

1 **Identification of mutations that cooperate with defects in B cell transcription**
2 **factors to initiate leukemia**

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4 Running title: **Cooperating mutations that initiate B cell leukemia**

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24 **Abstract**

25

26 The transcription factors EBF1 and PAX5 are frequently mutated in B cell acute lymphoblastic
27 leukemia (B-ALL). We demonstrate that *Pax5*^{+/-} x *Ebf1*^{+/-} compound heterozygous mice develop
28 highly penetrant leukemia. Similar results were seen in *Pax5*^{+/-} x *Ikzf1*^{+/-} and *Ebf1*^{+/-} x *Ikzf1*^{+/-}
29 mice for B-ALL, or in *Tcf7*^{+/-} x *Ikzf1*^{+/-} mice for T cell leukemia. To identify genetic defects that
30 cooperate with *Pax5* and *Ebf1* compound heterozygosity to initiate leukemia, we performed a
31 Sleeping Beauty (SB) transposon screen that identified cooperating partners including gain-of-
32 function mutations in *Stat5* (~65%) and *Jak1* (~68%), or loss-of-function mutations in *Cblb* (61%)
33 and *Myb* (32%). These findings underscore the role of JAK/STAT5 signaling in B cell
34 transformation and demonstrate unexpected roles for loss-of-function mutations in *Cblb* and
35 *Myb* in leukemic transformation. RNA-Seq studies demonstrated upregulation of a
36 PDK1>SGK3>MYC pathway; treatment of *Pax5*^{+/-} x *Ebf1*^{+/-} leukemia cells with PDK1 inhibitors
37 blocked proliferation in vitro. Finally, we identified conserved transcriptional variation in a
38 subset of genes between human leukemias and our mouse B-ALL models. Thus, compound
39 haploinsufficiency for B cell transcription factors likely plays a critical role in transformation of
40 human B cells and suggest that PDK1 inhibitors may be effective for treating patients with such
41 defects.

42

43

44 **Introduction**

45 Heterozygous deletions or loss of function mutations in a number of B cell transcription
46 factors are a common feature of human B cell acute lymphoblastic leukemia (ALL)[1].
47 This is clearly evident for three transcription factors - EBF1, PAX5 and IKZF1[1, 2].
48 Interestingly, alterations involving these transcription factors commonly occur
49 together[1, 3]. This is particularly pronounced in BCR-ABL⁺ leukemia in which 50% of
50 leukemias with *IKZF1* deletions also have mutations affecting *Pax5* expression or
51 function[4]. Therefore, an important question is whether compound haploinsufficiency
52 for these transcription factors drives transformation and which combinations of
53 transcription factors can promote transformation. Finally, the genetic alterations that
54 cooperate with haploinsufficiency for these transcription factors to drive transformation
55 have also not been comprehensively elucidated.

56

57 To address the questions above, we generated a set of mice that exhibited compound
58 haploinsufficiency for various combinations of *Ebf1*, *Pax5*, *Ikzf1*, *Cebpa*, and *Tcf7*.
59 Herein, we demonstrate that *Pax5*^{+/-} x *Ebf1*^{+/-}, *Pax5*^{+/-} x *Ikzf1*^{+/-}, and *Ebf1*^{+/-} x *Ikzf1*^{+/-} mice
60 all generated B cell leukemia, while *Tcf7*^{+/-} x *Ikzf1*^{+/-} mice generated T cell leukemia.
61 Furthermore, we used a SB Transposon screen to identify mutations that cooperate with
62 *Pax5*^{+/-} x *Ebf1*^{+/-} compound haploinsufficiency to promote transformation. Our findings
63 document the key role that compound haploinsufficiency for critical transcription factors
64 plays in leukemia transformation and identify mutations that cooperate with such
65 alterations to initiate transformation.

66

67 **MATERIALS AND METHODS**

68 **Mice and Cells**

69 All mice have been previously described [5-10]; the University of Minnesota IACUC
70 approved all animal experiments. Mice were monitored for up to 400 days for leukemia.
71 Spleen, lymph nodes, and bone marrow were isolated from tumor-bearing mice and
72 used for further experiments.

73

74 **RNA-Seq Analysis**

75 RNA-seq was performed on total RNA extracted from either column purified progenitor
76 B control cells (C57Bl/6, *Pax5*^{+/-}*x Ebf1*^{+/-}, *Pax5*^{+/-} or *Ebf1*^{+/-}) or leukemic cells from lymph
77 nodes of tumor-bearing mice using a RNeasy Plus kit (Qiagen). Fifty-three barcoded
78 TruSeq RNA v2 libraries were created and sequenced on a HiSeq 2500. A second set
79 of data were used for variant calling analysis. Eight barcoded libraries were sequenced
80 on the HiSeq 2000 to produce 100 bp paired end reads.

81 **Systematic identification of gene clusters**

82 Phase_2 BCCA and St Jude (2016) and phase_3 St Jude ALL(2018) Human mRNA
83 RNA-Seq data were downloaded from [https://ocg.cancer.gov/programs/target/data-](https://ocg.cancer.gov/programs/target/data-matrix)
84 matrix on April 23 2019 (dbGaP accession phs000218.v22.p8). The Value of 0.1 was
85 added to each value the data was mean centered and log transformed. A SD cutoff was
86 used to identify ~8500 genes in each of three datasets. Unsupervised hierarchical
87 clustering was used to define sets of genes which were defined by average linkage
88 correlation > 0.2 and more than 150 members. Statistical enriched Gene cluster

89 memberships across clusters were defined by Fisher exact test to identify “common”
90 clusters across datasets. For the mouse data, the tumors were treated in a similar
91 fashion except an SD cutoff of 1.0 was used. Statistical enriched gene cluster
92 memberships across clusters were defined by Fisher exact test to identify “common”
93 clusters across datasets using gene name matching.

94

95 **Data Analysis**

96 Data was analyzed using Prism 8 (Graphpad). A Shapiro-Wilk test was used to
97 determine normality of all data. Unpaired data that passed normality was analyzed by
98 an ordinary one-way ANOVA with Holm-Sidak’s multiple comparison test or by an
99 unpaired t-test; data that failed normality were analyzed using an unpaired Kruskal-
100 Wallace test with Dunn’s multiple comparison test. Kaplan-Meier Survival curves were
101 analyzed by Log-rank (Mantel-Cox) Test. Integrated Genomics Viewer was used to
102 view aligned sequences (Broad Institute).

103

104 **Accession Numbers**

105 RNA-Seq data was deposited with GEO and is accessible through GEO Series
106 accession number GSE148680
107 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148680>).

108

109 **Supplemental methods**

110 Supplemental Methods section includes detailed protocols of cell lines and culture
111 conditions, NGS, flow cytometry, qPCR, western blotting, Gene Set Enrichment

112 Analysis, SB Mutagenesis, Transposon Insertion Analysis, Reverse Phase Proteomics
113 and Inhibitor Assay.

114 **RESULTS**

115

116 **Reduced expression of transcription factors critical for lymphocyte development**
117 **leads to leukemia**

118 To explore whether compound haploinsufficiency for *Ebf1* and *Pax5* leads to B cell
119 transformation, we generated *Pax5*^{+/-} x *Ebf1*^{+/-} mice. As shown in Figure 1A, ~67% of
120 *Pax5*^{+/-} x *Ebf1*^{+/-} mice develop leukemia with a mean survival of 296 days. Flow
121 cytometry analysis from bone marrow, lymph nodes and spleens of these mice
122 demonstrated that leukemias resemble progenitor-B cells with a B220⁺CD19⁺IgM⁻
123 phenotype (Fig. 1B) and also express pre-BCR, CD43, IL7R, TSLPR, c-KIT, AA4.1 and
124 CD25 confirming their progenitor-B cell like phenotype (Sup Fig. 1). Although both male
125 and female mice developed leukemia in this model, female mice exhibited greater
126 penetrance (97% versus 71% at 400 days) and reduced median survival (265 days vs
127 298 days, p= 0.005; Sup Fig. 2). The effect of reduced expression of *Pax5* and *Ebf1* on
128 transformation was not limited to this combination of transcription factors as similar
129 results were observed in *Pax5*^{+/-} x *Ikzf1*^{+/-} and *Ebf1*^{+/-} x *Ikzf1*^{+/-} mice (Fig. 1A).
130 Compound haploinsufficiency for all three transcription factors (*Pax5*^{+/-} x *Ebf1*^{+/-} x *Ikzf1*^{+/-}
131 mice) resulted in 100% penetrance of leukemia and much shorter mean survival (202
132 days). We previously reported that *Pax5*^{+/-} or *Ebf1*^{+/-} mice do not develop leukemia[11].
133 In contrast, *Ikzf1*^{+/-} mice do develop leukemia with low penetrance (Fig. 1A)[12, 13];
134 however, these were always T cell leukemias (Fig. 1C). Deleting one copy of *Pax5* and
135 *Ebf1* not only increased the frequency of B cell leukemias in *Ikzf1*^{+/-} mice (none to
136 ~40%), but surprisingly, also resulted in a dramatic increase in T cell leukemias (~5% in

137 *Ikzf1*^{+/-} mice versus ~35% in *Pax5*^{+/-} *x* *Ebf1*^{+/-} *x* *Ikzf1*^{+/-} mice)(Fig. 1C,D). Thus, although
138 PAX5 and EBF1 are only expressed in B cells, reduced expression of these two
139 transcription factors paradoxically also promoted T cell leukemia.

140

141 We next examined whether compound haploinsufficiency for lineage determining
142 transcription factors was a general mechanism that could promote transformation of
143 multiple cell lineages. To this end, we generated *Tcf7*^{+/-} *x* *Ikzf1*^{+/-} mice, as *Tcf7*, which
144 encodes TCF1, and *Ikzf1*, are both required for T cell development[8, 9]. In addition, we
145 generated *Cebpa*^{+/-} *x* *Ikzf1*^{+/-} mice, as *Cebpa* and *Ikzf1* are both involved in myeloid cell
146 development[14]. *Cebpa*^{+/-} *x* *Ikzf1*^{+/-} mice did not develop myeloid leukemia and the
147 rate of T cell leukemia in *Cebpa*^{+/-} *x* *Ikzf1*^{+/-} mice was no higher than that observed for
148 *Ikzf1*^{+/-} mice. Thus, not all combinations of transcription factor haploinsufficiency
149 promote transformation. However, *Tcf7*^{+/-} *x* *Ikzf1*^{+/-} mice developed T cell leukemias
150 with high penetrance, comparable to that seen for B cell leukemias in *Pax5*^{+/-} *x* *Ebf1*^{+/-}
151 mice (Fig. 1A,C). Thus, compound haploinsufficiency for lineage defining transcription
152 factors can promote transformation in multiple cell lineages and may underlie many
153 types of leukemias.

