Brief Report

LYMPHOID NEOPLASIA

Activation of the *LMO2* oncogene through a somatically acquired neomorphic promoter in T-cell acute lymphoblastic leukemia

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Key Points
• Recurrent intronic mutations that create probable MYB, ETS1, and RUNX1 binding sites occur at the *LMO2* promoter in some T-ALL patients.
• CRISPR/Cas9-mediated disruption of the mutant MYB site in PF-382 cells markedly downregulates *LMO2* expression.

Somatic mutations within noncoding genomic regions that aberrantly activate oncogenes have remained poorly characterized. Here we describe recurrent activating intronic mutations of *LMO2*, a prominent oncogene in T-cell acute lymphoblastic leukemia (T-ALL). Heterozygous mutations were identified in PF-382 and DU.528 T-ALL cell lines in addition to 3.7% of pediatric (6 of 160) and 5.5% of adult (9 of 163) T-ALL patient samples. The majority of indels harbor putative de novo MYB, ETS1, or RUNX1 consensus binding sites. Analysis of 5′-capped RNA transcripts in mutant cell lines identified the usage of an intermediate promoter site, with consequential monoallelic *LMO2* overexpression. CRISPR/Cas9-mediated disruption of the mutant allele in PF-382 cells markedly downregulated *LMO2* expression, establishing clear causality between the mutation and oncogene dysregulation. Furthermore, the spectrum of CRISPR/Cas9-derived mutations provides important insights into the interconnected contributions of functional transcription factor binding. Finally, these mutations occur in the same intron as retroviral integration sites in gene therapy–induced T-ALL, suggesting that such events occur at preferential sites in the noncoding genome. (*Blood*. 2017;129(24):3221-3226)

Introduction

LIM-domain-only protein 2 (LMO2) plays a crucial bridging role in the formation of a large multimeric transcriptional complex that includes TAL1, LDB1, GATA, RUNX1, ETS1, and MYB.[1] In mice, *Lmo2* is progressively silenced after the early T-cell progenitor (ETP) stage of thymic development and leads to T-cell acute lymphoblastic leukemia (T-ALL) when overexpressed in transgenic models.[2-4] In human thymi, *LMO2* is similarly downregulated after commitment to the T-cell lineage as indicated by DNA microarray analyses.[5] Overexpression of *LMO2* in human hematopoietic stem cells also leads exclusively to preleukemic alterations in thymocytes and T cells but not in other lineages.[6] Reported mechanisms of aberrant *LMO2* expression in human T-ALL include recurrent chromosomal translocations, such as t(11;14)(p13;q11) and t(7;11)(q35;p13); cryptic deletions of an upstream negative regulatory region, as in del(11)(p12p13); and retroviral insertional mutagenesis at the *LMO2* locus during gene therapy.[7-11] Although ~50% of T-ALL patients overexpress *LMO2*, only about 10% of patients have a detectable cytogenetic lesion.[12] Notably, many of these patients will overexpress *LMO2* from a single allele, a feature reminiscent of *TAL1* overexpressing T-ALL cases driven by small somatic indel mutations that create binding

sites for MYB, thus generating a neomorphic enhancer.[13,14] We hypothesized that *cis*-acting mechanisms may account for T-ALL cases with monoallelic *LMO2* expression that lack abnormalities of the *LMO2* locus.[15,16]

