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Integrative methylome-transcriptome analysis unravels cancer cell vulnerabilities in infant MLL-rearranged B cell acute lymphoblastic leukemia

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B cell acute lymphoblastic leukemia (B-ALL) is the most common childhood cancer. As predicted by its prenatal origin, infant B-ALL (iB-ALL) shows an exceptionally silent DNA mutational landscape, suggesting that alternative epigenetic mechanisms may substantially contribute to its leukemogenesis. Here, we have integrated genome-wide DNA methylome and transcriptome data from 69 patients with de novo MLL-rearranged leukemia (MLLr) and non-MLLr iB-ALL leukemia uniformly treated according to the Interfant-99/06 protocol. iB-ALL methylome signatures display a plethora of common and specific alterations associated with chromatin states related to enhancer and transcriptional control in normal hematopoietic cells. DNA methylation, gene expression, and gene coexpression network analyses segregated MLLr away from non-MLLr iB-ALL and identified a coordinated and enriched expression of the AP-1 complex members FOS and JUN and RUNX factors in MLLr iB-ALL, consistent with the significant enrichment of hypomethylated CpGs in these genes. Integrative methylome-transcriptome analysis identified consistent cancer cell vulnerabilities, revealed a robust iB-ALL-specific gene expression–correlating dmCpG signature, and confirmed an epigenetic control of AP-1 and RUNX in reshaping the molecular network of MLLr iB-ALL. Finally, pharmacological inhibition or functional ablation of AP-1 dramatically impaired MLLr-leukemic growth in vitro and in vivo using MLLr-iB-ALL patient–derived xenografts, providing rationale for new therapeutic avenues in MLLr-iB-ALL.

Introduction
B cell acute lymphoblastic leukemia (B-ALL) is the most common childhood cancer (1). Current long-term survival rates for pediatric B-ALL approach approximately 85%. However, infant B-ALL (iB-ALL) (<1 year of age) is characterized by an aggressive early clinical manifestation, limited response to current therapies, and poor outcome (2). Chromosomal rearrangements involving the mixed-lineage leukemia gene (KMT2A, also known as MLL) account for approximately 80% of iB-ALL diagnostics (3), and the clinical outcome of B-ALL patients carrying MLL rearrangements (MLLr), especially t(4;11)/KMT2A-AFF1 (MLL-AF4+), is particularly dismal (4). MLL encodes for a H3K4 histone methyltransferase (HMT) required for normal hematopoiesis and HOX gene expression (5–7). Leukemia transformation by MLLr requires the recruitment of the H3K79 HMT Dot1L to the MLL transcriptional complex, rendering a H3K79 methylation pattern in MLLr leukemias (8–12).

In iB-ALL, MLLr occurs prenatally during embryonic/fetal hematopoiesis (13, 14) and represents an initiating oncogenic driver (15). This, coupled with the extremely short latency, suggests that MLLr might be sufficient for leukemogenesis. In line with this, genome-wide DNA-Seq studies in iB-ALL have revealed a silent mutational landscape irrespective of MLL status (9, 16, 17), reinforcing the assumption that MLLr-iB-ALL requires few cooperating mutations for overt leukemia. However, with the exception of a recent work that created a leukemogenic chimeric fusion between human MLL and murine AF4 (18), faithful recapitulation of the MLL-AF4+ iB-ALL disease phenotype remains challenging to model (19–23), suggesting that in the absence of

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DNA methylation profiling of iB-ALL through an integrative genome-wide DNA methylome, including whole-genome bisulfite sequencing (WGB-Seq) and transcriptome (RNA-Seq) profiling of 69 MLLr and non-MLLr iB-ALL leukemias uniformly treated according to the Interfant-99/06 protocol. Our study reveals an iB-ALL–specific gene expression–correlating dmCpG signature and suggests epigenetic control of AP-1 and RUNX family members in reshaping the molecular network of MLLr iB-ALL by regulating the methylation status of downstream target motifs. Pharmacological inhibition or functional ablation of AP-1 dramatically impairs MLLr-leukemic growth in vitro and in vivo, indicating a potential cancer cell vulnerability in MLLr iB-ALL. The elucidation of an intricate relationship between DNA methylation and gene expression underlying the pathobiology of MLLr and non-MLLr iB-ALL opens therapeutic avenues for iB-ALL.

Results

DNA methylation profiling of MLL-AF4+, MLL-AF9+, and non-MLLr iB-ALL. To depict the overall DNA methylation status of iB-ALL, we initially performed WGB-Seq in highly purified (>95%) CD19⁺CD34⁺ leukemic blasts from MLL-AF4⁺ (n = 2), MLL-AF9⁺ (n = 2), and non-MLLr (n = 2) iB-ALLs and in 2 pools of healthy CD34⁺CD19⁺ B cell progenitors (BCPs) as controls (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI138833DS1). Irrespective of MLL status, leukemic blasts from iB-ALL were characterized by a global significantly lower DNA methylation than in healthy BCPs (Figure 1, A and B). Analysis of the FLT3 gene, a classical MLL target overexpressed in MLLr iB-ALL (33), confirmed a specific loss of DNA methylation at target gene promoter regions in MLLr iB-ALL, thus validating the WGB-Seq data (Figure 1C). Next, we performed a differential methylation analysis and identified a total of 47,713, 54,409, and 50,127 differentially methylated regions (DMRs), mostly hypomethylated, between healthy BCPs and MLL-AF4⁺, MLL-AF9⁺, and non-MLLr iB-ALL patients, respectively (Figure 1D and Supplemental Table 2). DMRs, especially the hypomethylated sites, were enriched in multiple families of DNA repetitive elements (Figure 1E and Supplemental Table 3). WGB-Seq data were validated by DNA bisulfite pyrosequencing of long interspersed nucleotide element (LINE) and DNA methylation arrays in an extended cohort of n = 69 iB-ALLs and n = 6 pools of BCPs (Supplemental Figure 1, A and B, and Supplemental Table 4). Taking these data together, iB-ALL is characterized by a global loss of DNA methylation.

To understand the mechanisms underlying a particular epigenomic signature in iB-ALL, we performed a hypergeometric optimization of motif enrichment (HOMER) analysis with the information encoded by either DMRs “specific” for a given iB-ALL subtype or “common” to at least 2 iB-ALL subtypes (Supplemental Figure 2 and Supplemental Table 5). Analysis of hypomethylated DMRs revealed a significant enrichment in PU.1, EBF, SpiB, and ETS for iB-ALL, regardless of MLL status, suggesting that these regulators might either trigger a common transcriptional leukemogenic program or regulate normal B cell differentiation. In contrast, hypomethylated DMRs were specifically enriched in the AP-1 family transcription factors (TFs) FOS and JUN and in RUNX family members in MLLr iB-ALL.