154

155 **Genetic mutations that cooperate with Pax5 and Ebf1 heterozygosity to induce** 156 **leukemia**

157 Previous reports by Prasad and colleagues suggested that *Ebf1*^{+/-} mice have defects in
158 DNA repair with decreased expression of *Rad51* and increased γ H2AX foci[15]. These
159 studies further claimed that defects in DNA repair resulted in increased mutation rates in

160 *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias and that this accounts, in part, for progenitor B cell
161 transformation in those mice[15]. This suggestion is difficult to reconcile with the
162 relatively low frequency of somatic mutations reported in human in B-ALL[16]. We re-
163 examined this issue using *Ebf1*^{+/-} mice in our colony. In contrast to the previous study,
164 we found no difference in *Rad51*, *Rad51AP* or γ H2AX expression when examining log2
165 transformed FPKM values generated from two separate RNA-seq experiments (Sup.
166 Fig. 3A). In fact, the low level of variation paralleled that observed for a panel of
167 housekeeping genes (*B2m*, *Hprt*, and *Actb*; Sup. Fig. 3A). Since the previous studies
168 compared progenitor-B cells from WT and *Ebf1*^{+/-} mice that had been cultured
169 extensively in vitro we examined γ H2AX expression in long-term cultured progenitor-B
170 cells from WT and *Ebf1*^{+/-} mice; no significant difference was observed (Sup. Fig. 3B).
171 Further, we examined γ H2AX expression by flow cytometry in progenitor-B cells directly
172 from the bone marrow of WT and *Ebf1*^{+/-} mice. Again, we found no significant difference
173 in expression (Sup. Fig. 3C). Next, we examined whether genes involved in DNA
174 repair were enriched in *Ebf1*^{+/-} cells by Gene Set Enrichment Analysis (GSEA) using our
175 RNA-seq data. We saw a significant enrichment for DNA repair genes (Sup. Fig. 3D),
176 although it is unclear whether this reflects a direct effect of EBF1 on genes involved in
177 DNA repair or just a relative increase in cells stuck at a stage undergoing VDJ
178 recombination, as there is significant overlap between genes involved in DNA damage
179 response and VDJ recombination. Finally, we examined whether subsets of human B
180 cell leukemias exhibited increased mutation rates and if so, whether they were enriched
181 in those containing *Ebf1* mutations. As shown in figure 2A, ~16% of B-ALLs obtained
182 from the NIH TARGET ALL database showed significant levels of missense mutations.

183 We broke the total B-ALL samples down into smaller subsets, characterized by *ETV6-*
184 *RUNX1* or *TCF3-PBX1* translocations, or those with missense mutations in *CDKN2A*,
185 *PAX5*, *EBF1* or *IKZF1*. Leukemias expressing the *ETV6-RUNX1* or *TCF3-PBX1*
186 translocations, or *PAX5* missense mutations, were not enriched in hypermutated
187 leukemias (Fig. 2B,C). In contrast, leukemias with missense mutations in *CDKN2A*,
188 *EBF1* or *IKZF1* showed an increased percentage of leukemias with high numbers of
189 missense mutations (Fig.2B). The number of *CDKN2A*, *EBF1*, or *IKZF1* samples was
190 too small to assess whether the increased percentage of hypermutated leukemias was
191 statistically significant. However, mutations in *EBF1*, *IKZF1* and *CDKN2A* are all
192 enriched in *BCR-ABL*-like leukemias and when we pooled samples with these three
193 mutations together there was a clear enrichment in samples with high numbers of
194 missense mutations (Fig. 2C). In conclusion, leukemias with *EBF1* mutations may be
195 preferentially found in hypermutated B-ALL, but this is not a feature restricted to *EBF1*
196 as mutations in *CDKN2A* and *IKZF1* are also associated with this hypermutated
197 phenotype.

198
199 To discover novel genes that cooperate with *Pax5* and *Ebf1* heterozygosity to induce B-
200 ALL we employed a Sleeping Beauty (SB) transposon mutagenesis screen[17]. *Pax5*^{+/-}
201 *x Ebf1*^{+/-} *x Cd79a-cre* mice were crossed to mice expressing the mutagenic transposon
202 SB in a *Cd79a-Cre*-dependent, and hence B cell-specific, manner. We generated 34
203 mice that were heterozygous for both *Ebf1* and *Pax5* and expressed the mutagenic
204 transposon. Mice were housed for up to 400 days to allow them to develop leukemia.
205 We also included single heterozygous combinations (*Pax5*^{+/-} *x Cd79a-Cre* *x SB* and

206 *Ebf1*^{+/-} x *Cd79a-Cre* x *SB*) but neither of these cohorts developed leukemia within 400
207 days (data not shown). As seen in figure 3A, all of *Pax5*^{+/-} x *Ebf1*^{+/-} x *Cd79a-Cre* x *SB*
208 mice developed leukemia. Thus, the presence of the sleeping beauty transposon
209 increased penetrance of leukemia from 67% to 100% and decreased the median age of
210 death from 296 to 205 days. Thus, other genes mobilized or silenced by SB
211 transposition clearly cooperate with *Pax5* and *Ebf1* heterozygosity to initiate
212 transformation.

213

214 To identify which genes were targeted by the transposon, we performed RNA-seq
215 analysis on 31 SB induced leukemias. The SB transposon contains a unique 5' leader
216 sequence with a splice donor site that allows for splicing into transcripts. In addition, the
217 SB transposon also has a splice acceptor and SV40 polyA tail that allows for splicing of
218 upstream exons to the SV40 poly A sequence, thereby allowing for premature
219 termination. These unique 5' SB sequences and 3' SV40 polyA signal sequences can
220 be identified by RNA-Seq as novel fusion proteins. This allowed us to map SB fusions
221 and determine how transposon insertions altered specific gene expression[18]. We
222 carried out RNA-Seq analysis on progenitor B cells (CD19⁺B220⁺Igκ/λ⁻) from WT,
223 *Ebf1*^{+/-}, *Pax5*^{+/-}, and *Pax5*^{+/-} x *Ebf1*^{+/-} pre-leukemic mice (~6-12 weeks of age), as well
224 as spontaneous *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias (Fig. 3B). Differential gene expression
225 analysis showed that WT and pre-leukemic samples all clustered distinctly from the SB-
226 induced and spontaneous leukemias. The spontaneous *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias
227 were interspersed among the SB-induced leukemias demonstrating that the SB-induced
228 leukemias shared gene expression signatures with the spontaneous leukemias. Finally,

229 the leukemias were clearly heterogenous with a number of distinct subsets harboring
230 distinct gene signatures (Fig. 3C).

231

232 **RNA fusion analysis defines genes that cooperate with *Pax5* x *Ebf1***

233 **heterozygosity to induce leukemia.**

234 To identify the targets of transposon mutagenesis, we performed an RNA-Seq-based
235 analysis of transposon fusions to identify genes targeted in our screen. The fusion
236 transcripts are detected either directly as unique gene fusions or can be imputed from
237 paired end reads that have one end derived from SB and the second end derived from
238 the target gene sequence (called bridging fusions)[18]. There were 758 unique gene
239 fusions or bridging fusions that were used to identify recurrent fusion events in 27 of 31
240 leukemias. Figure 3D lists all the reoccurring RNA fusions identified in our screen.
241 Consistent with the heterogeneity of the gene expression profiles in distinct B-ALL
242 subsets (Fig 3C), many of the targeted genes were only found in a fraction of the
243 leukemias (Fig. 3E). The most notable exception was that almost all leukemias had SB
244 insertions involving either *Jak1* or *Stat5b* (Fig. 3E). SB RNA fusion analysis
245 demonstrated that the SB 5' leader UTR sequence typically fused to the first 1-4 coding
246 exons of *Stat5b* or *Jak1* genes (Fig. 4A, Sup. 4A). This suggested that a full-length or
247 nearly full-length coding transcript would be generated for both *Jak1* and *Stat5b*. *Stat5b*
248 mRNA was expressed at significantly higher levels in leukemic samples harboring a SB
249 transposon insertion (Fig. 4B,C). Consistent with data for *Stat5b* mRNA, there was a
250 clear increase in the expression of STAT5 protein in samples with an SB insertion in the
251 *Stat5b* gene (Fig. 4D-E). In contrast, the spontaneous *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias did

252 not exhibit significant increases in *Stat5b* expression (Fig. 4B,C). However, when we
253 examined levels of phosphorylated STAT5 we found that *Pax5*^{+/-} x *Ebf1*^{+/-} leukemic cells
254 expressed significantly higher levels of activated STAT5 than WT control mice, either
255 directly ex vivo (Fig. 4F), or following in vitro stimulation with IL7 (Fig. 4G). This change
256 represents a significant increase in phospho-STAT5 (p-STAT5) as it was equal to or
257 higher than seen in mice expressing a constitutively active form of STAT5b in progenitor
258 B cells (Fig. 4F,G)[19]. This result illustrated that there were increased levels of pStat5
259 expression in our leukemic cells. We next looked at known targets of STAT5b - *Cish*
260 and *Socs2* - to determine if there is increased *Stat5b* activity in these leukemias. We
261 saw increased expression of both *Cish* and *Socs2* in leukemic cells from both *Pax5*^{+/-} x
262 *Ebf1*^{+/-} and *Pax5*^{+/-} x *Ebf1*^{+/-} x *Cd79a-Cre* x *SB* leukemias, which suggests that pStat5 is
263 active in the leukemic cells (Fig. 4H, I). Similar expression results were seen for *Jak1*.
264 We detected a significant increase in *Jak1* mRNA in mice harboring insertions in the
265 *Jak1* locus (Sup. Fig. 4B,C). This increase in *Jak1* transcription significantly increased
266 expression of JAK1 protein in leukemic samples with an SB transposon insertion in the
267 *Jak1* gene locus (Sup. Fig. 4D,E). Our findings are consistent with the high rate of
268 STAT5 activation observed in both human and murine B-ALL[11, 20] and underscore
269 the critical role of JAK/STAT5 signaling in B cell leukemia – particularly those with
270 reduced expression of *Pax5* and *Ebf1*.

271

272 **Loss of *Cblb* cooperates with reduced expression of *Pax5* and *Ebf1* to more**
273 **rapidly induce leukemia**

274 The other top hit in our mutagenesis screen was *Cblb*, which was targeted in almost 2/3
275 of our leukemias. Transposon insertional analysis from RNA-seq suggested that *Cblb*
276 expression would be reduced as the majority of the SB gene fusions detected involved
277 splicing in exons 6-9 (Fig. 5A). Consistent with this idea, both spontaneous *Pax5*^{+/-} x
278 *Ebf1*^{+/-} leukemias, and SB-induced *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias with an SB insertion in
279 *Cblb*, showed significantly reduced *Cblb* mRNA expression (4.6-fold) compared to WT
280 controls (Fig. 5B). Similar results were seen for CBLB protein expression as SB-induced
281 leukemias with an SB insertion in the *Cblb* gene exhibited significantly lower expression
282 of CBLB protein (1.8-fold) than SB-induced leukemias without an insert (Fig. 5C,D). To
283 determine the role of reduced *Cblb* expression in leukemic transformation, we crossed
284 our *Pax5*^{+/-} x *Ebf1*^{+/-} mice to *Cblb*^{-/-} mice. *Pax5*^{+/-} x *Ebf1*^{+/-} x *Cblb*^{-/-} mice developed B-
285 ALL and died significantly faster than *Pax5*^{+/-} x *Ebf1*^{+/-} mice, demonstrating that *Cblb*
286 acts as a tumor suppressor in progenitor B cells (Fig. 5E).