Study design

Detailed methods are described in the supplemental Data available on the *Blood* Web site. Chromatin immunoprecipitation sequencing (ChIP-seq) was performed on T-ALL cell lines after immunoprecipitation with antibodies against MYB and acetylated H3K27 (H3K27ac). Analysis of motif enrichment was used to confirm enrichment of MYB motifs in the MYB ChIP-seq data (supplemental Tables 1 and 2). *LMO2* messenger RNA levels were quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Mutation screening of primary T-ALL samples was achieved by denaturing high-performance liquid chromatography of *LMO2* intron 1 PCR products. Luciferase reporter constructs consisting of 469 bp PCR products inserted upstream of an SV40 promoter and firefly luciferase gene were electroporated into Jurkat cells. CRISPR/Cas9 genome editing was used to target the *LMO2* intron 1 mutations in the PF-382 T-ALL cell line.
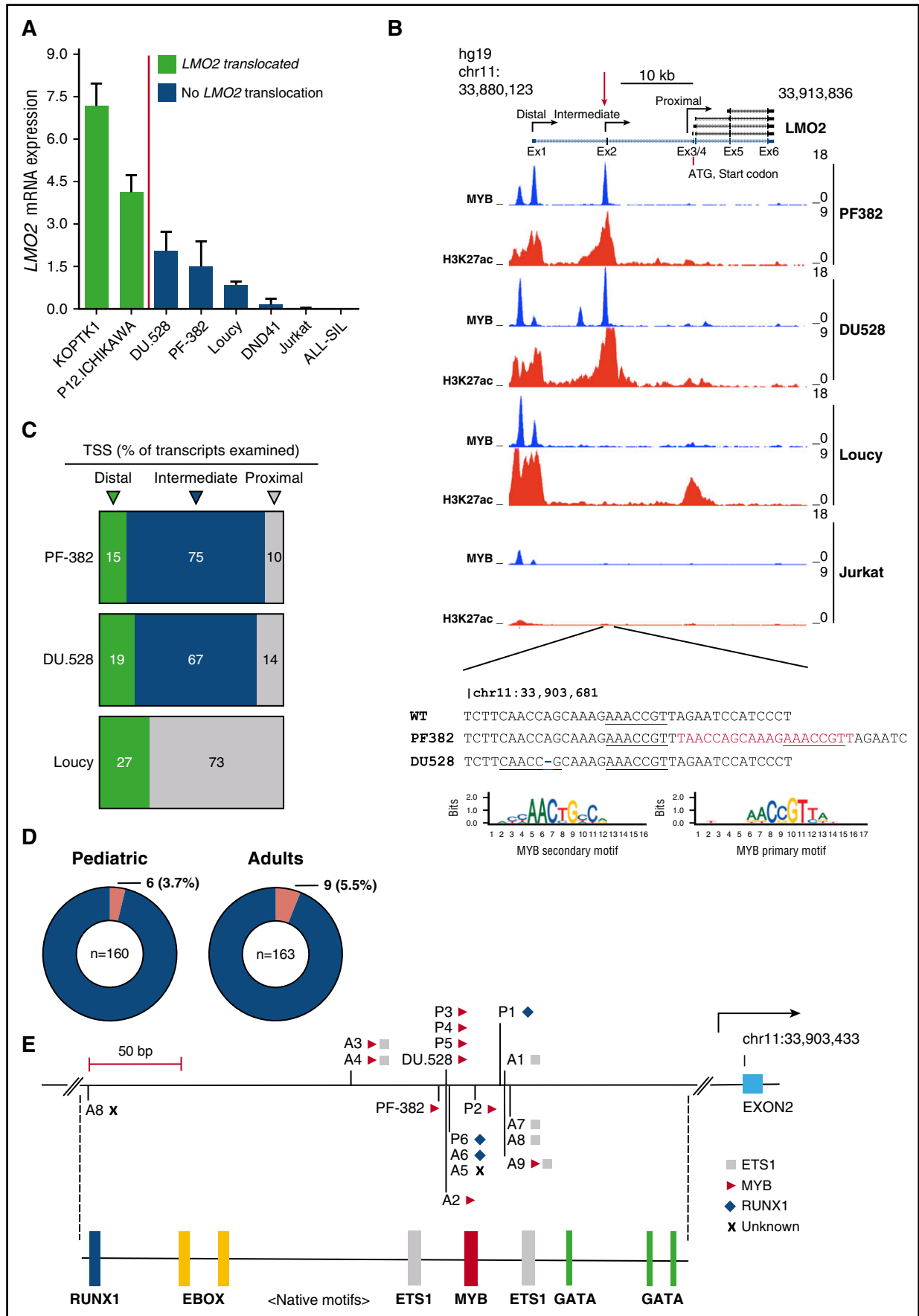
Figure 1.