Different DNA methylation patterns in iB-ALL subtypes according to the genomic location and CpG context. To further capture the epigenetic heterogeneity of iB-ALL, we studied a larger cohort of 37 MLL-AF4⁺, 12 MLL-AF9⁺, and 20 non-MLLr iB-ALL using high-content DNA methylation arrays. Six healthy BCPs and naive B cells (34) were analyzed as normal B cell counterparts accounting for DNA methylation changes naturally occurring during B cell differentiation (Figure 2A and Supplemental Table 4). Importantly, DNA methylation arrays and WGB-Seq displayed a very robust correlation (Supplemental Figure 3, A and B). An unsupervised principal component analysis (PCA) segregated iB-ALL methylomes from BCP/naive B cell methylomes (Figure 2B), and a hierarchical clustering of the 10,000 most variable CpG sites distinguished the iB-ALL subtypes, including MLL-AF4⁺ and MLL-AF9⁺ subgroups, suggesting that specific MLL fusions might differentially reshape the methylome landscape of iB-ALL (Figure 2C).

We next performed a differential methylation analysis comparing iB-ALLs and healthy naive B cells (34) with healthy BCPs and found 77,596 and 35,157 differentially methylated CpGs (dmCpGs) in iB-ALL and naive B cells, respectively (Figure 2D). Intriguingly, 53% (18,470) of the dmCpGs observed in naive B cells were shared with iB-ALL samples, indicating that they represent methylation changes naturally occurring during B cell differentiation and were therefore subtracted from iB-ALL–specific dmCpGs, providing an iB-ALL–specific dmCpG signature (Figure 2D and Supplemental Table 6). In line with WGB-Seq data, hypomethylated CpGs surpassed hypermethylated CpGs in most of the comparisons (Figure 2D). However, hypermethylated CpGs were substantially abundant in MLLr iB-ALL.

To define the overall relatedness among the different iB-ALL subtypes, we performed a global pairwise correlation analysis using the aforementioned iB-ALL–related 77,596 dmCpGs. MLL-AF4⁺ and non-MLLr leukemias were the least related leukemia subtypes at these aberrant loci (Pearson’s correlation, 0.63; Figure 2E). However, while MLL-AF9⁺ leukemias tend to display a higher correlation with MLL-AF4⁺ samples (Pearson’s correlation, 0.77), they also shared multiple alterations with non-MLLr samples (Pearson’s correlation, 0.75), indicating that the MLL-AF9⁺ subtype displays overlapping features with MLL-AF4⁺.
pattern specific for $MLL-AF4^+$ iB-ALL. Healthy naive B cells displayed no substantial enrichment of hypermethylated CpGs at either CpG islands or promoter regions (Figure 3, A and B; statistics available in Supplemental Table 7). Of note, hypermethylated CpGs at CpG islands and promoter regions were more enriched in $MLL-AF4^+$ iB-ALL than in $MLL-AF9^+$ and non-$MLLr$ iB-ALL subgroups, defining a methylene and non-$MLLr$ iB-ALLs. A detailed inspection of the genomic distribution of the dmCpG sites revealed an enrichment of hypomethylated CpGs at open sea locations and intronic regions, while hypermethylated CpGs were enriched in CpG islands, CpG shores, and promoter areas, regardless of the iB-ALL subtype (Figure 3, A and B; statistics available in Supplemental Table 7).

We next performed a transcription factor binding site (TFBS) enrichment analysis to predict the involvement of potential TFs enriched in dmCpGs. We used the Gene Transcription Regulation Database (GTRD), which includes the most complete

Figure 1. Global DNA methylation status of the different iB-ALL subtypes. (A) Violin plots reflecting the global DNA methylation levels of CpG sites identified by WGB-Seq. Graph represents the percentages of CpG methylation distribution of the genome segmented in 10 Kbp genomic windows. The DNA methylation status significantly differs among all iB-ALL subtypes and the average methylation of healthy BCPs. *** $P < 0.001$, 2-sided Wilcoxon’s rank sum test. (B) Circos-plot representation of DNA methylation levels along the genome. CpG methylation was averaged in 10 Mbp genomic windows and the average DNA methylation value for each iB-ALL subtype is represented as a histogram track. Inner lines identify $MLL-AF4^+$ (blue) and $MLL-AF9^+$ (green) translocation events. (C) Line plots depicting the DNA methylation profile for the FLI3 gene. The CpG context and the CpG site location are mapped to the corresponding genomic coordinates, as indicated in the lower panels. Areas with significant ($q < 0.05$) differential methylation between $MLL-AF4^+$/ $MLL-AF9^+$ and non-$MLLr$ iB-ALL or healthy BCPs methyleomes are shaded. *** $P < 0.001$. (D) Venn diagrams representing the total number of DMRs with consistent hyper- or hypomethylation changes for each of the indicated comparisons. (E) Heatmap indicating the log2 odds ratio enrichment of different DNA repetitive regions for significant hyper- or hypomethylated DMRs, respectively ($q < 0.05$).
collection of uniformly processed TF ChIP-Seq data available to date (35). In line with WGB-Seq data, analysis of hypomethylated CpGs revealed a significant differential enrichment in MLL-AF4+ iB-ALLs for the AP-1 family TFs FOS and JUN (Figure 3C and Supplemental Table 8), suggesting they represent MLL-AF4+–specific differentially hypomethylated TFBS.

Aberrant DNA methylation in iB-ALL is associated with a distinctive repertoire of chromatin signatures. To unveil the potential functional implications of aberrant DNA methylation in iB-ALL, we performed a comprehensive region set enrichment analysis by using 6 publicly available histone data sets (H3K4me1, H3K4me3, H3K27ac, H3K27me3, H3K36me3, H3K9me3) comprising a total of 5 reference B-lineage hematopoietic epigenomes from the Roadmap and ENCODE epigenome consortia (36, 37). We found a differential enrichment of iB-ALL–specific, but not normal B cell–specific, hypermethylated CpGs at locations decorated with the H3K4me3 mark (Figure 4A and Supplemental Table 9). This was more evident in MLL-AF4+ iB-ALL. Hypomethylated CpGs were not differentially enriched in iB-ALL for any of the histone marks analyzed (Figure 4A).

Given the complexity of the histone code, its widespread distribution, and the plausible colocalization of some of these histone marks, we next performed an enrichment analysis based on chromatin segmentation data to facilitate the interpretation of the potential role of these leukemia alterations in normal tissue. This type of analysis integrates histone mark information from the aforementioned tissues in order to fractionate the genome into functionally related chromatin states (38). The functional distribution of both hyper- and hypomethylated CpG sites differed between iB-ALL subgroups and normally differentiated B cells (Figure 4B and Supplemental Table 10). Hypermethylated CpG sites were significantly enriched in iB-ALL at genomic locations associated with flanking and bivalent transcription start sites (TSSs) (states 2, 4, 14). These enrichments were more consistent for MLL-AF4+–specific hypermethylated CpGs. In contrast, DNA hypermethylation enrichment at enhancer regions (states 9–11) associated with normal B cell differentiation was lost in iB-ALL, suggesting that the precise control of these regulatory elements is impaired in iB-ALL, regardless of MLL status. Hypomethylated CpG sites
with different molecular mechanisms involved in the epigenetic remodeling of the distinct iB-ALL subtypes.