287

288 **Reduced levels of *Myb* cooperate with *Pax5* and *Ebf1* heterozygosity to more**
289 **rapidly induce leukemia**

290 *Myb* was another frequent target of our mutagenesis screen. SB transposon insertions
291 were scattered throughout the *Myb* gene locus, suggesting that this would result in
292 decreased expression of *Myb* (Fig. 6A). Spontaneous *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias
293 showed a clear trend towards reduced *Myb* expression. Consistent with this
294 observation, in SB-induced *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias we saw a decrease in *Myb*
295 expression in leukemias that lacked an SB insertion in *Myb* (1.5-fold, Fig. 6B) and an
296 additional significant decrease in leukemias with an SB insertion in the *Myb* (2.3-fold,

297 Fig. 6C). Thus, downregulation of *Myb* appears to be a general feature of *Pax5^{+/-}x*
298 *Ebf1^{+/-}* leukemias. In SB-induced *Pax5^{+/-} x Ebf1^{+/-}* leukemias with insertions in the *Myb*
299 gene, there was also a significant reduction (2.8 fold, Fig. 6D, E) at the protein level.
300 Importantly, we found that *Myb* expression as assessed by RNA-Seq correlated with
301 age of death - leukemias with less *Myb* were more aggressive resulting in earlier
302 lethality (Fig. 6F).

303

304 **PDK1-signaling pathway is deregulated in *Pax5^{+/-} x Ebf1^{+/-}* leukemias**

305 In addition, to gene alterations directly regulated by SB transposition, we also noted a
306 number of genes whose expression was significantly altered in *Pax5^{+/-} x Ebf1^{+/-}*
307 leukemias relative to non-transformed controls. These included genes such as the
308 tumor suppressor *Bach2*, which was significantly reduced (Sup. Fig. 5A). Intriguingly,
309 we also noted dramatic (5.7 fold) downregulation of Asparagine synthetase in these
310 leukemias, which may explain their susceptibility to L-Asparaginase[21](Sup. Fig. 5B).
311 Other genes were strikingly upregulated including *Pdk1* (3.0-fold) and its downstream
312 targets *Sgk3*, and *Rheb1* (Fig. 7A,B,C). Conversely *Tsc2*, which inhibits this pathway,
313 was downregulated (Sup. Fig. 5C,D); this pathway has been previously shown to
314 enhance mTORC1 function and ultimately MYC expression[22, 23]. To determine
315 whether PDK1 plays a critical role in maintaining viability of *Pax5^{+/-} x Ebf1^{+/-}* leukemias,
316 we treated two cell lines generated from *Pax5^{+/-} x Ebf1^{+/-}* leukemias in vitro with either
317 vehicle control or the PDK1 inhibitor (GSK2334750). Treatment of these cell lines with
318 the PDK1 inhibitor resulted in a dose-dependent decrease in the survival of these cell

319 lines suggesting that this might be a useful treatment for B-ALL subsets with reduced
320 *Pax5* and *Ebf1* expression (Fig. 7D).

321

322 To examine a possible role for PDK1 expression in human ALL, we examined ALL
323 patient samples using a reverse phase proteomics approach[24]. PDK1 was expressed
324 in five subsets of B-ALL but expression varied widely (Sup. Fig. 5E). We examined
325 PDK1 expression in the two largest cohorts B-NOS and BCR-ABL+ leukemias. PDK1
326 levels did not correlate with overall survival in B-NOS patients (data not shown). In
327 contrast, BCR-ABL+ patients with the highest levels of PDK1 expression did
328 significantly better than those with lower PDK1 expression (Fig. 7E). The difference in
329 overall survival appears to be driven most strongly by young adults, as they showed the
330 most dramatic difference in overall survival (Fig. 7F). Finally, we examined PDK1
331 expression in patients with BCR-ABL and B-NOS leukemia based on relapse status. In
332 both subsets of leukemia, lower levels of PDK1 correlated with relapse (Fig. 7G,H).
333 Thus, PDK1 appears to play an important role in B-ALL survival or proliferation, but
334 patients with the highest level of PDK1 expression respond better to therapy.

335

336 **Pax5 x Ebf1 leukemia show common transcriptional variation patterns across**
337 **mouse and human**

338 To determine if the murine leukemias that developed in our sleeping beauty screen are
339 similar to subsets of human B-ALL, we quantified inter-leukemia transcriptional variation
340 using our newly developed gene cluster expression summary score (GCESS)[25].

341 Using this approach, we first examined inter-leukemia transcriptional variation in distinct

342 human leukemia datasets. There was notable heterogeneity between human B-ALL
343 subsets. However, we could identify clusters with similar variations in gene expression
344 (Fig. 8A). We used the GCESS approach to establish transcriptional similarity between
345 these human leukemias and our murine sleeping beauty transposon induced leukemias.
346 As shown in figure 8B, there were two distinct transcriptional variants in our SB dataset.
347 One of these SB leukemia subsets showed a similar gene expression signature to one
348 of the conserved human leukemia subsets (fishers exact test, $p=4.3e-07$). Thus, the
349 leukemias that developed in our SB system are similar to human leukemias, thereby
350 validating our approach as a useful model of human leukemia.

351
352 To better characterize these leukemias, we utilized ENRICH to examine gene lists
353 from the GCESS of each of the datasets from the conserved murine and human
354 leukemias (Sup. Table 1)[26, 27]. Consistent with other findings in this study, we found
355 upregulation of cytokines and cytokine receptor genes, as well as genes involved in
356 JAK/STAT5 signaling. In addition, NF κ B signaling was significantly altered, which is
357 consistent with work from multiple groups on NF κ B in B cell development and leukemia
358 [20, 28, 29]. A surprising observation was a strong myeloid gene signature in the human
359 and murine leukemias. There are two potential reasons for this. First, these leukemias
360 could be infiltrated with myeloid cells. Alternatively, the leukemias could have lost
361 lineage fidelity and begun to express myeloid genes. Since PAX5, EBF1 and IKZF1 all
362 play key roles in enforcing B cell lineage fidelity, and our murine B cell leukemias were
363 relatively pure populations of leukemic blasts, we favor this later possibility. Thus,
364 subsets of *Pax5*^{+/-} x *Ebf1*^{+/-} leukemia exhibit some myeloid characteristics.

365

366 **Discussion**

367 Genes encoding the transcription factors *PAX5*, *EBF1* and *IKZF1* frequently exhibit loss
368 of one allele or express loss-of-function mutations in human B cell leukemia[2, 4, 30].

369 However, their role in B cell transformation is not entirely clear. It is likely that loss of
370 function mutations in these transcription factors affect B cell differentiation. Previous

371 studies using inducible *Pax5* mutants in murine models of B cell leukemia suggest that
372 this plays a role[31]. However, *Pax5*^{+/-} and *Ebf1*^{+/-} mice do not develop leukemia, while

373 *Ikzf1*^{+/-} mice have only been shown to develop T cell leukemia. This raises the question
374 of how mutations in these genes promote transformation. We previously demonstrated

375 that combining loss of one allele of either *Pax5* or *Ebf1* with a constitutively active *Stat5*
376 transgene (referred to as *Stat5b-CA*) led to rapid onset of B cell leukemia. A key

377 feature of these leukemias is that *Ebf1* expression was reduced ~50% in *Stat5b-CA* x
378 *Pax5*^{+/-} leukemias, while *Pax5* expression was comparably reduced in *Stat5b-CA* x

379 *Ebf1*^{+/-} leukemias[11]. These findings suggested that perhaps compound

380 haploinsufficiency for these transcription factors might be key for promoting

381 transformation. Herein we demonstrated that this is the case as *Pax5*^{+/-} x *Ebf1*^{+/-} mice

382 develop B cell leukemia with high penetrance. Importantly, this was not a phenomenon

383 restricted to this pair of transcription factors as we saw qualitatively similar onset of B

384 cell leukemia in *Pax5*^{+/-} x *Ikzf1*^{+/-} and *Ebf1*^{+/-} x *Ikzf1*^{+/-} mice. Nor was this observation

385 restricted to B cell leukemia as we observed that *Tcf7*^{+/-} x *Ikzf1*^{+/-} mice gave rise to

386 highly penetrant T cell leukemia. Thus, compound haploinsufficiency for transcription

387 factors that play key roles in either B cell or T cell development can promote
388 transformation.

389

390 The mechanism by which compound haploinsufficiency promotes transformation
391 remains unclear. Previous studies suggested that this may be due to defective DNA
392 repair upon reduced *Ebf1* expression. We were unable to validate defects in *Rad51*,
393 *Rad51ap* expression or increased γ H2AX expression in pre-B cells from *Ebf1*^{+/-} mice.
394 However, we did observe that a subset of human leukemias expressed a hypermutated
395 phenotype and that leukemias with *CDKN2A*, *EBF1* and *IKZF1* missense mutations
396 were enriched in this subset. It is possible that the newly described BCR-ABL-like
397 subset of B-ALL (which is enriched in leukemias with mutations in *CDKN2A*, *IKZF1* and
398 *EBF1*) might be characterized by the hypermutated phenotype. This should be
399 examined further and if confirmed suggests that these leukemias may be more
400 susceptible to immunotherapy-based treatments.

401

402 An alternative mechanism by which compound haploinsufficiency promotes
403 transformation could involve loss of lineage fidelity. Consistent with this hypothesis,
404 *EBF1* and *PAX5* have both been shown to play important roles in restricting cells to the
405 B cell lineage. Consistent with this observation, B cell progenitors in *Pax5*^{+/-} *x* *Ebf1*^{+/-}
406 mice retain T cell lineage potential[32]. Moreover, we found that compound
407 haploinsufficiency for *Pax5* and *Ebf1* promotes increased penetrance of B cell and T cell
408 leukemia on an *Ikzf1*^{+/-} background. Thus, decreased expression of transcription factors
409 that are only expressed in B cells (*PAX5* and *EBF1*) paradoxically enhance the

410 development of T cell leukemia in *Ikzf1*^{+/-} mice. Although the mechanism remains
411 unclear, it is possible that in some cases T-ALL may emerge from a B cell progenitor.
412 This may have implications for how such leukemias develop resistance to therapy if the
413 key progenitor is more closely linked to B cell rather than T cell development. Finally,
414 our finding that a subset of murine *Pax5*^{+/-} x *Ebf1*^{+/-} leukemias, as well as their similar
415 human B-ALL counterparts, exhibit a strong myeloid gene signature also suggests that
416 loss of lineage fidelity may be a key feature of this disease. Alternatively, the myeloid
417 signature could arise due to preferential infiltration of this type of leukemia with myeloid
418 cells. This is certainly a possibility, especially for leukemias in the human datasets.
419 However, our murine leukemias are strongly enriched for B lineage cells. Thus, we
420 favor an explanation in which the myeloid gene signature arises due to aberrant
421 expression of myeloid genes in B cell leukemic blasts.