Results and discussion

To test this hypothesis, we first assessed *LMO2* expression by qRT-PCR in several T-ALL cell lines arrested at different stages of thymic differentiation. The ETP-like T-ALL cell line Loucy expressed *LMO2* at levels significantly higher than the more mature T-ALL cell lines (DND-41, ALL-SIL, Jurkat), reflecting physiological expression of *LMO2* at the ETP stage of thymic development (Figure 1A). The *TAL1*-positive cell lines DU.528 and PF-382 both exhibited upregulated *LMO2* expression, yet crucially have no reported chromosomal lesions affecting this locus (Figure 1A).[17,18] In contrast to Loucy cells, aberrant H3K27ac marks (indicative of active chromatin) were identified before and encompassing the noncoding exon 2 of the *LMO2* gene by ChIP-seq in PF-382 and DU.528 T-ALL cell lines (Figure 1B; supplemental Figure 1). Sequencing across these peaks revealed a heterozygous 20 bp duplication in PF-382 cells and a heterozygous 1 bp deletion in DU.528 cells located close to a region recently described as an intermediate promoter for reasons that were not then apparent (Figure 1B).[19] Notably, the mutations were not described as normal germ line variants in the Single Nucleotide Polymorphism Database. In silico analysis of the reference sequence identified a high-confidence primary MYB binding motif (AACCGTT) that was duplicated in the PF-382 cell line, whereas the single bp deletion in DU.528 cells creates a CAACCGC sequence that closely resembles a secondary MYB binding motif (Figure 1B; supplemental Tables 3 and 4).

To assess whether the mutations form aberrant sites of MYB binding, we performed ChIP-seq for MYB and analyzed peaks of MYB enrichment at the *LMO2* locus. There was a complete absence of MYB binding at the intermediate promoter in cells that were wild-type at this locus, suggesting that the presence of the single native MYB motif in itself is insufficient to recruit MYB. In contrast, both PF-382 and DU.528 cells that harbor dual MYB motifs displayed precisely aligned MYB binding at the mutation site (Figure 1B). To determine whether the mutations affected promoter usage, we performed rapid amplification of 5′ complementary DNA ends in *LMO2* mutant and wild-type cell lines by using a common primer in exon 6 capable of capturing the transcription start site of all *LMO2* isoforms. Whereas the majority (73%) of 5′ capped transcripts in Loucy cells originated from the proximal promoter, both PF-382 and DU.528 cells demonstrated preferential usage of the recently described intermediate promoter (75% and 67% of transcripts, respectively; Figure 1C).

Our observations were not limited to T-ALL cell lines because heterozygous mutations at *LMO2* intron 1 were detected in diagnostic samples from 3.7% of pediatric (6 of 160) and 5.5% of adult (9 of 163) T-ALL patients (Figure 1D). Absence of the mutations in 7 available patient-matched remission samples confirmed that they were somatic (supplemental Figure 2). Notably, the mutations were densely distributed around highly conserved native ETS1, MYB, and GATA motifs (supplemental Figure 3). Including the cell lines, 7 mutations

introduced an additional MYB site, resulting in 2 MYB motifs spaced 10 or 20 bp apart, equivalent to 1 or 2 helical coils of DNA, respectively (Figure 1E). Three mutations created potential binding sites for both MYB and ETS1, 3 formed potential ETS1 sites, and 3 produced potential new RUNX1 binding sites (Figure 1E; supplemental Tables 3 and 4). Given that *NOTCH* and *TAL1* have been shown to collaborate with *LMO2* to promote leukemogenesis in murine models of T-ALL, it is noteworthy that of the 15 patients with *LMO2* promoter mutations, 7 had *NOTCH-1* mutations and 8 had *TAL1* activating lesions, including 2 with *TAL1*-enhancer mutations (both creating new MYB motifs; supplemental Table 5).[20,21] Such collaboration between *TAL1*, *LMO2*, and *NOTCH-1* has also been described in gene therapy–induced T-ALL, including 1 patient who harbored both a retroviral integration upstream of *LMO2* and an episomal reintegration at the *TAL1* locus.[9,13,22]