The transcriptional program of MLLr iB-ALL is governed by members of the AP-1 complex. Aberrant DNA methylation in iB-ALL associates to the function of chromatin states related to transcriptional control (Figure 3B). To identify relevant cellular pathways displaying transcriptional changes, we next performed a comprehensive transcriptomic analysis of iB-ALL patients (n = 40) and healthy BCPs (n = 5) from whom paired RNA-Seq DNA methylation data were available (Figure 5A). An unsupervised PCA of the RNA-Seq data readily distinguished iB-ALL patients from healthy naive B cells and BCPs (Figure 5B). Hierarchical clustering based on DNA methylation data was more homogeneous than that based on gene expression, particularly for MLL-AF4+ iB-ALL (Figure 5C), as gene expression clustering yielded 2 separate subgroups in MLL-AF4+ patients. However, both DNA methylation and gene expression could generally cluster non-MLLr apart from MLLr patients, indicating that both the methylome and transcriptome landscape of non-MLLr and MLL-AF4+ iB-ALL are differentially governed.
We next performed a differential expression analysis of MLL-AF4+, MLL-AF9+, non-MLLr iB-ALLs, and naive B cells (Supplemental Table 12). Our pipeline identified 5170 and 3919 differentially expressed genes (DEGs) specific for iB-ALL and naive B cells, respectively (Figure 5D). MLL-AF4+ iB-ALLs displayed a larger expression variability (Figure 5E), consistent with the gene expression heterogeneity observed in the hierarchical clustering (Figure 5C), indicative of distinct molecular subgroups within MLL-AF4+ iB-ALL patients (9). To explore the molecular networks underlying the transcriptional rewiring of iB-ALL, we performed a gene coexpression network analysis using the 663 most variable genes from the RNA-Seq and found 3 substantially different gene expression modules (Figure 5F and Supplemental Figure 5A). Modules 1 and 2 were enriched in MLL-AF4+ and MLL-AF9+ subgroups and barely represented in healthy naive B cells and BCPs, while module 3 mainly distinguished MLL-AF4+ iB-ALL from MLL-AF9+ and non-MLLr iB-ALL (Figure 5F). A detailed inspection of these modules revealed a differential gene set enrichment of gene ontology categories related to cell activation, inflammatory response, and cell division for modules 1, 2, and 3, respectively (Supplemental Figure 5, B and C). In addition, module 2 showed a significant enrichment of gene sets related to NF-κB, p53, and STAT signaling pathways, while module 3 displayed an enrichment of gene sets associated with G2M checkpoint, E2F targets, and mitotic spindle. A different set of TFs was coexpressed in each module (Supplemental Figure 5, D–F). Importantly, module 2, which best segregated MLLr iB-ALLs from non-MLLr iB-ALLs and normal naive B cells and BCPs, showed a coordinated and enriched expression of FOS and JUN, TFs of the AP-1 complex (Supplemental Figure 5E), consistent with the significant enrichment of hypomethylated CpGs in FOS and JUN for MLLr iB-ALL, indicating that an epigenetic control of these AP-1 members may contribute to the pathogenesis of MLLr iB-ALL.

Integrative analysis reveals a gene expression–correlating dmCpG signature and an epigenetic control of AP-1 members in MLLr iB-ALL. We used ELMER (40) to integrate paired DNA methylation and RNA-Seq data in iB-ALL. ELMER correlations were calculated using those dmCpGs specific for a given iB-ALL subtype or common for iB-ALL (shared by > 2 iB-ALL subgroups), and all the genes expressed in the RNA-Seq (Figure 6A). Up to 10%, 17%, 33%, and 25% of the common, MLL-AF4+, MLL-AF9+, or non-MLLr dmCpGs, respectively, showed a robust correlation with gene expression (Supplemental Table 13). To facilitate data interpretation, we focused on those correlations supported by the classical model of epigenetic regulation and found that DNA
methylation correlated well with gene expression; hypermethylation changes were associated with gene repression, while hypomethylation changes correlated with gene activation (Figure 6, B and C). Gene expression–correlating dmCpG probes were enriched at CpG islands and distal promoters, but significantly underrepresented at open sea locations and intergenic regions (Supplemental Figure 6, A and B; statistics available in Supplemental Table 7). Moreover, the distribution of these CpG sites was associated with histone marks and chromatin states linked to active transcription (Supplemental Figure 6, C and D, and Supplemental Tables 14 and 15), identifying gene expression–associated dmCpGs in iB-ALL. For instance, hypomethylation of the CpG probe cg07893009 in iB-ALL correlated with increased expression of LMO2 (ref. 41 and Figure 6D), while hypermethylation of the CpG probe cg06365535 was significantly associated with repression of BRIP1 in iB-ALL (ref. 42 and Figure 6E). Additionally, reatome pathway enrichment analysis using all gene expression–correlating dmCpG probes showed a link between gene repression and hypermethylated CpGs in MLLr iB-ALL, associated with impairment of cellular pathways related to cell cycle, mitosis, and DNA replication and repair (Figure 6F).

Then we sought to determine the contribution of master TFs interacting with gene expression–correlating dmCpGs. Enrichment of TFBS within these dmCpGs was performed using ELMER and publicly available human binding models from the HOCOMOCO database (43). A marked enrichment of hypermethylated CpGs was seen in SP and E2F TFs (Figure 6G). Strikingly, however, many FOS/JUN members and RUNX proteins were substantially enriched in hypomethylated CpGs, specifically for MLL-AF4+ iB-ALL patients, further suggesting that an epigenetic control of AP-1 members reshapes the molecular network of MLL-AF4+ iB-ALL.

AP-1– and RUNX-interacting factors reshape the regulatory network of iB-ALL by regulating the methylation status of downstream target
Importantly, the average DNA methylation of E2F5 motifs substantially increased in MLL-AF4+ iB-ALL (Figure 7D), suggesting that the absence of E2F5 facilitated methylation at these particular loci. Furthermore, the expression of E2F5 targets was below that expected by chance in all iB-ALL groups (Figure 7E). As expected, the expression of E2F5 targets was also repressed in naive B cells, as E2F5 is a transcriptional repressor in terminally differentiated B cells.