422

423 To gain a better understanding of the molecular alterations that cooperate with *Pax5*
424 and *Ebf1* haploinsufficiency to promote transformation we carried out an unbiased SB
425 transposon screen in *Pax5*^{+/-} x *Ebf1*^{+/-} mice. These studies identified two major
426 pathways that cooperate with *Pax5* and *Ebf1* haploinsufficiency to drive transformation.
427 First, we found gain-of-function mutations for *Jak1* or *Stat5b* in almost all of our
428 leukemias in this screen. This finding underscores in an unbiased way the critical role
429 of JAK/STAT5 signaling in B cell transformation. In a previous SB mutagenesis study
430 targeting the STAT5 pathway, we were able to induce more rapid leukemia onset than
431 SB mice with only *Pax5*^{+/-} x *Ebf1*^{+/-} predisposing mutations (average onset of leukemia
432 ~72 versus 302 days, respectively) [33]. This suggests that changes needed to activate

433 STAT5 may take longer to arise than secondary loss-of-function mutations to *Pax5*,
434 *Ebf1*, or other transcription factors.

435

436 The second major pathway identified involves CBL-B, and to a much lesser extent the
437 related family member CBL. The mechanism by which *Cblb* loss-of-function affects
438 transformation is unclear. However, the fact that these are loss-of-function mutations is
439 supported by the observation that crossing *Cblb*-deficiency onto the *Pax5*^{+/-} x *Ebf1*^{+/-}
440 background accelerated the onset of leukemia and increased overall penetrance. Our
441 SB screen suggest that *CBLB* mutations should be examined in greater detail in human
442 B-ALL.

443

444 A number of other target genes were identified in our SB screen, although none were as
445 prevalent as the mutations in *Jak1*, *Stat5b* or *Cblb*. These include SB insertions in
446 several cytokine/receptor genes that have previously been shown to be involved in
447 transformation including *Il2rb*, *Gh*, *Csf2*, as well as the histone acetyltransferase *Ep300*.
448 Finally, we noted relatively frequent mutations in the transcription factor *Myb*. The
449 finding that *Myb* was targeted by SB in our leukemias was initially not surprising as *Myb*
450 has previously been identified as an oncogene. However, what was surprising is that
451 the mutations in *Myb* were loss-of-function mutations resulting in reduced *Myb*
452 expression. This leads to the somewhat surprising observation that *Myb* acts
453 operationally as a tumor suppressor in this context and parallels our previous
454 observation that NFκB also acts as a functional tumor suppressor in progenitor B

455 cells[20, 34]. Since MYB plays a role in B cell differentiation[35] this likely reflects a role
456 for MYB in blocking differentiation at the highly proliferative pre-B cell stage.

457

458 In addition to targets directly identified by SB integration, our RNA-Seq analysis of
459 *Pax5^{+/-} x Ebf1^{+/-}* and *SB x Pax5^{+/-} x Ebf1^{+/-}* leukemias also identified other deregulated
460 signaling pathways. One of the most prominently deregulated pathways involved the
461 serine/threonine kinase PDK1. PDK1 expression was modestly but significantly
462 upregulated in *Ebf1^{+/-}* and *Pax5^{+/-} x Ebf1^{+/-}* pre-leukemic progenitor B cells, and
463 significantly further elevated in *Pax5^{+/-} x Ebf1^{+/-}* leukemias. The mechanism by which
464 increased PDK1 expression promotes transformation is unclear. However, previous
465 studies have shown that PDK1 interacts with SGK1/3 to inhibit TSC2 function and
466 expression[22]. This results in increased function of RHEB or RHEBL1, which in turn
467 promote mTOR function and ultimately MYC expression[22]. Consistent with this model
468 we found that *Tsc2* expression levels were reduced in *Pax5^{+/-} x Ebf1^{+/-}* leukemias while
469 *Sgk3*, *Rheb1* and *Myc* levels were increased. An alternative pathway that could also
470 be affected by PDK1 involves PDK1-dependent activation of PLK1, which in turn has
471 been shown to phosphorylate and activates MYC[23]. Thus, there are a number of
472 potential mechanisms by which increased PDK1 expression could promote
473 transformation. What is clear is that PDK1 inhibitors effectively blocked proliferation of
474 *Pax5^{+/-} x Ebf1^{+/-}* primary leukemia cell lines in vitro. Since there are currently a number
475 of PDK1 inhibitors available with some demonstrating efficacy in preclinical trials[36,
476 37], our findings suggest that PDK1 inhibition might be an effective strategy for treating
477 B cell leukemias that exhibit reduced expression of *Pax5* and *Ebf1*.

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493

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510 **Conflict of interest**

511 The authors declare no conflicts of interest.

512 **References**

513

- 514 1 Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD *et al.*
515 Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia.
516 *Nature* 2007; 446: 758-764.
517
- 518 2 Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB *et al.* Deletion of
519 IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med* 2009; 360:
520 470-480.
521
- 522 3 Mullighan CG. The genomic landscape of acute lymphoblastic leukemia in
523 children and young adults. *Hematology Am Soc Hematol Educ Program* 2014;
524 2014: 174-180.
525
- 526 4 Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J *et al.* BCR-ABL1
527 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* 2008;
528 453: 110-114.
529
- 530 5 Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the
531 transcription factor EBF. *Nature* 1995; 376: 263-267.
532
- 533 6 Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M. Complete block of
534 early B cell differentiation and altered patterning of the posterior midbrain in mice
535 lacking Pax5/BSAP. *Cell* 1994; 79: 901-912.
536
- 537 7 Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ *et al.* Cbl-b
538 regulates the CD28 dependence of T-cell activation. *Nature* 2000; 403: 216-220.
539
- 540 8 Wang JH, Nichogiannopoulou A, Wu L, Sun L, Sharpe AH, Bigby M *et al.*
541 Selective defects in the development of the fetal and adult lymphoid system in
542 mice with an Ikaros null mutation. *Immunity* 1996; 5: 537-549.
543
- 544 9 Verbeek S, Izon D, Hofhuis F, Robanus-Maandag E, te Riele H, van de Wetering
545 M *et al.* An HMG-box-containing T-cell factor required for thymocyte
546 differentiation. *Nature* 1995; 374: 70-74.
547
- 548 10 Lee YH, Sauer B, Johnson PF, Gonzalez FJ. Disruption of the *c/ebp* alpha gene
549 in adult mouse liver. *Mol Cell Biol* 1997; 17: 6014-6022.
550
- 551 11 Heltemes-Harris LM, Willette MJ, Ramsey LB, Qiu YH, Neeley ES, Zhang N *et al.*
552 *Ebf1* or *Pax5* haploinsufficiency synergizes with *STAT5* activation to initiate acute
553 lymphoblastic leukemia. *The Journal of experimental medicine* 2011; 208: 1135-
554 1149.
555

- 556 12 Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene
557 leads to rapid development of leukemia and lymphoma. *Cell* 1995; 83: 289-299.
558
- 559 13 Papathanasiou P, Perkins AC, Cobb BS, Ferrini R, Sridharan R, Hoyne GF *et al.*
560 Widespread failure of hematolymphoid differentiation caused by a recessive
561 niche-filling allele of the Ikaros transcription factor. *Immunity* 2003; 19: 131-144.
562
- 563 14 Heath V, Suh HC, Holman M, Renn K, Gooya JM, Parkin S *et al.* C/EBPalpha
564 deficiency results in hyperproliferation of hematopoietic progenitor cells and
565 disrupts macrophage development in vitro and in vivo. *Blood* 2004; 104: 1639-
566 1647.
567
- 568 15 Prasad MA, Ungerback J, Ahsberg J, Somasundaram R, Strid T, Larsson M *et al.*
569 Ebf1 heterozygosity results in increased DNA damage in pro-B cells and their
570 synergistic transformation by Pax5 haploinsufficiency. *Blood* 2015.
571
- 572 16 Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV *et*
573 *al.* Signatures of mutational processes in human cancer. *Nature* 2013; 500: 415-
574 421.
575
- 576 17 Starr TK, Largaespada DA. Cancer gene discovery using the Sleeping Beauty
577 transposon. *Cell cycle (Georgetown, Tex)* 2005; 4: 1744-1748.
578
- 579 18 Temiz NA, Moriarity BS, Wolf NK, Riordan JD, Dupuy AJ, Largaespada DA *et al.*
580 RNA sequencing of Sleeping Beauty transposon-induced tumors detects
581 transposon-RNA fusions in forward genetic cancer screens. *Genome research*
582 2016; 26: 119-129.
583
- 584 19 Goetz CA, Harmon IR, O'Neil JJ, Burchill MA, Farrar MA. STAT5 activation
585 underlies IL7 receptor-dependent B cell development. *J Immunol* 2004; 172:
586 4770-4778.
587
- 588 20 Katerndahl CDS, Heltemes-Harris LM, Willette MJL, Henzler CM, Fietze S, Yang
589 R *et al.* Antagonism of B cell enhancer networks by STAT5 drives leukemia and
590 poor patient survival. *Nature immunology* 2017; 18: 694-704.
591
- 592 21 Oettgen HF, Old LJ, Boyse EA, Campbell HA, Philips FS, Clarkson BD *et al.*
593 Inhibition of leukemias in man by L-asparaginase. *Cancer Res* 1967; 27: 2619-
594 2631.
595
- 596 22 Castel P, Ellis H, Bago R, Toska E, Razavi P, Carmona FJ *et al.* PDK1-SGK1
597 Signaling Sustains AKT-Independent mTORC1 Activation and Confers
598 Resistance to PI3Kalpha Inhibition. *Cancer Cell* 2016; 30: 229-242.
599
- 600 23 Tan J, Li Z, Lee PL, Guan P, Aau MY, Lee ST *et al.* PDK1 signaling toward
601 PLK1-MYC activation confers oncogenic transformation, tumor-initiating cell