To ascertain whether *LMO2* promoter mutations in T-ALL led to aberrant expression compared with its matched thymic counterpart, we assessed *LMO2* expression by qRT-PCR in thymic subsets sorted for different levels of thymic differentiation.[5] Validating earlier reports that used microarrays, *LMO2* expression was highest in the most immature, precommitment stages of T-cell development and was expressed at low levels from the double-negative stage onward, when thymocytes had undergone biallelic TCR-γ rearrangement (Figure 2A).[5] To determine the level of differentiation arrest of the 15 mutant patient samples, we analyzed the TCR-γ locus by qPCR (supplemental Figure 4); 12 of the 15 samples (including 5 of the 6 patients with available RNA) had biallelic TCR-γ deletion (position weight matrices; supplemental Table 5), indicating that maturation arrest occurred after the pro–T-cell stage of differentiation, and that the majority of patients did not have the ETP ALL phenotype. Thus, compared with their physiological counterparts, those patients with RNA available for *LMO2* qRT-PCR exhibited aberrant *LMO2* overexpression ($P < .002$ vs double-negative and double-positive subsets; Figure 2A). Although we were unable to confirm *LMO2* overexpression in all mutant samples because of the unavailability of RNA, all classes of mutation (additional MYB, ETS1, RUNX1, or MYB+ETS1 sites) were represented in the 6 patients with *LMO2* overexpression. Exploiting a heterozygous germ line single nucleotide polymorphism (rs3740617), DU.528 cells and 3 of 4 informative patient samples displayed skewed allelic expression of *LMO2* (Figure 2B). The observation of biallelic expression in sample A1 suggests a potential lesion on the second allele that remains undefined. Consistent with their *cis*-activating potential, ≥96% of reads from MYB ChIP-seq performed in DU.528 and PF-382 cells aligned to the mutant rather than the wild-type allele (Figure 2C; supplemental Figure 5). Furthermore, the gain-of-function nature of the mutations was confirmed by luciferase reporter assays conducted in Jurkat cells where all mutations markedly activated luciferase activity compared with the wild-type sequence (Figure 2D; supplemental Figure 6A).

To assess causality between the mutations and *LMO2* dysregulation, we used CRISPR/Cas9 genome editing with a guide