We next explored whether master TFs epigenetically control gene expression in iB-ALL. We first mapped the genomic context of E2F5, a TF enriched in hypermethylated CpG sites (Figure 6G), and found 11 highly correlated CpG probes within 1 Mbp upstream of the E2F5 TSS (Figure 7A). This genomic region was specifically hypermethylated in iB-ALL (Figure 7B), and the E2F5 expression was repressed, particularly in MLL-AF4+ iB-ALL (Figure 7C).
genes and highly correlated CpGs. Top network nodes were characterized by the presence of hypomethylated FOSL2-binding motifs and upregulation of corresponding FOSL2 targets (Supplemental Figure 7A). We further explored 2 nodes of the FOSL2 regulatory network, DUSP10 and CD44, which are protu-
morigenic genes highly expressed in iB-ALL (44, 45). DUSP10-
and CD44-related dmCpGs were significantly hypomethylated (Supplemental Figure 7, B and C) and DUSP10/CD44 inversely overexpressed in iB-ALL, particularly in MLL
r iB-ALL (Supplemental Figure 7, D and E).

In order to demonstrate the functional impact of FOSL2 upreg-
ulation in the methylation status of MLL-AF4+ leukemias, we
performed functional assays in CRISPR-Cas9–mediated FOSL2
knockout MLL-AF4+ SEM cells (Figure 8A). Overall, we observed
a significant global hypermethylation of the genome upon FOSL2
ablation (Figure 8B), and a total of 12,218 hypermethylated and

Then we focused on AP-1 members, which were highly
enriched at hypomethylated CpG sites in MLL-AF4+ iB-ALL
(Figure 6G). Five highly correlated enhancer-linked CpG probes
were found distributed across 0.35 Mbp downstream of the
FOSL2 promoter (Figure 7F). These dmCpG loci were robustly
demethylated in iB-ALL, especially in MLL-AF4+ patients (Fig-
ure 7G). Hypomethylation of such dmCpGs was accompanied by
a significant increase in FOSL2 expression in iB-ALL (Figure
7H). Accordingly, the average DNA methylation of binding
motifs dramatically decreased in iB-ALL, particularly in MLL-
AF4+ patients (Figure 7I), and the expression of FOSL2 targets
in iB-ALL was higher than in naive B cells and that expected by
chance in iB-ALL (Figure 7J).

To confirm that FOSL2 plays a role in reshaping the epigen-
etic landscape of iB-ALL, we performed a deeper network anal-
ysis to validate the link between the expression of FOSL2 target

Figure 7. E2F and AP-1 interacting factors control the methylation status of downstream target motifs. (A) Ideogram representing the genomic location of E2F5 expression–correlating dmCpG sites. n denotes the number of correlating dmCpGs identified with ELMER algorithm. (B) Boxplot depicting the average methylation of the significant E2F5 expression–correlating CpG probes across different groups. (C) Boxplot reflecting the expression of E2F5 in the indicated groups. (D) Scatter plot showing the correlation between average DNA methylation of E2F5 motif targets and the expression of E2F5. Colored dots: blue, BCP; red, MLL-AF4+; green, MLL-AF9+; yellow, non-MLLr. (E) Violin plots indicating the distribution of gene expression changes (log2 fold change of the indicated groups versus healthy BCPs) of target genes with E2F5 motifs obtained with ELMER algorithm.*P < 0.05; ***P < 0.001, 2-sided Wilcoxon’s rank sum test. All correlated genes with E2F5 motifs included in any of the iB-ALL subgroups were used for the representation of the B cell gene expression distribution. The “random” group includes a random sampling of the same number of genes included in the B cell group, but using the original gene expression matrix including all genes with detectable expression in the RNA-Seq data set. (F–J) Same as A–E, but for the TF FOSL2.

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Supplemental Table 17). To further validate these observations at the gene expression level, we performed RNA-Seq in SEM WT and SEM-FOSL2KO cells. We observed that genes associated with the activation of the AP-1 pathway were significantly impoverished in the context of FOSL2 depletion (Figure 8E). In addition, FOSL2 hypomethylated sites were identified between FOSL2KO and SEM WT cells (mean $\beta$ difference $>0.40$). A subsequent HOMER motif enrichment analysis highlighted the top enriched TFBSs most likely associated with members of the AP-1 complex (Figure 8D and Supplemental Table 17). To further validate these observations at the gene expression level, we performed RNA-Seq in SEM WT and SEM-FOSL2KO cells. We observed that genes associated with the activation of the AP-1 pathway were significantly impoverished in the context of FOSL2 depletion (Figure 8E). In addition, FOSL2
targets were significantly downregulated in FOSL2KO cells (Figure 8F). In the context of FOSL2 ablation in MLL-AF4+ cells, further analyses of the FOSL2-related dmCpGs and their potential contribution to gene expression were exemplified with NUDT21 and FSCN1 genes (Figure 8, G and H). Upon FOSL2 ablation, a CpG island in the vicinity of NUDT21 TSS was hypermethylated, which led to an increased expression of this 3’ end RNA cleavage and polyadenylation factor. In contrast, DNA hypermethylation of a FOSL2 motif located near the TSS of FSCN1 was correlated with the downregulation of such genes in SEM cells. These experiments validate the role of FOSL2 observed in the in silico network approach.

We next explored the regulatory network of RUNX1 in order to expand our observations toward another classical molecular target of MLL-AF4+ aberrantly expressed in MLL-AF4+ iB-ALL (46). We found 4 highly correlated enhancer-linked CpG sites distributed along the genomic loci of RUNX1 (Supplemental Figure 8A). Similar to FOSL2, these dmCpG loci were robustly demethylated in iB-ALL, especially in MLL-AF4+ patients (Supplemental Figure 8B). Hypomethylation of such dmCpGs was accompanied by a significant increase in RUNX1 expression (Supplemental Figure 8C). In addition, the average DNA methylation of RUNX1 binding motifs dramatically decreased in iB-ALL (Supplemental Figure 8D), and the expression of RUNX1 targets in iB-ALL was higher than in naive B cells and that expected by chance in iB-ALL (Supplemental Figure 8E). A network analysis of RUNX1 top target genes revealed that the RUNX homolog RUNX2 was modulated in a similar fashion in MLLr iB-ALL (Supplemental Figure 8F). RUNX2-related CpGs were significantly hypomethylated in MLLr iB-ALL (Supplemental Figure 8G) and RUNX2 inversely overexpressed in MLLr iB-ALL (Supplemental Figure 8H). Collectively, an intricate relationship between DNA methylation and gene expression reshapes the regulatory network of iB-ALL through master molecular hubs, such as members of the AP-1 complex and RUNX1.