- 602 activation, and resistance to mTOR-targeted therapy. *Cancer Discov* 2013; 3:
603 1156-1171.
- 604
- 605 24 Kornblau SM, Tibes R, Qiu YH, Chen W, Kantarjian HM, Andreeff M *et al.*
606 Functional proteomic profiling of AML predicts response and survival. *Blood*
607 2009; 113: 154-164.
- 608
- 609 25 Scott MC, Temiz NA, Sarver AE, LaRue RS, Rathe SK, Varshney J *et al.*
610 Comparative Transcriptome Analysis Quantifies Immune Cell Transcript Levels,
611 Metastatic Progression, and Survival in Osteosarcoma. *Cancer research* 2018;
612 78: 326-337.
- 613
- 614 26 Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV *et al.* Enrichr:
615 interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC*
616 *Bioinformatics* 2013; 14: 128.
- 617
- 618 27 Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z *et al.*
619 Enrichr: a comprehensive gene set enrichment analysis web server 2016 update.
620 *Nucleic Acids Res* 2016; 44: W90-97.
- 621
- 622 28 Xiao X, Yang G, Bai P, Gui S, Nyuyen TM, Mercado-Uribe I *et al.* Inhibition of
623 nuclear factor-kappa B enhances the tumor growth of ovarian cancer cell line
624 derived from a low-grade papillary serous carcinoma in p53-independent
625 pathway. *BMC Cancer* 2016; 16: 582.
- 626
- 627 29 Chen F, Castranova V. Nuclear factor-kappaB, an unappreciated tumor
628 suppressor. *Cancer research* 2007; 67: 11093-11098.
- 629
- 630 30 Kuiper RP, Waanders E, van der Velden VH, van Reijmersdal SV,
631 Venkatachalam R, Scheijen B *et al.* IKZF1 deletions predict relapse in uniformly
632 treated pediatric precursor B-ALL. *Leukemia* 2010; 24: 1258-1264.
- 633
- 634 31 Liu GJ, Cimmino L, Jude JG, Hu Y, Witkowski MT, McKenzie MD *et al.* Pax5 loss
635 imposes a reversible differentiation block in B-progenitor acute lymphoblastic
636 leukemia. *Genes & development* 2014; 28: 1337-1350.
- 637
- 638 32 Ungerback J, Ahsberg J, Strid T, Somasundaram R, Sigvardsson M. Combined
639 heterozygous loss of Ebf1 and Pax5 allows for T-lineage conversion of B cell
640 progenitors. *The Journal of experimental medicine* 2015; 212: 1109-1123.
- 641
- 642 33 Heltemes-Harris LM, Larson JD, Starr TK, Hubbard GK, Sarver AL, Largaespada
643 DA *et al.* Sleeping Beauty transposon screen identifies signaling modules that
644 cooperate with STAT5 activation to induce B-cell acute lymphoblastic leukemia.
645 *Oncogene* 2015.
- 646

- 647 34 Xia Y, Shen S, Verma IM. NF-kappaB, an active player in human cancers.
648 *Cancer Immunol Res* 2014; 2: 823-830.
649
- 650 35 Fahl SP, Crittenden RB, Allman D, Bender TP. c-Myb is required for pro-B cell
651 differentiation. *J Immunol* 2009; 183: 5582-5592.
652
- 653 36 Emmanouilidi A, Fyffe CA, Ferro R, Edling CE, Capone E, Sestito S *et al.*
654 Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition
655 in pancreatic cancer. *J Exp Clin Cancer Res* 2019; 38: 191.
656
- 657 37 Barile E, De SK, Pellicchia M. PDK1 inhibitors. *Pharm Pat Anal* 2012; 1: 145-
658 163.
659
660

661 **Figure Legends**

662 **Figure 1.** Compound haploinsufficiency for transcription factor genes drives B cell
663 or T cell leukemia. **A** Kaplan-Meier survival analysis of mice of the indicated
664 genotype. **B** Flow cytometric analysis of control C57Bl/6 bone marrow (BM) cells or
665 bone marrow, lymph node (LN), and spleen cells from *Pax5*^{+/-} *x* *Ebf1*^{+/-} leukemic mice.
666 Representative flow cytometric analysis of B220, CD19, and IgM expression is
667 shown. Doublets were gated out and a lymphocyte gate was set based on side and
668 forward scatter properties. All gates shown are based on bone marrow isolated from
669 control C57Bl/6 mice. **C** Pie charts showing the number of leukemias from each
670 genotype that were either of the B cell (Blue) or T cell (Red) phenotype; grey
671 represents mice that either failed to develop leukemia or developed mixed lineage
672 leukemia (grey). **D** Flow cytometric analysis of bone marrow cells from control
673 C57Bl/6 and *Pax5*^{+/-} *x* *Ebf1*^{+/-} *x* *Ikzf1*^{+/-} leukemic mice. Representative flow cytometric
674 analysis of B220, CD3, CD4, and CD8 expression on bone marrow cells is shown.
675 Doublets were gated out and a lymphocyte gate was set based on side and forward
676 scatter properties. All gates shown are based on bone marrow isolated from control
677 C57Bl/6 mice.

678 **Figure 2.** Missense mutations in primary human ALL. **A** Histogram showing the
679 distribution of missense mutations per sample across 486 primary human B-ALL
680 samples from the TARGET phase-2 study. SNV/indel variants were called across all
681 genes by MuTect2. Samples were classified as high or low mutation burden based
682 on the natural break in the bimodal distribution (low < 669, high > 863

683 variants/sample). The percentage of samples in the high category is labeled inside
684 the plot. **B** Bar graph summarizing the percentage of samples with high mutation
685 burden compared to low for all B-ALL samples or various subsets. **C** Bar graph
686 summarizing the total number of primary human B-ALL samples displaying high or
687 low missense mutation burden. Samples affected by loss of function alleles in
688 various genes (*CDKN2a*, *EBF1*, *PAX5*, and *IKZF1*) or contain certain gene-fusions
689 (*ETV6-RUNX1*, *TCF3-PBX1*) were shown as subsets. * $p < 0.0114$ Fisher's exact test.

690

691 **Figure 3.** Sleeping Beauty mutagenesis screen to identify genes that cooperate
692 with *Pax5* and *Ebf1* heterozygosity to induce leukemia. **A** Kaplan-Meier survival
693 analysis of mice comparing *Pax5*^{+/-} x *Ebf1*^{+/-} leukemic mice (n=51) and SB *Pax5*^{+/-} x
694 *Ebf1*^{+/-} (n=34) leukemic mice to control mice SB x *Cd79a-Cre* (n=17). P-value
695 compares *Pax5*^{+/-} x *Ebf1*^{+/-} versus SB *Pax5*^{+/-} x *Ebf1*^{+/-} mice. **B** Table indicating all of
696 the samples used in RNA-seq analysis. The table indicated the status, type and
697 number of samples utilized for the RNA-Seq. Control samples represent progenitor
698 B cell pools from 7-8 mice. **C** Hierarchical clustering of all leukemic and control
699 samples. **D** Fusions identified from RNA-seq analysis of our sleeping beauty
700 mutagenesis screen. This chart identifies recurrent insertions found in 27 of the 31
701 samples tested and indicate how many mice had each specific gene insertion. **E**
702 Matrix analysis of individual mice by gene identified in fusion analysis.

703

704 **Figure 4. Increased Expression of Stat5b in leukemia. A** Map of common

705 insertion sites in the *Stat5b* gene; numbers refer to number of insertions at a
706 particular site. **B** Quantitative Real Time PCR (qRT-PCR) for *Stat5b* normalized to
707 *Actin* in progenitor B cells isolated from the bone marrow of WT (black, n=4) mice,
708 and leukemic cells isolated from the lymph nodes of *Pax5^{+/-} x Ebf1^{+/-}* (purple, n=6)
709 and SB *Pax5^{+/-} x Ebf1^{+/-}* mice. The samples from the SB *Pax5^{+/-} x Ebf1^{+/-}* mice were
710 split between those with (blue, n=15) or without (red, n=10) an insertion in the *Stat5b*
711 locus. The normalized values were log2 transformed and an ordinary one-way
712 ANOVA with Holm-Sidak's multiple comparison test was used to determine
713 significance. The line represents the median value. **C** Log2 transformed fragments
714 per kilobase of exon model per million reads mapped (FPKM) values from WT (black
715 filled, n=3), *Pax5^{+/-}* (green filled, n=4), *Ebf1^{+/-}* (green open, n=4), *Pax5^{+/-} x Ebf1^{+/-}* pre-
716 leukemic (purple open, n=4), *Pax5^{+/-} x Ebf1^{+/-}* leukemic (purple filled, n=7), and SB
717 *Pax5^{+/-} x Ebf1^{+/-}* leukemic samples with (blue filled, n=20) or without (red filled, n=11)
718 a transposon insertion in *Stat5b* locus. A Kruskal-Wallis test with Dunn's multiple
719 comparison test was used to test for significance. The line represents the median
720 value. **D** Western blot analysis showing increased expression of STAT5. The + or -
721 indicates the presence or absence of a SB transposon insertion in each
722 representative sample. **E** Plotted ratio of STAT5 to actin from the western blot.
723 Samples were plotted according to transposon insert status where those samples
724 without a transposon insert are red (n=7) and those with a transposon insert are blue
725 (n=6). Significance was determined using an unpaired student t-test and the line
726 represents the median. **F** Flow cytometric analysis of bone marrow cells from *Pax5^{+/-}*
727 *x Ebf1^{+/-}* leukemic mice. Representative flow cytometric analysis of pSTAT5

728 expression in cells where doublets were gated out, a lymphocyte gate was applied,
729 and cells were further gated on B220 and AA4.1. This is representative of 5
730 independent experiments. **G** Flow cytometric analysis of leukemic B cells from
731 *Pax5^{+/-} x Ebf1^{+/-}* leukemic mice. Lymph node cells from leukemic mice or bone
732 marrow cells from WT mice were activated with IL-7 for 30 minutes and subjected to
733 flow cytometric analysis for pSTAT5 expression. Doublets were gated out, a
734 lymphocyte gate was applied, and cells were further gated on B220 and AA4.1. This
735 is a representative plot of 4 independent experiments. **H** Log2 transformed FPKM
736 values for *Cish* from WT (black filled, n=3), *Pax5^{+/-}* (green filled, n=4), *Ebf1^{+/-}* (green
737 open, n=4), *Pax5^{+/-} x Ebf1^{+/-}* pre-leukemic (purple open, n=4), *Pax5^{+/-} x Ebf1^{+/-}*
738 leukemic (purple filled, n=7), and SB *Pax5^{+/-} x Ebf1^{+/-}* leukemic (blue filled, n=31)
739 samples. An ordinary one-way ANOVA with multiple comparisons was used to test
740 for significance. The line represents the median value. **I** Log2 transformed FPKM
741 values for *Socs2* from WT (black filled, n=3), *Pax5^{+/-}* (green open, n=4), *Ebf1^{+/-}* (green
742 filled, n=4), *Pax5^{+/-} x Ebf1^{+/-}* pre-leukemic (purple open, n=4), *Pax5^{+/-} x Ebf1^{+/-}*
743 leukemic (purple filled, n=7), and SB *Pax5^{+/-} x Ebf1^{+/-}* leukemic (blue filled, n=31)
744 samples. An ordinary one-way ANOVA with Holm-Sidak's test for multiple
745 comparisons was used to test for significance. The line represents the median value.

