform poorly in competitive culture conditions

A. MafB expression measured by qRT-PCR in T6E cells 48 hours after treatment with indicated siRNAs relative to that in cells cultured with transfection reagent (untreated). * $p < 0.05$, ** $p < 0.005$ by unpaired t-test. **B.** Relative expression analysis by qRT-PCR of Notch signaling and Myc signaling (CAD) targets and expression controls (GAPDH) in MafB-siRNA#1-treated cells relative to each in ScrmB-siRNA-treated cells. **C.** Chromatin immunoprecipitation (ChIP) assay measuring occupancy of P300 at the *Hes1* promoter and at the Notch-dependent Myc enhancer (NDME) in MafB-shRNA#3-treated T6E cells. Genomic DNA sequence of GAPDH is used as non-specific control. *** $p < 0.0005$ by unpaired t-test. **D.** Pie chart representation of Notch-dependent gene expression stratified on the basis of differential H3K27Ac abundance measured by ChIP-seq analysis of T6E cells transfected with shScrmB or shMafB#3. Of the 59 Notch-dependent transcripts, as determined by GSI-washout, 37 have nearby MafB-dependent H3K27Ac regions, which display a fold-change of -1.5 (dark-gray portion of pie chart). **E.** Genome tracts of

individual examples of MafB-dependent H3K27Ac for Notch target genes (Dtx1 top, Myc bottom) in T6Es transfected with shScrmB or shMafB#3. **F.** Schematic representation of experimental design for MafB shRNA-treated T-ALL cells. The cells were sorted for their respective markers and subsequently cultured in equal numbers directly afterward. Every three days, the percentage of the cellular population bearing each marker was determined in an aliquot of the competition culture. **G.** Competition assay of T6E cells treated with GFP⁺ or NGFR⁺ scrambled shRNA plated in equal numbers (2.5×10^5) at Day 0. n=3; no significant difference. Competition assay of T6E cells treated with GFP⁺ scrambled shRNA or one of two NGFR⁺ MafB shRNAs plated in equal numbers (2.5×10^5) at Day 0. n=3; p=0.02 and 0.03, respectively.

Figure 4. Ectopic MAFB expression in T-ALL cells enhances Notch target gene expression and limits the effect of GSI treatment

A. Western blot for MafB (with anti-MafB antibody; Abcam) and loading control (GAPDH) in whole cell lysates (10 μ g) from T6E cells expressing Flag-MAFB or HA-MAFB. MigR1, empty vector. **B and C.** *Hes1* (B) and *Myc* (C) expression measured by qRT-PCR in T6E cells overexpressing MAFB. **D and E.** *Hes1* (D) and *Myc* (E) expression measured by qRT-PCR in T6E cells treated with DMSO or GSI (0.1 μ M) for 24 hours and subsequently washed out for 6 hours. Black bars: control cells; grey bars: MAFB-overexpressing cells. N=3–6 experiments (Panels A–E). **F.** Proliferation of MigR1-transfected control or MAFB-overexpressing T6E cells (2.5×10^5) plated in media containing DMSO or GSI (10 nM). n=2 experiments with 3 technical replicates per experiment. * p 0.05, ** p 0.005, *** p 0.0005, ****p 0.00001, and NS not significant by unpaired t-test.

Figure 5. MAFB interacts with ETS proteins and recruits cofactors to Notch/RBPJ binding sites
A. Schematic representation of the experimental design for the oligo-immunoprecipitation (OIP) assay in U2OS cells. A biotin-tagged oligo from the TP1 Notch reporter was used as the bait to immunoprecipitate proteins interacting at the CSL or EBS regions. **B.** Representative results of OIP assay in U2OS cells transfected with MigR1, ETS2, Flag-MAFB, or ETS2 + Flag-MAFB, and probed with ETS2 and Flag antibodies following OIP. 10% loading of nuclear lysates indicates protein abundance; GAPDH is the loading control. **C.** Schematic representation of the tagged MafB and MafB- bZIP mutant. Right: Western blot on mixed lysates of HA-MAFB and HA-MAFB- bZIP immunoprecipitated from doubly transduced T6E cells to show relative protein size and HA-pulldown efficiency. **D.** Representative results of HA- or FLAG-immunoprecipitation assessing MAFB interactions with ETS1, ETS2, P300 and PCAF in T6E T-ALL cells. **E.** Representative HA-immunoprecipitation in T6E cells transduced with MigR1, HA-MAFB or HA-MAFB- bZIP and probed for HA, ETS2, PCAF and P300. The background bands in the HA 10% loading control likely result from the blot being probed and restriped multiple times. **F.** Representative HA-immunoprecipitation in T6E cells transduced with HA-MafB and probed for cleaved-Notch1 (Val1744). **G.** Representative FLAG-immunoprecipitation in T6E cells transduced with MigR1, FLAG-MAFB- bZIP, or FLAG-MAFB and probed for RBPJ and

FLAG. **H.** Notch TP-1 reporter assay in T6E cells transfected with LP P and the indicated plasmids. EV is the vector control. ** $p < 0.005$, NS (not significant), by unpaired t-test; $n=3$. **I.** Kaplan-Meier plot comparing percent of T-ALL-free survival after BMT with progenitors transduced with LP P-only, LP P+MAFB or LP P + MAFB- bZIP domain deletion mutant. LP P $n=6$, LP P+MAFB $n=12$, LP P+ MAFB bZIP $n=10$. Animals that succumbed to non-T-ALL conditions, such as bone marrow failure/anemia (LP P-only, $n=1$) were excluded from analysis. P value calculated by Mantel-Cox test. *** $p < 0.0001$.

Figure 6. Loss of MafB suppresses Notch target gene expression and cell proliferation in human T-ALL

A. MafB mRNA expression in human T-ALL cell lines (U2OS cells used as negative control). **B.** MafB mRNA expression after MafB knockdown in KOPT-K1 cells by each of two shRNAs. **C.** Notch target gene expression in sorted KOPT-K1 cells 72 hours after transduction with shRNA against MafB relative to KOPT-K1 cells treated with ScrmB-shRNA. **D.** Proliferation of sorted KOPT-K1 cells after transduction with shRNAs against MafB. Growth is compared to cells treated with ScrmB-shRNA $n=3$. **E.** Representative western blotting for ETS1 and ETS2 in human T-ALL cell lines. GAPDH is the loading control. **F.** Representative FLAG-immunoprecipitation in KOPT-K1 cells transduced with Migr1 or FLAG-MAFB and probed for FLAG, ETS2 and RBPJ. 10% loading of lysates indicates RBPJ and ETS2 abundance. GAPDH is the loading control. ** $p < 0.005$, *** $p < 0.001$.