MLL-AF4 regulates the expression of E2F5, AP-1, and RUNX family members. The silent mutational landscape of MLLr iB-ALL suggests that MLL-AF4+ translocation may suffice to initiate leukemogenesis. To explore whether MLL-AF4 can itself activate the transcriptional program observed in iB-ALL patients, we analyzed RNA-Seq data from a recent study by Lin and colleagues that created a leukemogenic chimeric fusion between MLL-AF4 and FOSL1 genes (18). We found that expression of E2F5, FOSL2, and RUNX1 was similarly regulated in MLL-AF4+ transduced CD34+ cells (Figure 9A) and in human primary MLL-AF4+ iB-ALL patients (Figure 7), indicating that MLL-AF4 triggers an analogous transcriptomic rewiring in the absence of further genomic mutations. Moreover, a similar gene activation signature was observed for multiple members of the AP-1 complex, including FOS, FOSL1, FOSB, JUN, JUNB, and JUND (Supplemental Figure 9A). Of note, we found direct binding of MLL-AF4 in CD34+ cells or MLLN/AF4C in SEM cells to the promoter region of FOSL2 and RUNX1 (Figure 9B), FOS, FOSL1, JUN, JUNB, and JUND (Supplemental Figure 9B), suggesting a direct regulation of these target genes by MLL-AF4.

To validate the regulation of these targets in the context of the endogenous human translocation, we generated CRISPR/Cas9-mediated locus-specific t(4;11)/MLL-AF4+ in CD34+ cells, as recently reported by Secker and colleagues (47). Expression of FOSL2 and RUNX1 (Figure 9C), but also the other members of the AP-1 family, including FOS, FOSL1, FOSB, JUNB, and JUND, were upregulated upon the endogenous MLL-AF4+ translocation event (Supplemental Figure 9C). We next performed a comprehensive analysis of the DNA methylome upon t(4;11)/MLL-AF4+ translocation in these CD34+ cells and observed a total of 5011 hypermethylated and 6752 hypomethylated CpGs upon the induction of the translocation event (Figure 9D and Supplemental Table 18). Intriguingly, more than 80% of the dmCpG sites identified upon t(4;11)/MLL-AF4+ induction in CD34+ cells were located at the same methylated genes affected by MLL-AF4 in iB-ALL patients, yielding an exact positional overlap of approximately 25% of CpG sites (Figure 9E). Nonetheless, AP-1 members were among the most significant TFBSs identified at t(4;11)/MLL-AF4+ hypomethylated sites (Figure 9F and Supplemental Table 19). Collectively, MLL-AF4 controls the expression of AP-1 members in the absence of other genetic insults and is directly associated with the observed demethylation at their binding motifs, indicating that the presence of AP-1 members may protect from DNA methylation at these particular loci.

AP-1 complex sustains cell proliferation and tumorigenic capacity of MLL-AF4+ B-ALL cells in vitro and in vivo. The AP-1 complex acts as a central player in the epigenetic and transcriptional remodeling observed in MLLr iB-ALL patients. Thus, we were next prompted to explore in vitro and in vivo whether targeting AP-1 may represent a therapeutic opportunity for MLLr B-ALL. We perturbed AP-1 activity in SEM cells by either transduction of a dominant negative isofom of FOS (dnFOS) (ref. 48, Figure 10A) or CRISPR/Cas9-mediated ablation of FOSL2 (Figure 8A). dnFOS expression was impaired in in vitro cell proliferation (Figure 10A) and reduced 4-fold the clonogenic capacity (Figure 10B) and reduced 4-fold the clonogenic capacity of dnFOS-SEM cells in vivo (Figure 10, C–E). Even a more robust impairment in proliferation and clonogenic and tumorigenic capacity was obtained upon CRISPR-mediated FOSL2 ablation in SEM cells both in vitro and in vivo (Figure 10, F–J).

We then performed a pharmacological inhibition of AP-1 via the available chemical inhibitors SR11302 or T5224 and observed an impaired proliferation of t(4;11)/MLL-AF4+CD34+ cells in a dose-dependent manner (Figure 11A). To validate our observations, we expanded our in vitro pharmacological analyses of the T5224 AP-1 inhibitor in a panel of additional MLLr and non-MLLr leukemic cell lines. Of note, we confirmed a specific and dose-dependent effect of T5224 in inhibiting the proliferation of MLLr, but not non-MLLr, cell lines (Figure 11, B and C).

Finally, to validate the in vivo efficacy of our pharmacological approach, the T5224 inhibitor was combined with the B-ALL standard-of-care treatment, based on vincristine, dexamethasone, and L-asparaginase (VXL treatment), and administered to preclinical B-ALL PDX models well established in our laboratory (refs. 49, 50; Figure 12A). The levels of minimal residual disease (MRD) after 2 complete cycles of VXL and subsequent relapse rates were measured in BM, spleen, and PB.
The therapeutic improvement mediated by T5224 became more evident at endpoint analysis. The proportion of mice with BM leukemic graft was lower in the T5224-treated group (10%) compared to VXL (12%) and VXL+T5224 (18%).

engraftment was determined before treatment in order to randomize mice based on BM leukemic burden to receive VXL or VXL+T5224 (Figure 12B). After 2 complete cycles of VXL (day 20), we observed 8 of 10 and 9 of 10 MRD negative mice (% blasts <5%) for VXL and VXL+T5224 treatment, respectively (Figure 12C). The therapeutic improvement mediated by T5224 became more evident at endpoint analysis. The proportion of mice with BM leukemic graft was lower in the T5224-treated group (10%) compared to VXL (12%) and VXL+T5224 (18%).

Figure 9. MLL-AF4 regulates the expression of AP-1 members and RUNX1 and reshapes the methylome landscape of CD34+ cells. (A) Boxplot depicting the average expression of E2F5, FOSL2, and RUNX1 genes in healthy untransduced CD34+ cells or in CD34+ cells transduced with either human:mouse chimeric MLL-AF4 or human MLL-AF9. ***P < 0.001, 2-sided Welch's t test. n = 3; n = 6; n = 3, respectively. (B) UCSC Genome Browser tracks representing the binding pattern of MLL-AF4 (in CD34+ cells) or MLLN/AF4C (in SEM cells) in the vicinity of E2F5, FOSL2, or RUNX1 genes. Data represents ChIP-Seq signals obtained from NCBI database Gene Expression Omnibus [GEO] GSE84116 and GSE74812, respectively. (C) Barplots indicating RT-PCR relative fold-change of E2F5, FOSL2, or RUNX1 expression between nonedited CD34+ cells (CD34CONTROL) and CRISPR-edited CD34+ cells carrying locus-specific t(4;11)/MLL-AF4+ (CD34CRISPR t(4;11)). Barplots represent mean ± SD. *P < 0.05, 2-sided Welch's t test. (D) Scatterplot indicating the number of hyper- or hypomethylated CpG sites observed upon CRISPR t(4;11)/MLL-AF4+ edition in CD34+ cells (mean β difference > 0.20). (E) Venn diagrams representing the number of overlapping genes decorated with dmCpGs (top) or the number of overlapping dmCpGs (bottom, positional overlap) between MLL-AF4+ patients and CD34+ t(4;11)/MLL-AF4+ (CD34CRISPR t(4;11)) cells, as compared with healthy BCPs or nonedited CD34+ cells, respectively. P < 0.001. One-tailed hypergeometric test was used for all the comparisons. (F) Plots displaying enrichment of TFBS in the context of hypo- and hypermethylated CpGs in CD34+ t(4;11)/MLL-AF4+ (CD34CRISPR t(4;11)) cells as determined by the information obtained from the GTRD database. The y axis represents the –log10 adjusted P value enrichment of particular TFBS data set as compared with the background distribution of the EPIC platform.
iB-ALL, especially t(4;11)/MLL-AF4+-expressing iB-ALL, remains an outlier acute leukemia during childhood characterized by early clinical manifestation, limited response to current therapies, and dismal outcome (2). iB-ALL is a well-established developmental cancer in which the recurrent initiating genetic/molecular alterations arise prenatally (13, 14, 51, 52). Despite improved genetically modified mouse and human xenograft models, the historical challenge to faithfully recapitulating the latency and phenotype of MLLr iB-ALL disease has hampered clinical/therapeutic advances. Importantly, recent elegant genome-wide DNA-Seq studies in iB-ALL revealed a silent mutational landscape regardless of MLL status (9, 16, 17). This together with the extremely short latency of the disease indicates that MLLr might be sufficient for leukemogenesis or that iB-ALL may require very few cooperating DNA mutations for overt leukemia. The nature of both the cell