Figure 1. *LMO2* intron 1 mutations in pediatric and adult human T-ALL. (A) *LMO2* expression as determined by qRT-PCR in *LMO2* translocated T-ALL cell lines (KOPT-K1 and P12-Ichikawa) and nontranslocated T-ALL cell lines (DU.528, PF-382, Loucy, DND41, Jurkat, and ALL-SIL). (B) ChIP-Seq tracks at the *LMO2* locus for MYB and H3K27ac in PF-382, DU.528, Loucy, and Jurkat T-ALL cell lines. Y-axis values are reads per bin per million mapped reads. Mutations are shown below as identified by Sanger sequencing of PF-382 and DU.528 DNA, with inserted sequences shown in red and MYB motifs underlined. The position weight matrices for the primary and secondary MYB binding sites are from UniPROBE.[27] (C) Pie chart summarizing the percentage of *LMO2* transcripts identified by rapid amplification of 5′ complementary DNA ends that start from the distal, intermediate, and proximal promoters for the PF-382, DU.528, and Loucy T-ALL cell lines. A total of 20, 21, and 22 *LMO2* transcripts was examined for PF-382, DU.528, and Loucy T-ALL cell lines, respectively. (D) Pie chart summarizing mutation recurrence within pediatric and adult human T-ALL cohorts. (E) Indels mapped to the *LMO2* intron 1 mutation hotspot labeled with the associated de novo consensus site as aligned to the UniPROBE or HOCOMOCO position weight matrices, in which MYB, ETS1, and RUNX1 sites are marked as a triangle, square, and diamond, respectively. Below, motif analysis of the region shows the native binding sites for members of the TAL1 complex, including RUNX1, E-box (for TAL1 binding), ETS1, MYB, and GATA. TSS, transcription start site.
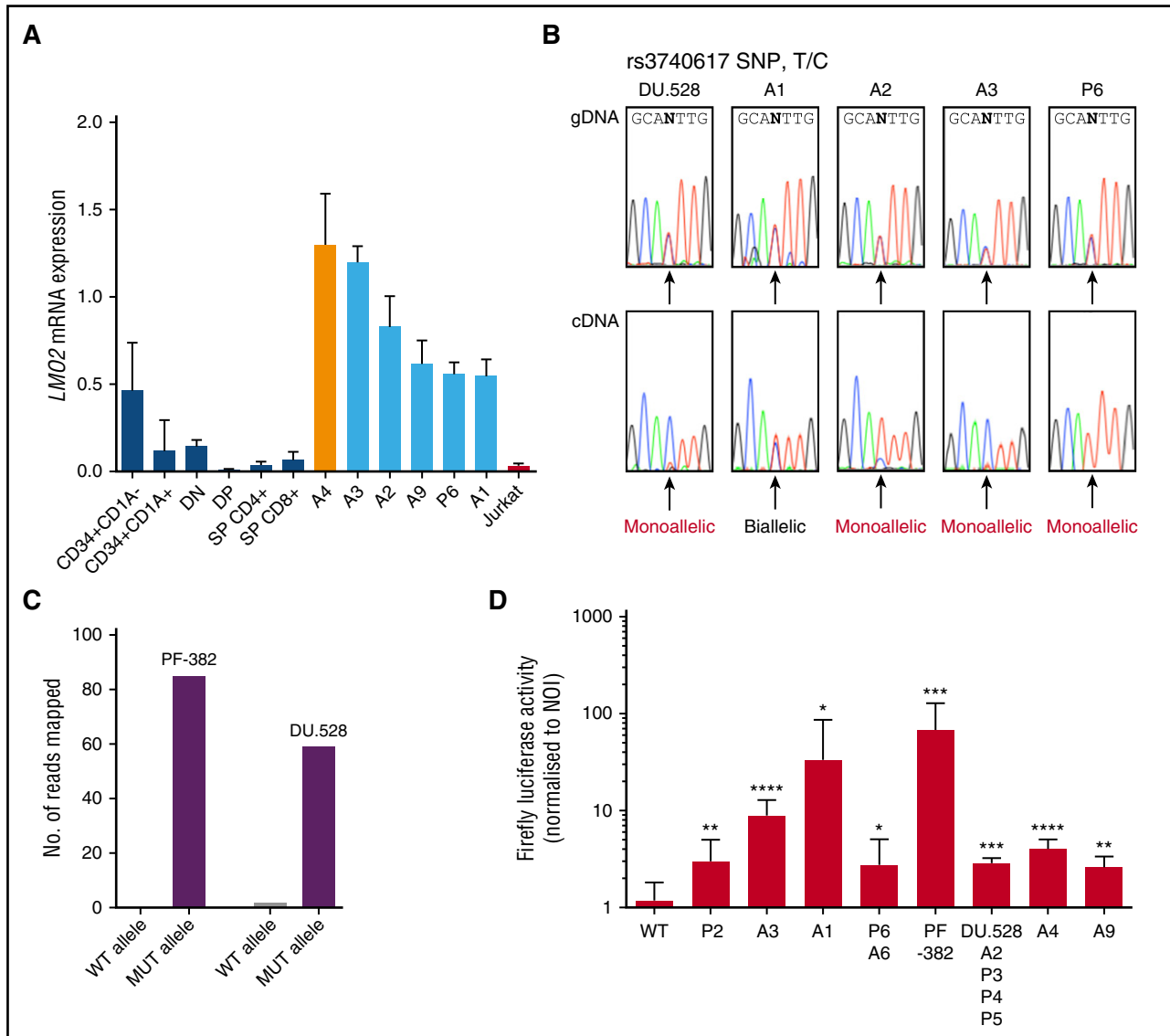
Figure 2. *LMO2* intron 1 indels are predominantly monoallelically activating, and CRISPR/Cas9-mediated knockout of the PF-382 mutant allele downregulates *LMO2* expression. (A) *LMO2* expression as determined by qRT-PCR in human sorted thymic subsets, primary patient samples with *LMO2* intron 1 indels, and the wild-type Jurkat cell line. $P < .002$ for samples A1, A2, A3, A9, and P6 vs double negative and double positive by two-tailed t test. Primary patient samples were assessed for the absence of biallelic TCR-γ deletion (ABD), of which patient sample A4 (orange bar) exhibited ABD, whereas all other patients were non-ABD. (B) The informative single nucleotide polymorphism (SNP) (rs3740617) was amplified in 4 patient samples and the DU.528 cell line from both genomic DNA (gDNA) and complementary DNA (cDNA) templates to infer monoallelic expression. To do this, if one chromatogram peak is detected at a heterozygous SNP within the cDNA, the expression can be interpreted as coming from one allele. (C) Quantification of the number of reads mapped to the wild-type (WT) or mutant (MUT) allele where 54 of 56 reads and 85 of 85 reads mapped to the mutant alleles for DU.528 and PF-382, respectively. (D) Firefly luciferase activity after renilla and no-insert vector normalization for patient-derived indels. Data shown are from ≥3 independent experiments performed in triplicate. Values are mean ± standard deviation and P values were calculated by a two-tailed Student t test. (E) The yellow highlighted sequence is the target region for the CRISPR/Cas9 guide RNA. Aligned sequences are from CRISPR/Cas9-edited PF-382 single-cell clones showing the associated genomic edits generated. Red sequences are inserted sequences, blue are altered, and dashes represent deleted bases. Underlined region shows the presence of the native and mutant MYB binding sites. (F) Gene expression of *LMO2* for each PF-382 clone, as determined by qRT-PCR. Data are expressed as fold change relative to the mean expression of the unedited clones in arbitrary units (AU). Clones are labeled as "unedited" when CRISPR/Cas9 did not edit the region targeted by the guide RNA and "edited" when successful targeting led to the formation of an indel. *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, and ****$P \leq .0001$. NOI, no-insert control; T/C, T to C single nucleotide polymorphism.