746

747 **Figure 5. Loss of *Cblb* accelerates the onset of B cell ALL.** **A** Map of common
748 insertion sites in the *Cblb* gene; numbers represent number of insertions at a specific
749 site. **B** qRT-PCR for *Cblb* normalized to *Actin* in progenitor B cells isolated from the

750 bone marrow of WT (black, n=4) mice, and leukemic cells isolated from the lymph
751 nodes of *Pax5^{+/-} x Ebf1^{+/-}* (purple, n=6) and SB *Pax5^{+/-} x Ebf1^{+/-}* mice. The samples
752 from the SB *Pax5^{+/-} x Ebf1^{+/-}* mice were split between those with (blue, n=8) or without
753 (red, n=17) an insertion in the *Cblb* locus. The normalized values were log₂
754 transformed and significance was determined using a Kruskal-Wallis test with Dunn's
755 multiple comparison test. The line represents the median value. **C** Western blot
756 analysis showing expression of CBLB. The + or - indicates the presence or absence
757 of a SB transposon insertion in each representative sample. **D** Plotted ratio of CBLB
758 to actin from the western blot. Samples were plotted according to transposon insert
759 status where those samples without a transposon insert are red (n=6) and those with
760 a transposon insert are blue (n=8). Significance was determined using an unpaired
761 student t-test and the line represents the median. **E** Kaplan-Meier survival analysis of
762 mice comparing *Pax5^{+/-} x Ebf1^{+/-}* leukemic mice (n=51) and *Cblb^{-/-} x Pax5^{+/-} x Ebf1^{+/-}*
763 (n=13) leukemic mice.

764

765 **Figure 6.** Loss of MYB expression results in worse outcome in ALL. **A** Map of
766 common insertion sites in the *Myb* gene; numbers refer to number of insertions at a
767 specific site. **B** qRT-PCR for *Myb* normalized to *Actin* in progenitor B cells isolated
768 from the bone marrow of WT (black, n=4) mice, and leukemic cells isolated from the
769 lymph nodes of *Pax5^{+/-} x Ebf1^{+/-}* (purple, n=6) and SB *Pax5^{+/-} x Ebf1^{+/-}* mice. The
770 samples from the SB *Pax5^{+/-} x Ebf1^{+/-}* mice were split between those with (blue, n=8)
771 or without (red, n=17) an insertion in the *Myb* locus. The significance was tested
772 using an ordinary one-way ANOVA with Holm-Sidak's multiple comparison test and

773 the lines represent median. **C** Log₂ transformed fragments per kilobase of exon
774 model per million reads mapped (FPKM) values from WT (black filled, n=3), *Pax5*^{+/-}
775 (green filled, n=4), *Ebf1*^{+/-} (green open, n=4), *Pax5*^{+/-} x *Ebf1*^{+/-} pre-leukemic (purple
776 open, n=4), *Pax5*^{+/-} x *Ebf1*^{+/-} leukemic (purple filled, n=7), and SB *Pax5*^{+/-} x *Ebf1*^{+/-}
777 leukemic samples with (blue filled, n=10) or without (red filled, n=21) a transposon
778 insertion. Significance was tested using a Kruskal-Wallis test with Dunn's multiple
779 comparison test and the line represents median. **D** Western blot analysis showing
780 decreased expression of MYB in SB leukemia samples harboring a transposon
781 insertion. The + or - indicates the presence or absence of a SB transposon insertion
782 in each representative sample. **E** Plotted ratio of MYB to actin from the western blot
783 in panel d. Samples were plotted according to transposon insert status where those
784 samples without a transposon insert are red (n=4) and those with a transposon insert
785 are blue (n=8). Significance was determined using an unpaired student t-test and the
786 line represents the median. **F** Linear regression analysis comparing date of death
787 versus FPKM value for leukemic samples harboring a transposon insertion. The
788 dashed lines represent 95% confidence bands.

789

790 **Figure 7.** Inhibition of PDK1 blocks leukemic proliferation. **A** Log₂ transformed
791 FPKM values for *Pdk1* from the RNA-seq datasets. Significance was determined by
792 an ordinary one-way ANOVA with Holm-Sidak's multiple comparison test. **B** Log₂
793 transformed FPKM values for *Sgk3* from the RNA-seq datasets. Significance was
794 tested using a Kruskal-Wallis test with Dunn's multiple comparison test. **C** Log₂
795 transformed FPKM values for *Myc* from the RNA-seq datasets. Significance was

796 determined by an ordinary one-way ANOVA with Holm-Sidak's multiple comparison
797 test. **D** PDK1 inhibitor blocks growth. MTT assay was performed on two *Pax5^{+/-}* x
798 *Ebf1^{+/-}* leukemic cell lines generated from lymph node cells from leukemic mice. The
799 cells were subjected to differing concentrations of GSK2334470. Each dot
800 represents the average of two biological replicates - each biological replicate
801 represents the mean of triplicate technical replicates within an experiment. Error
802 bars represent the range between experiments. **E** Overall survival of *Bcr-Abl*
803 patients. *Bcr-Abl* patients were separated into three equal groups representing
804 higher (black line, n=14), intermediate (red line, n=28) and lower (blue line, n=14)
805 levels of PDK1. Patients with lower levels of PDK1 did significantly worse than
806 patients with higher PDK1 (p=0.04, Log Rank test for trend). **F** Overall survival of
807 *Bcr-Abl* young adult patients. Young adult patients were separated into two equal
808 groups representing higher (red line, n=9) and lower (blue line, n=9) levels of PDK1.
809 Patients with lower levels of PDK1 did significantly worse than patients with higher
810 PDK1 (p=0.02, Log Rank (Mantel-Cox) Test). **G** PDK1 expression of *Bcr-Abl* patients
811 split by relapse status. *Bcr-Abl* patients were separated into two groups
812 representing no relapse (blue dots, n=14), and relapse (red dots, n=14) levels of
813 PDK1 were graphed. Patients with lower levels of PDK1 did significantly worse than
814 patients with higher PDK1 (p<0.01, unpaired T-test). **H** PDK1 expression of *B-Nos*
815 patients split by relapse status. Patients were separated into two groups
816 representing no relapse (blue dots, n=14), and relapse (red dots, n=14) and levels of
817 PDK1 were graphed. Patients with lower levels of PDK1 did significantly worse than

818 patients with higher PDK1 ($p < 0.02$, unpaired T-test)

819

820 **Figure 8.** Transcriptome profiles from leukemic progenitor B cells show common
821 interleukemic transcriptional variation across human and mouse samples. **A** Common
822 transcriptional patterns identified in RNA-Seq from human leukemias. Top ~8000 gene
823 values defined by SD in ph2_SJ and ph2_BCCA separately identify conserved
824 transcriptional patterns to be present. Red bars indicate highly significantly enriched
825 sets of conserved genes to be present across Human ALL datasets via Fisher exact
826 test comparison of gene cluster membership. Gene transcript values derived from
827 human leukemias were log transformed and mean centered within each species.
828 Invariant (low SD) genes were removed prior to unsupervised average linkage
829 clustering. Conservation was apparent despite the fact that the SJ set was summarized
830 as gene symbols while the BCCA set was summarized to ENSEMBL ids. Transcripts
831 with increased levels are shown in yellow, while transcripts with decreased levels are
832 shown in blue. **B** Gene cluster overlap analyses comparing clusters derived from
833 human and mouse tumors show that the variation present in our mouse dataset
834 represents one of the clear variations present and conserved in all of the human
835 samples. Gene lists for the conserved cluster from each dataset are provided in
836 Supplementary Table S2.

Figure 1

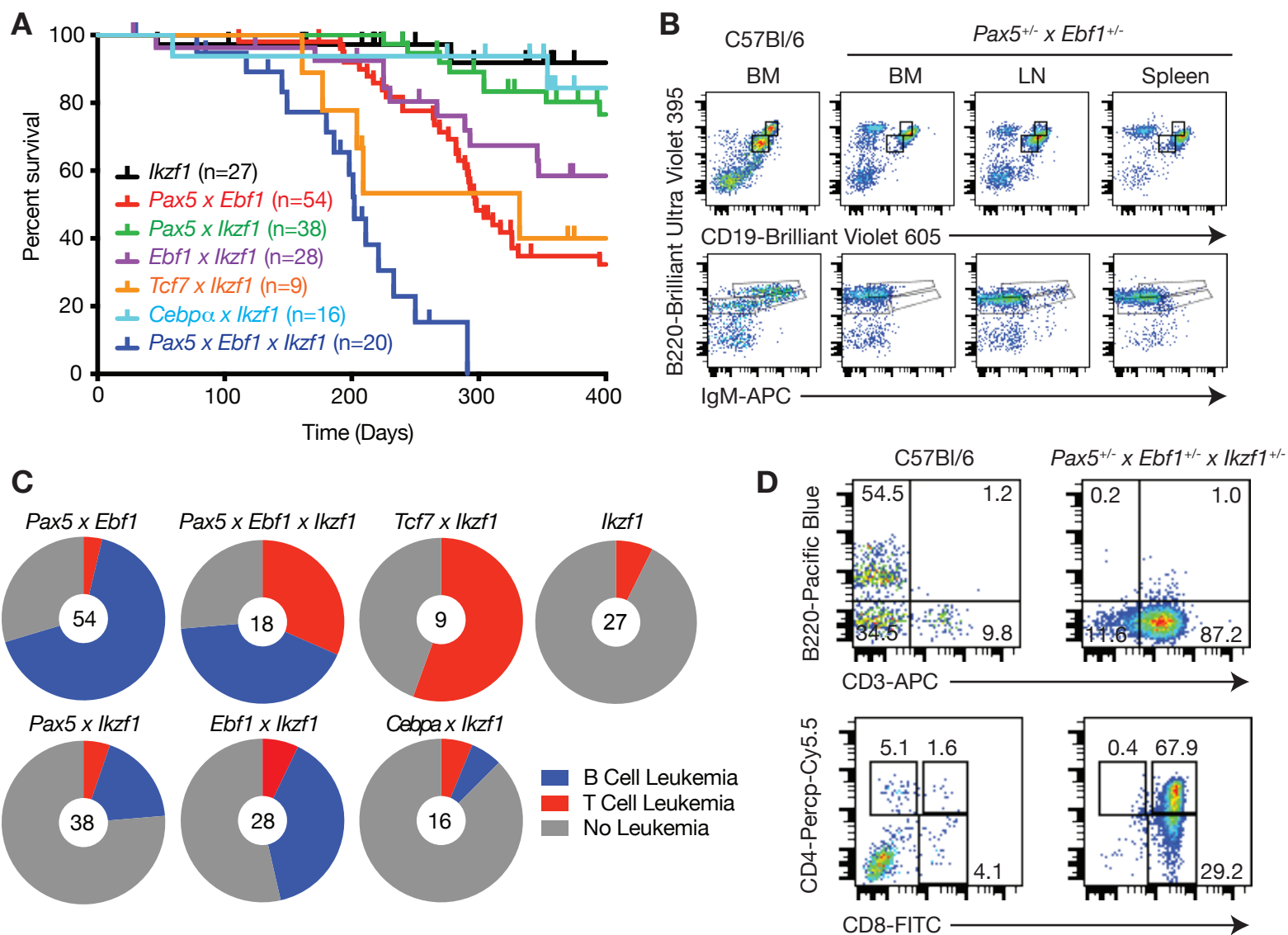
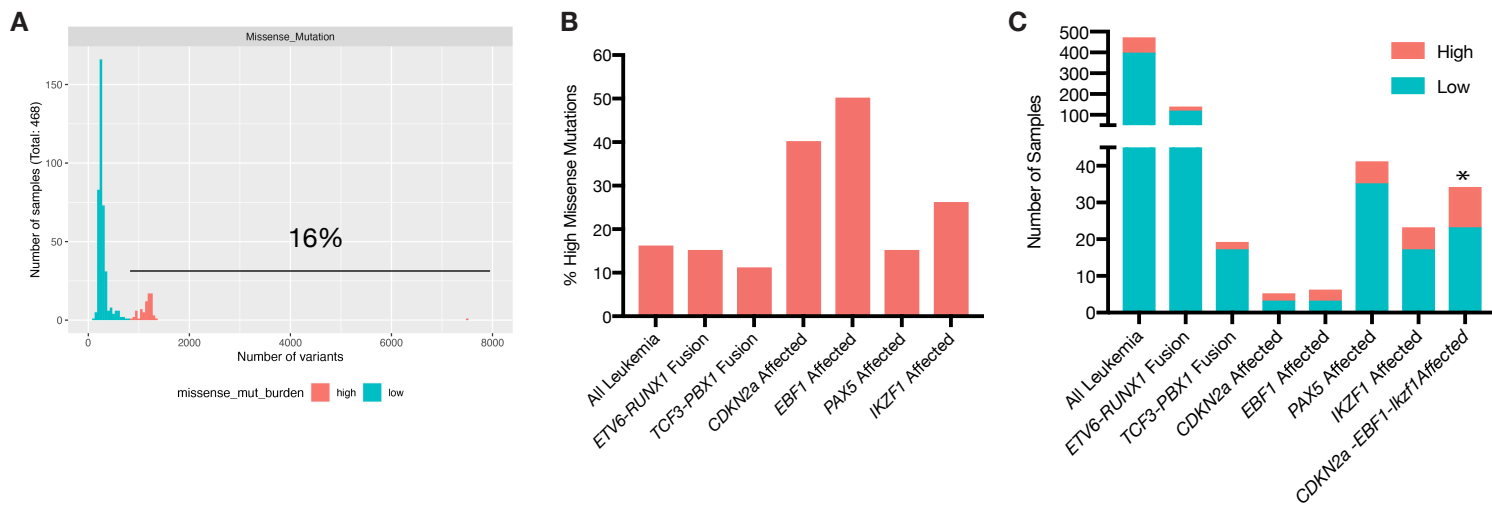