disruption of the AP-1 complex of Tfs is central in the pathogenesis and leukemogenesis of MLL-AF4+ iB-ALL and may offer an unexplored clinical therapeutic opportunity for MLLr iB-ALL.

Discussion

iB-ALL, especially t(4;11)/MLL-AF4+-expressing iB-ALL, remains an outlier acute leukemia during childhood characterized by early clinical manifestation, limited response to current therapies, and dismal outcome (2). iB-ALL is a well-established developmental cancer in which the recurrent initiating genetic/molecular alterations arise prenatally (13, 14, 51, 52). Despite improved genetically modified mouse and human xenograft models, the historical challenge to faithfully recapitulating the latency and phenotype of MLLr iB-ALL disease has hampered clinical/therapeutic advances. Importantly, recent elegant genome-wide DNA-Seq studies in iB-ALL revealed a silent mutational landscape regardless of MLL status (9, 16, 17). This together with the extremely short latency of the disease indicates that MLLr might be sufficient for leukemogenesis or that iB-ALL may require very few cooperating DNA mutations for overt leukemia. The nature of both the cell
determine the contribution of global DNA methylation to the pathogenesis of iB-ALL.

We report a global DNA hypomethylation in iB-ALL samples, similar to that found in many human tumors (56). This is supported by a recent whole-genome methylome study in leukemic twins with concordant iB-ALL who displayed a strikingly similar genome-wide methylome landscape, supportive of a convergent epigenetic evolution of the concordant iB-ALL (52). Of note, we identified that a marked proportion of the epigenetic aberrations found in iB-ALL coincided with the orchestrated changes observed during naturally occurring B cell maturation. This is consistent with previous findings in CLL (54) and other B cell neoplasms (53), indicating that regulated DNA methylation changes occurring in physiological processes, such as B cell differentiation, represent confounding noise challenging the discovery of bona fide cancer-specific methylation changes. Indeed, a reliable discrimination between the methylomes of the distinct iB-ALL subtypes was achieved by analyzing healthy BCPs and naive B cells, thus adopting a “filtered-out” strategy in which methylation changes

of origin in which MLLr arises and the downstream transformed hematopoietic stem and progenitor cells (HSPCs) capable of initiating leukemia and fueling disease progression is still a matter of debate (9, 23). Furthermore, in the absence of DNA mutations, other nongenetic oncogenic insults are required to initiate leukemogenesis of such aggressive leukemia in newborns (15).

In this study, we speculated that a whole-genome deconstruction of the DNA methylome landscape of this mutationally silent iBCP-ALL will likely provide unique insights into the disease pathobiology. Recent advances in computational and next-generation sequencing approaches have been crucial to uncovering the complex relationship among the genome, the epigenome, and the transcriptome of other B cell malignancies, including adult B-ALL, diffuse large B cell lymphoma (53), chronic lymphocytic leukemia (CLL) (54), and mantle cell lymphoma (55). Here, we have integrated genome-wide DNA methylome and transcriptome profiling data from a total of 69 MLLr and non-MLLr iB-ALL leukemia patients uniformly treated according to the Interfant-99/06 protocol in order to determine the contribution of global DNA methylation to the pathogenesis of iB-ALL.

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naturally occurring during normal B cell differentiation were subtracted, thus defining an iB-ALL–specific methylation signature.

A key aspect in the diagnosis and treatment of leukemia patients relies on the identification of common and specific alterations between defined leukemia subtypes. Here, we observed that while the aberrant DNA methylation landscape of MLL-AF4+ and non-MLLr leukemias substantially differs, MLL-AF9+ leukemias correlate with both MLL-AF4+ and non-MLLr subtypes. Despite the limited number of MLL-AF9+ cases included in this study, this observation indicates that MLL-AF9+ iB-ALL may display shared aberrant features and that the contribution of MLL rearrangement in this scenario may account for only a fraction of the observed aberrations in MLL-AF9+ iB-ALL. These results are in agreement with data from Stumpel and colleagues (31) showing that differential DNA hypermethylation at promoter regions was observed between MLL-AF4+ and MLL-AF9+ patients.

Despite the intrinsic limitations of the Human Methylation EPIC platform, our study uncovers a potential association between aberrant DNA methylation in MLLr iB-ALL and chromatin states related to enhancer and transcriptional control of healthy hematopoietic cells, suggesting that the orchestrated molecular homeostasis of transcriptional programs key for normal hematopoiesis are epigenetically disrupted in iB-ALL. MLL protein is a H3K4 HMT found in COMPASS-like complexes, which regulate the RNA–pol II–mediated transcriptional initiation of MLL target genes (57). It is therefore plausible that the observed methylome alterations at MLL target motifs are the consequence of altered MLL activity in iB-ALL cells, in agreement with a recent study reporting DNA hypomethylation at
enhancer elements in pediatric MLLr B-ALL (58). Moreover, a recent study by Prange and colleagues (59) proposed that MLL-AF4 + and MLL-AF9 + fusion proteins can bind to a distinct enhancer repertoire, thus supporting the divergent distribution of altered DNA methylation patterns in these leukemia subtypes.