RNA designed to target the duplicated MYB site in PF-382 cells (supplemental Figure 6B). Crucially, clone 4F11, which had a single T>C substitution disrupting the MYB binding site, and clone 1A8, in which the mutant allele had been reverted to wild-type, resulted in the most dramatic downregulation of *LMO2* (Figure 2E-F; supplemental Figure 7). Interestingly, 2 clones (4H12 and 6D4) that increased the distance between the native and the mutant MYB sites resulted in a marked reduction in *LMO2* expression, supporting the hypothesis that MYB binding is augmented when additional motifs are orientated on the same side

of the DNA helix.[23] This was further validated by the lack of reduction in *LMO2* expression in a clone (5F10) in which the sequence between the 2 MYB sites was altered but the spacing distance was unchanged.

In conclusion, we identified and functionally validated a novel recurrent mutation hotspot occurring in a noncoding site that drives *LMO2* overexpression from a neomorphic promoter in a substantial proportion of both adult and pediatric T-ALL patients. Remarkably, the mutations create potential binding sites for MYB, ETS1, or RUNX1, all of which are members of a highly oncogenic TAL1-LMO2 complex in
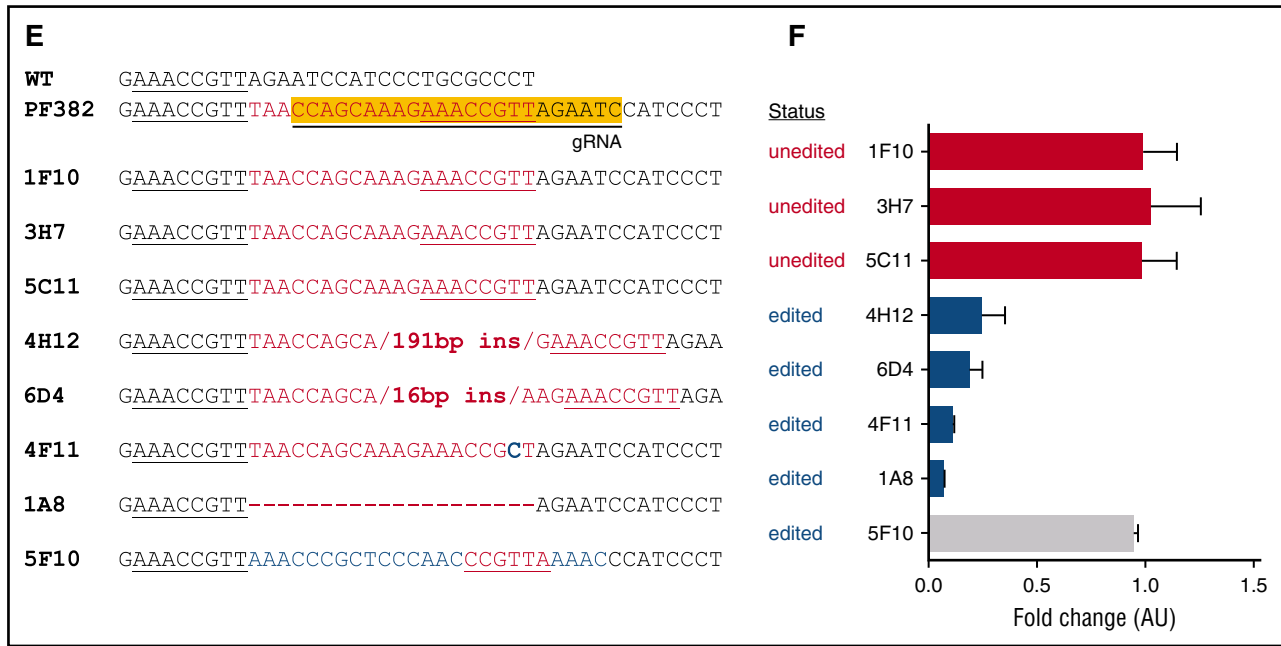
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WT      GAAACCGTTAGAATCCATCCCTGCGCCCT
PF382   GAAACCGTTTAACCAGCAAAGAAACCGTTAGAATCCATCCCT
                                            gRNA