Figure 2



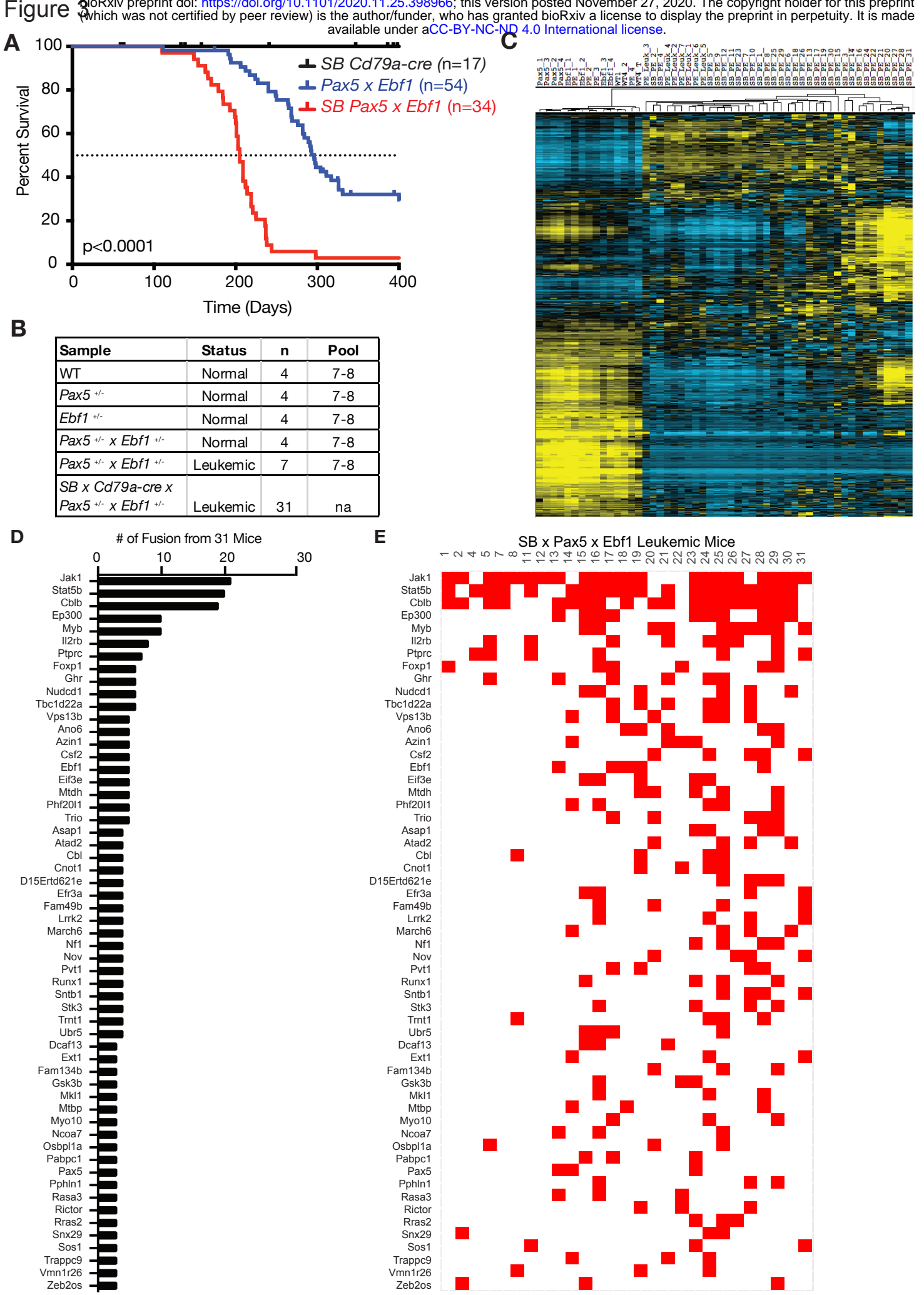


Figure 4

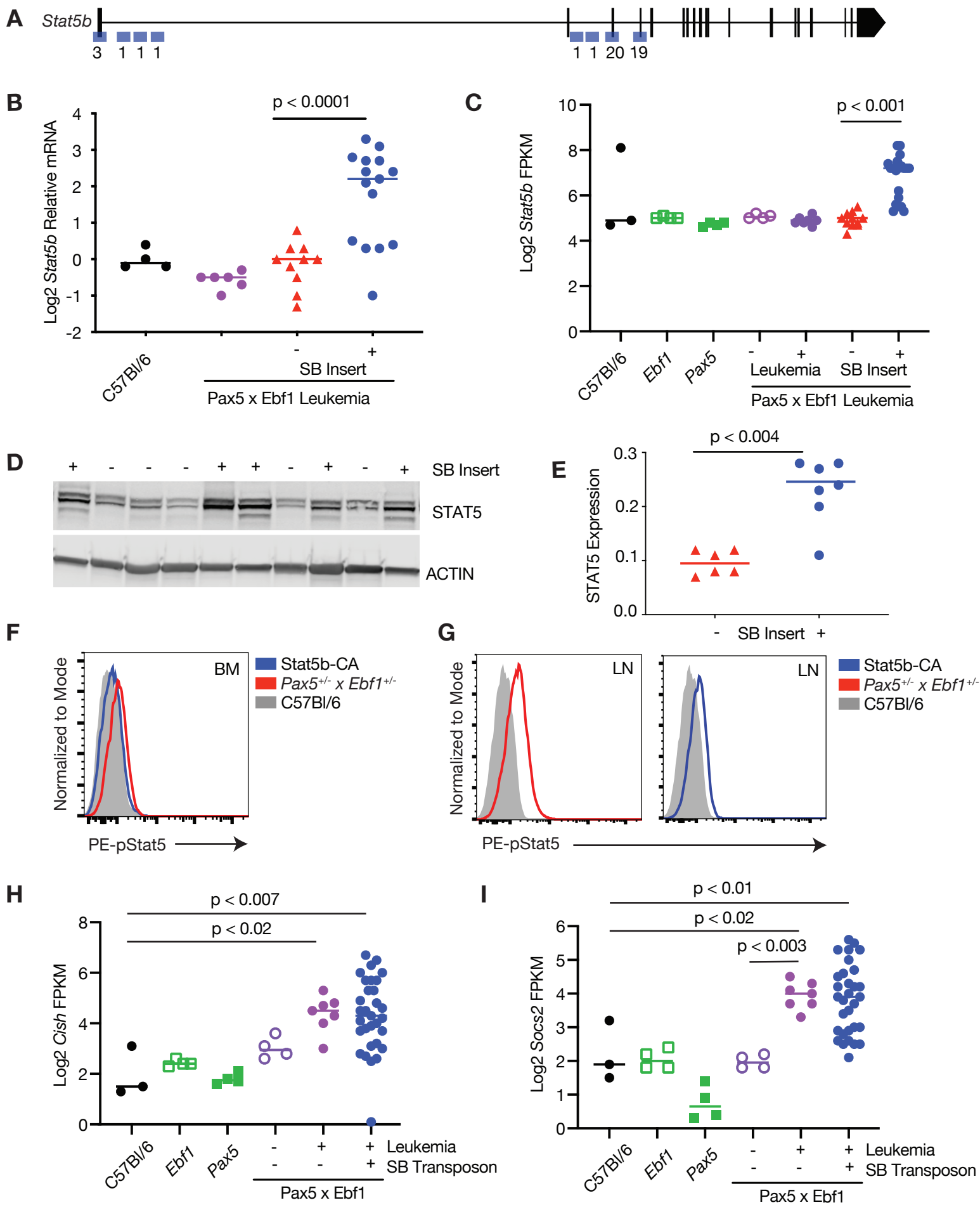


Figure 5