As none of the known DNA methyltransferases (DNMTs) have a defined sequence specificity beyond CpG sites, the establishment of aberrant, yet defined DNA methylation patterns in cancer must be achieved by other mechanisms (60). Among them, the absence of DNA methylation at given loci mediated by the steric effects of histone modifications or DNA-bound TFs could impair proper DNMT recruitment at these regions and thus may play an important role in rewiring the methylation status of iB-ALL. In this context, a mutually exclusive relationship between H3K4 modifications and DNA methylation (61) may potentially explain the differential enrichment at H3K4me3 regions observed in our analyses. De novo methylation mediated by DNMT3L is best achieved in the context of nonmethylated H3K4 (62), but binding of the DNMT3L is impeded in the presence of H3K4me3 (63), suggesting that H3K4 methylation may prevent de novo DNA methylation at given genomic regions. As DNA methylation changes are suggested to be a late event that coincides with loss of TF binding (64), our DNA methylation results may reflect the epigenetic rewiring driven by the relocation of multiple TFs including, but not restricted to, MLLr. A dedicated epigenetic profiling of multiple histone marks from iB-ALL samples would be of extreme relevance to further elucidating the complex relationship among DNA methylation, histone modifications, and chromatin accessibility in iB-ALL.

The comprehensive transcriptional profiling of iB-ALL cohorts allowed the identification of deregulated transcriptional programs (9, 17). Of note, differential gene expression analysis revealed that MLL-AF4 + iB-ALL displayed a larger expression variability, suggesting the existence of distinct molecular subgroups within MLL-AF4 + iB-ALL patients (9). In this context, the reciprocal fusion AF4-MLL +, which is expressed only in half of the (4;11)+ iB-ALL patients, was shown to be required for expression of HOXA cluster genes and cooperates with MLL-AF4 to promote the emergence of hematopoietic precursors (9, 65). Importantly, the expression of AF4-MLL/HOXA is an independent prognostic factor and identifies (4;11)+ iB-ALL patients with improved overall and event-free survival (9).

Network-based coexpression analysis revealed the presence of predominant molecular hubs differentially altered in MLLr iB-ALL subtypes. We observed that FOS and JUN, members of the AP-1 family of TFs, displayed a pivotal role in the establishment of a specific transcriptional signature in MLLr iB-ALL, particularly in MLL-AF4 + patients. Indeed, components of the AP-1 complex have a well-established contribution in multiple types of human neoplasms (66), and a recent study demonstrated that FOS/JUN-related proteins are crucial players in the pathogenesis of acute myeloid leukemia (AML) (48). Further integration of DNA methylation and gene expression data, coupled with an extensive validation by multiple orthogonal approaches, confirmed that FOS/JUN proteins are not only aberrantly expressed in iB-ALL, but exert a key role in reshaping the DNA methylation landscape in iB-ALL, suggesting an intricate relationship between TF expression and the establishment of defined epigenetic programs, which are particularly imbalanced in the context of the disease. Moreover, our integrated strategy allowed us to define specific regulatory networks by linking the functional consequence between the methylation status/expression of TFs and their downstream target genes, leading to the discovery of robust cancer cell vulnerabilities in the context of iB-ALL.

Multiple epigenetic and transcriptomic events perturb the regulatory network required for proper B cell differentiation. The identification and functional in vitro and in vivo validation of such molecular hubs deregulated in MLLr iB-ALL, including the AP-1 axis, provide insights toward a better understanding of the pathogenesis and initiation of MLLr iB-ALL, opening up potential therapeutic avenues for MLLr iB-ALL through the combination of targeting approaches. Future studies should address whether the cancer cell vulnerabilities here described through a genome-wide integrative methylome-transcriptome analysis and the potential therapeutic opportunity for targeting AP-1 members in MLLr iB-ALL may be extended to adult MLLr B-ALL and MLLr AML.

**Methods**

**Patients and control samples.** BM samples from 69 infants (<12 months old) diagnosed with B-ALL were used in this study (percentages of leukemic blasts: 90% ± 12%, range: 67%-100%). Patients were cytogenerically subgrouped into 3 groups: t(4;11)/MLL-AF4 + (n = 37), t(9;11)/MLL-AF9 + (n = 12), and without MLLr (non-MLLr B-ALL without numerical or structural chromosomal abnormalities, n = 20). MLLr was confirmed by fluorescence in situ hybridization. All patients were enrolled in the Interfant International Treatment Study (ClinicalTrials.gov NCT00015873). BM samples were collected at Erasmus MC-Sophia Children’s Hospital (Rotterdam, the Netherlands), Armand Trousseau Hospital (Paris, France), and San Gerardo Pediatric Hospital (Monza, Italy). Supplemental Table 4 shows the clinic-biological features of the patients. As controls, CD34+CD19+ healthy BCPs were FACS purified from 6 human fetal livers (FLs) (18 to 22 weeks old) obtained from the MRC/Wellcome Trust Human Developmental Biology Resource Center. Samples were subjected to the Infinium HumanMethylationEPIC 850K beadchip platform (n = 75; n = 69 patients and n = 6 BCP controls), WGB-Seq (n = 8; n = 6 patients and n = 2 BCP controls), and RNA-Seq (n = 45; n = 40 patients and n = 5 BCP controls).

WGB-Seq, microarray-based DNA methylation analyses, and RNA-Seq experiments. Further details concerning next-generation sequencing protocols, computational pipelines, data preprocessing, and all subsequent downstream analyses are provided as Supplemental Data. This information has been also deposited at the Zenodo repository (https://doi.org/10.5281/zenodo.3695640).

Integration of DNA methylation and gene expression data. Enhancer linking by methylation-expression relationships was performed with the R/Bioconductor package ELMER (v.2.6.3; ref. 40). Methylation of DMPs from the HumanMethylationEPIC platform was correlated with expression of their 10 most proximal nearby genes to identify functional connections between the methylation status of a given region and their potential transcriptional targets. Paired DNA methylation loci-gene expression targets were identified using the supervised mode of the get.pair function (permutation size = 100000, Pe = 0.001) for hyper- or hypomethylated probes. Only classical inverse relationships between DNA methylation and gene expression were
ing/t(4;11)+CD34+ HSPCs were generated by CRISPR-mediated instructions. CD34+ cells were isolated from human FL samples using AF4C in SEM cells. ChIP-Seq tracks corresponding to MLL-AF4 and (CB) CD34+ cells using TRIzol (Sigma-Aldrich) according to the FOSLKO and t(4;11)/MLL-AF4+ CD34+ cells. The SEM cell line was purchased from the DSMZ Cell Line Bank and maintained according to the manufacturer’s instructions. CD34+ cells were isolated from human FL samples using the CD34 MicroBead Kit (Miltenyi Biotec), as previously described (9, 21, 68). The Flag-tagged dominant-negative FOS (dnFOS) construct was provided by Conny Bonifer (Institute of Biomedical Research, College of Medicine and Dentistry, University of Birmingham, Birmingham, United Kingdom; ref. 69). dnFOS-expressing lentiviruses were used to stably transduce SEM cells that were then FACS selected based on GFP expression (21). SEM-FOSL2KO and MLL-AF4-expressing/t(4;11)/CD34+ HSPCs were generated by CRISPR-mediated genome editing as described by our group (70) and another (47) using the Cas9 protein/tracrRNA/crRNA complex (IDT). The intrinsic crRNAs used for this study are listed in Supplemental Table 20.