1F10    GAAACCGTTTAACCAGCAAAGAAACCGTTAGAATCCATCCCT

3H7     GAAACCGTTTAACCAGCAAAGAAACCGTTAGAATCCATCCCT

5C11    GAAACCGTTTAACCAGCAAAGAAACCGTTAGAATCCATCCCT

4H12    GAAACCGTTTAACCAGCA/191bp ins/GAAACCGTTAGAA

6D4     GAAACCGTTTAACCAGCA/16bp ins/AAGAAACCGTTAGA

4F11    GAAACCGTTTAACCAGCAAAGAAACCGCTAGAATCCATCCCT

1A8     GAAACCGTT--------------------AGAATCCATCCCT

5F10    GAAACCGTTAAACCCGCTCCCAACCGTTAAAACCCATCCCT
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F

Figure 2. (Continued).

T-ALL; this indicates that LMO2 is a component of an autoregulatory self-sustaining positive feedback loop in these cells, analogous to the autoregulation of *TAL1* we recently described in Jurkat cells.[14,24] To prove that the newly formed ETS1 and RUNX1 sites are sufficient to drive *LMO2* expression, we attempted to but ultimately were unable to knock in these mutations in vitro. Thus, the oncogenic potential of these particular mutations remain an area of ongoing study. There is still a question regarding exactly how various members of the TAL1 complex position themselves on DNA with regard to spacing, orientation, and order of motifs, so-called syntax.[25] Thus, identifying gain-of-function noncoding mutations that have been selected for during tumorigenesis in vivo offers important insights into the optimal DNA syntax required for nucleation of such multiprotein transcription factor complexes. For instance, it may become apparent why a single MYB binding site is sufficient to drive expression from certain loci, such as at the *TAL1* enhancer, whereas others require dual MYB motifs. Finally, we note that these mutations occur within the same intron as retroviral integration sites described in 2 cases of gene therapy–induced T-ALL (supplemental Figure 8).[22,26] This raises the possibility that formation of aberrant promoters and enhancers, either by mutation or retroviral insertion, occur at preferred rather than random sites in the noncoding genome.

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Authorship

Contribution: S.R., M.M., T.E.L., N.F., A.P., Z.L., S.B., C.A., T.P., K.P.-O., L.G.-P., and B.J.A. performed experimental work; K.Z.A., R.J.M., T.N., A.K.F., R.E.G., K.P.-O., L.G.-P., and F.J.T.S. provided primary samples; S.R., M.M., T.E.L., N.F., D.C.L., R.A.Y., F.J.T.S., and A.T.L. analyzed data; S.R., R.E.G., F.J.T.S., D.C.L., and M.R.M. wrote the manuscript; M.R.M. designed the study; and all authors approved the final manuscript.

Conflict-of-interest disclosure: R.A.Y. is a founder and member of the Board of Directors of Syros Pharmaceuticals, which develops therapies that target gene regulatory elements. The remaining authors declare no competing financial interests.

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