RNA extraction, reverse transcription, real-time qPCR, and immunoblotting. RNA extraction and quantitative PCR (qPCR) were performed as previously described (71) with some modifications. Briefly, total RNA was extracted from human FL CD34+ cells or cord blood (CB) CD34+ cells using TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions, followed by a treatment with RNase-free DNase (Roche Applied Science). cDNA was synthesized from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Amounts of specific mRNAs in samples were quantified by quantitative reverse-transcription PCR (qRT-PCR) using QuantStudio 7 (Applied Biosystems) and SYBR Green Master Mix (Thermo Fisher Scientific). qRT-PCR was performed in 384-well microtest plates (Applied Biosystems) with 0.5 units of Taq Polymerase (Applied Biosystems) per well and 35 to 40 cycles. In all experiments, mRNA amounts were normalized to the total amount of cDNA by using amplification signals for GUSB endogenous control. Each sample was determined in triplicate, and at least 3 independent samples of each cell lysate were used. Primer sequences and PCR conditions are listed in Supplemental Table 20. For immunoblotting, whole-cell extracts were resolved on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes (Bio-Rad). Fra2 protein (FOSL2, 40 kDa) was detected with the Enhanced Chemiluminescence Detection System (GE Healthcare) using an anti-Fra2 antibody (1:1000 dilution; rabbit mAb, catalog 19967; Cell Signalling Technology).

Proliferation and clonogenic assays. SEM cells (WT, dnFOS, and FOSLKO) and t(4;11)/MLL-AF4+ CD34+ cells were plated at 1 x 10^4 cells/cm^2 in the presence of different concentrations of the API inhibitors T5224 (APEXBIO) or SR13302 (TOCRIS Bioscience), and cell proliferation was assessed every 3 to 4 days. These experiments were extended using a panel of MLLr (SEM, THP-1, MV4;11) and non-MLLr (REH, KG1a, Kasumi) cell lines in the presence of different concentrations of T5224. All cell lines were obtained from the DSMZ Cell Line Bank and genetically validated in our laboratory. To assess the clonogenic capacity of WT, dnFOS, and FOSLKO SEM, single cells were deposited into individual wells of 96-well plates using FACSAria Fusion Cell Sorter equipped with the Automated Cell Deposition Unit (ACDU) (BD Bioscience). Three weeks later, individual cells were scored for clonal cell expansion. The wells were inspected by light microscopy the day after cell seeding and those wells containing 2 or more cells were discarded from the analysis (65).

In vivo xenograft models for MLL-AF4+ B-ALL. Eight- to twelve-week-old NOD-Cg-Prkdcscid Il2gtml1Wjl/SzJ (where NOD indicates nonobese diabetic) (NSG) mice (Jackson Laboratory) were bred and housed under pathogen-free conditions in the animal facility of the Barcelona Biomedical Research Park. Mice (4 to 6/condition) were i.v. transplanted with 2 x 10^5 Luc-GFP-expressing WT, dnFOS, or FOSLKO SEM cells, and tumor burden was monitored weekly by BLI using the Xenogen IVIS 50 Imaging System (PerkinElmer; ref. 72). SEM engraftment in PB and splenomegaly were also determined at sacrifice.

To test the efficacy of the pharmacological inhibition of AP-1 in vivo, primary MLL-AF4+ iB-ALL cells were transplanted intratibially into sublethally (2.25 Gy) NSG mice, as described (49, 50). Leukemia engraftment was monitored through weekly PB analysis, and the leukemic graft was immunophenotyped by flow cytometry. When human engraftment was more than 0.5% in PB, mice were homogeneously distributed into different groups for treatment initiation. Treatment schedules were as follows: vincristine (V, 0.15 mg/kg) once weekly intraperitoneally for 2 weeks, dexamethasone (X, 5 mg/kg), and l-asparaginase (L, 1000 U/kg) daily intraperitoneally during 5 days for 2 weeks (50). The AP-1 inhibitor T5224 (100 mg/kg) was orally administered daily for 2 weeks.

Data availability. Raw HumanMethylationEPIC data including IDAT files from naive B cells, BCPs, MLL-AF4+, MLL-AF9+ and non-MLLr iB-ALLs have been deposited in ArrayExpress (E-MTAB-8505). IDAT files from SEM-WT, SEM-FOSL2KO, and t(4;11)/MLL-AF4+ CD34+ cells were deposited in ArrayExpress (E-MTAB-10090). Raw WGB-Seq data (Fastq files) corresponding to FL-BCP and iB-ALL cases have been deposited in the European Genome-Phenome Archive (EGA EGAD00001005010). Access to WGB-Seq data will be granted upon request. RNA-Seq data have been deposited at the European Nucleotide Archive (ENA PRJEB23605) as described (9). RNA-Seq data from SEM-WT and SEM-FOSL2KO have been deposited at ENA (PRJEB42796). Other datasets and source scripts required to reproduce the content of this manuscript have been deposited at Zenodo repository (10.5281/zenodo.3695640).

Statistics. Statistical analyses were performed using R programming language and publicly available software from the R/Bioconductor and CRAN repositories. Details on the statistical methods used are provided in Results and in the figure legends of the manuscript. Described details of additional computational analyses, including CpG island status and genomic region analyses (Supplemental Tables 6, 7, 11, 16, and 18), region set enrichment analyses (Supplemental Tables 8, 9, 10, 14, 15, and 19), integration of B cell enhancers (Supplemental Table 11), modular coexpression analyses, pathway enrichment analyses and network representation, are available as Supplemental Data.

Study approval. Human samples were collected upon informed consent and approval by the Barcelona Clinic Hospital research ethics
Author contributions

MFF, AFF, and PM conceived the study, designed experiments, analyzed and interpreted the data, wrote the manuscript, and financially supported the study. JRT designed experiments, analyzed and interpreted the data, and wrote the manuscript. CB conceived the study, designed experiments, analyzed and interpreted the data, and financially supported the study. MV, PP, AAD, IC, RTR, GFB, RFP, SLT, FGA, PSO, and IV analyzed and interpreted the data. MKO, MB, GC, PB, PS, and RWS provided clinical samples and biological data. JRT and CB have largely contributed to the manuscript in terms of computational and experimental analyses, respectively, so both deserve a shared first authorship. The final order of the authors was agreed on and approved by all the coauthors based on merit and time dedication to both data generation and data analysis and was not influenced by the race, gender, or religion of the individuals.

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