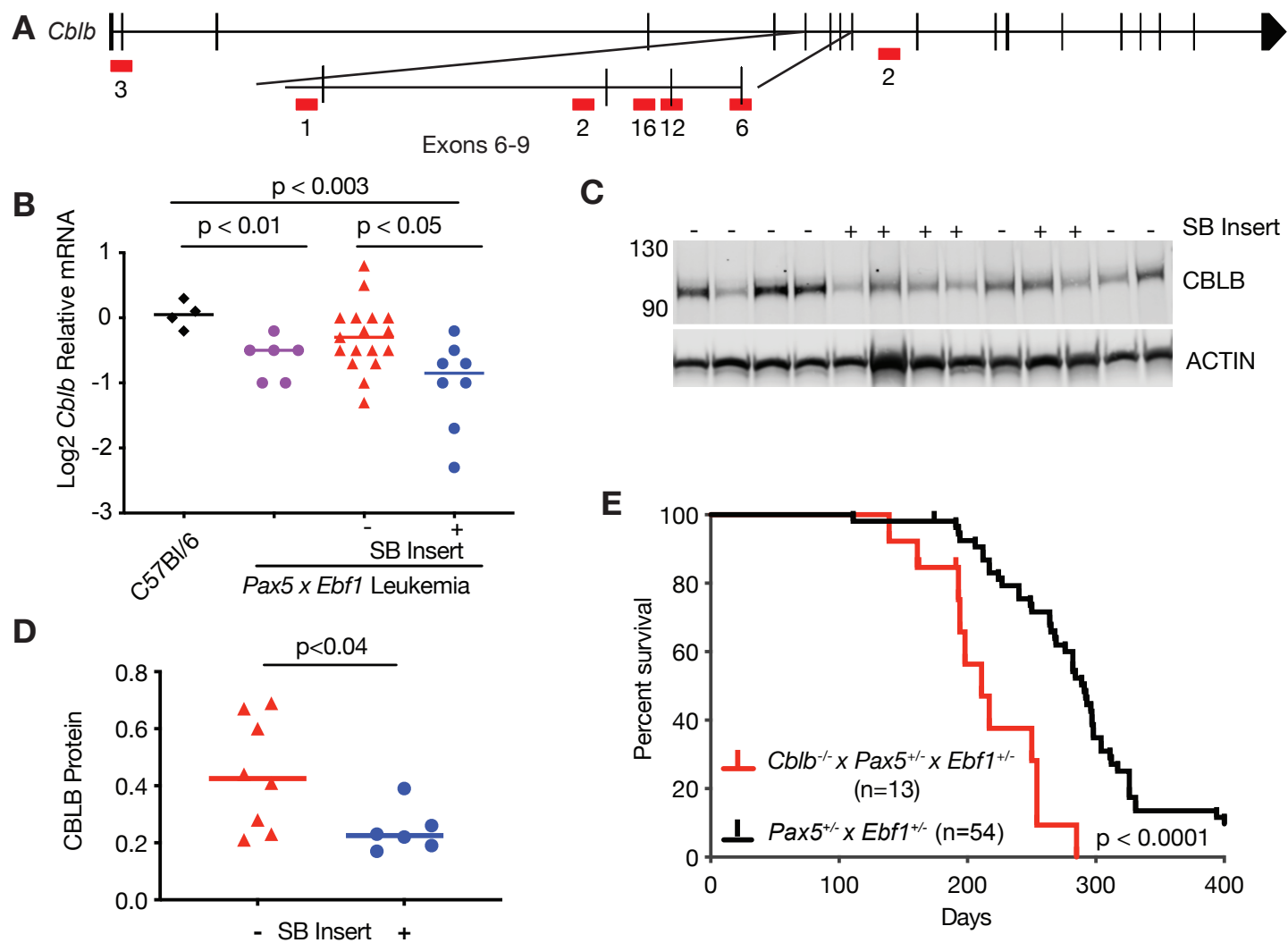
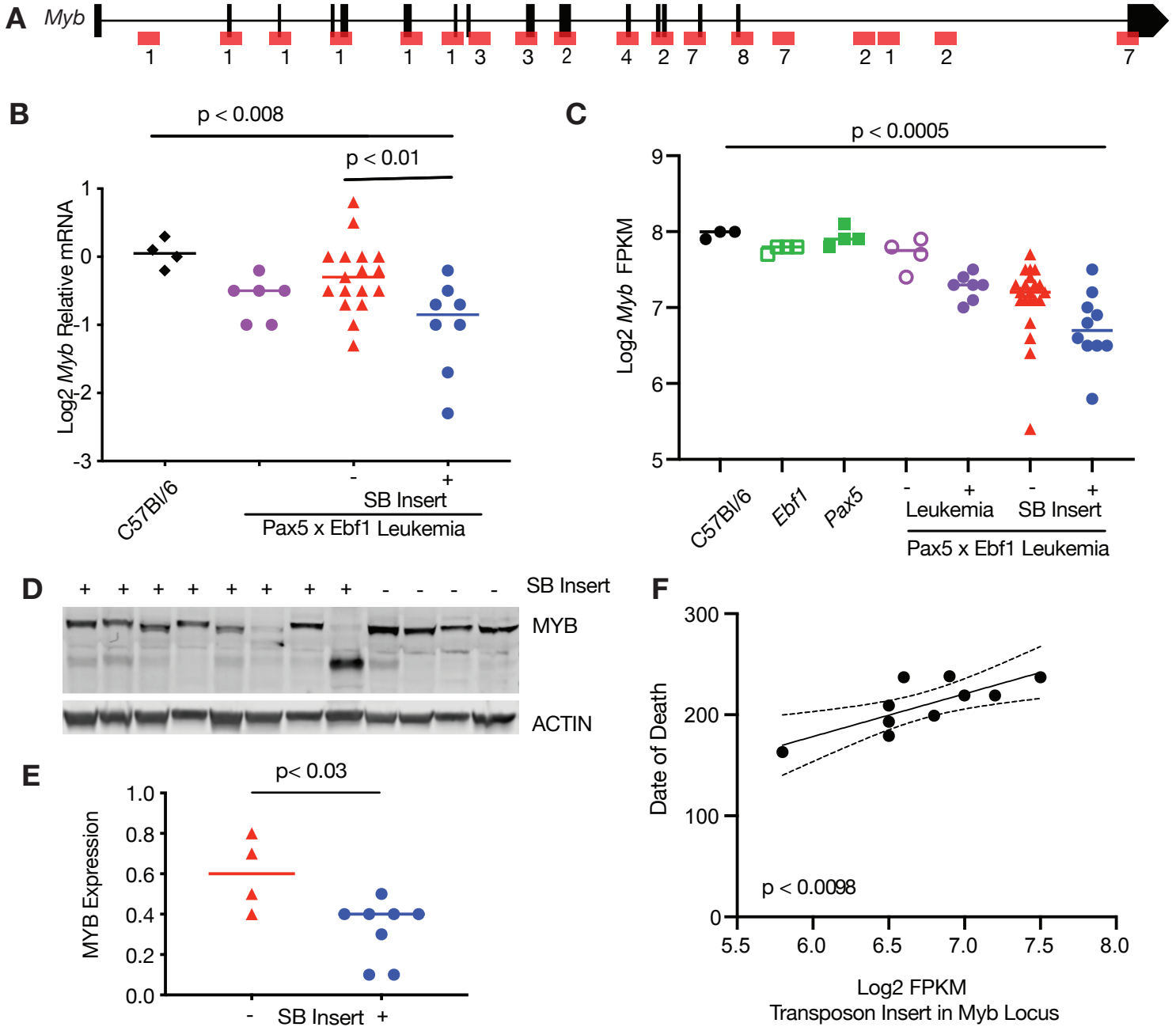


Figure 6



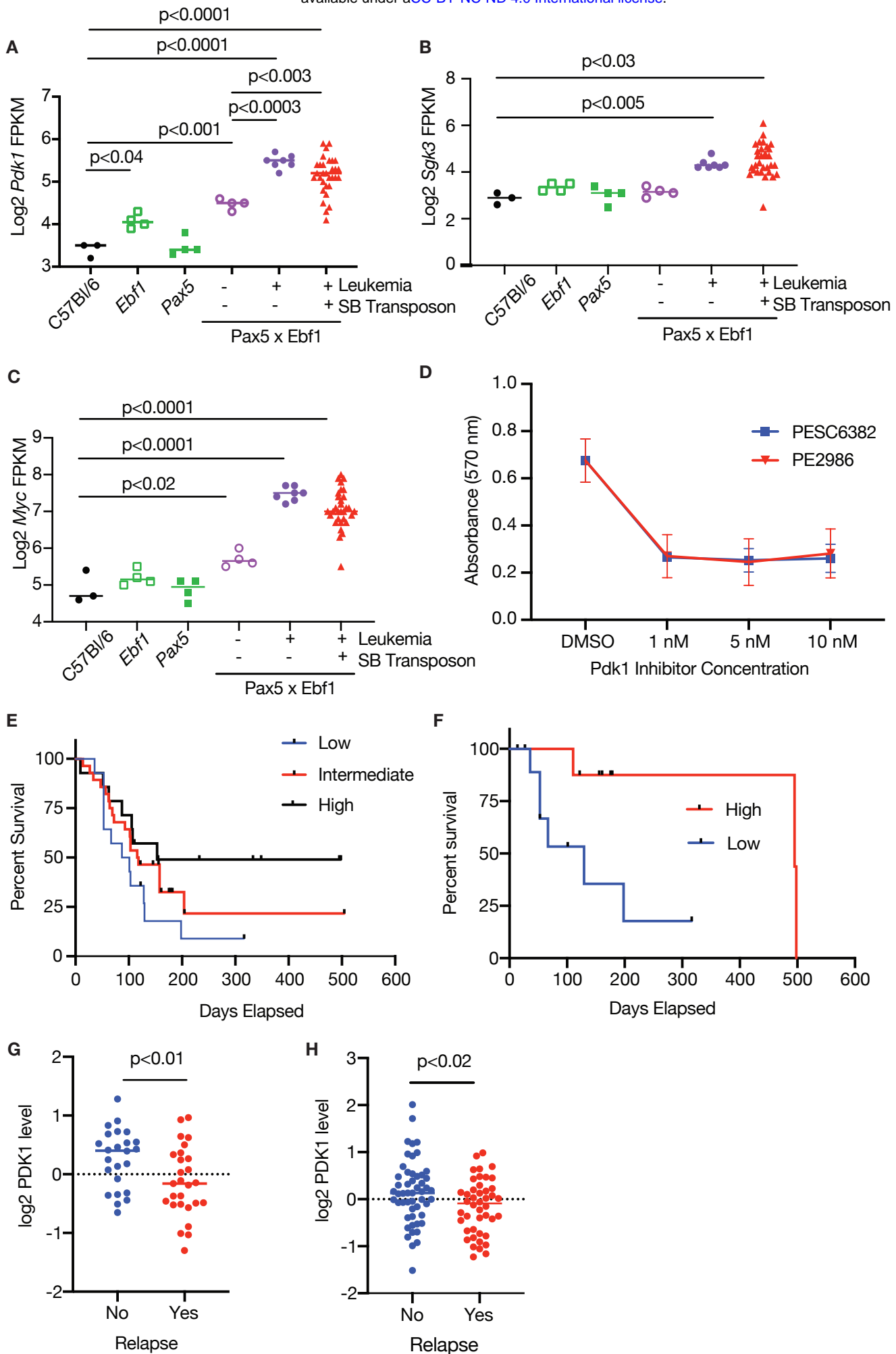
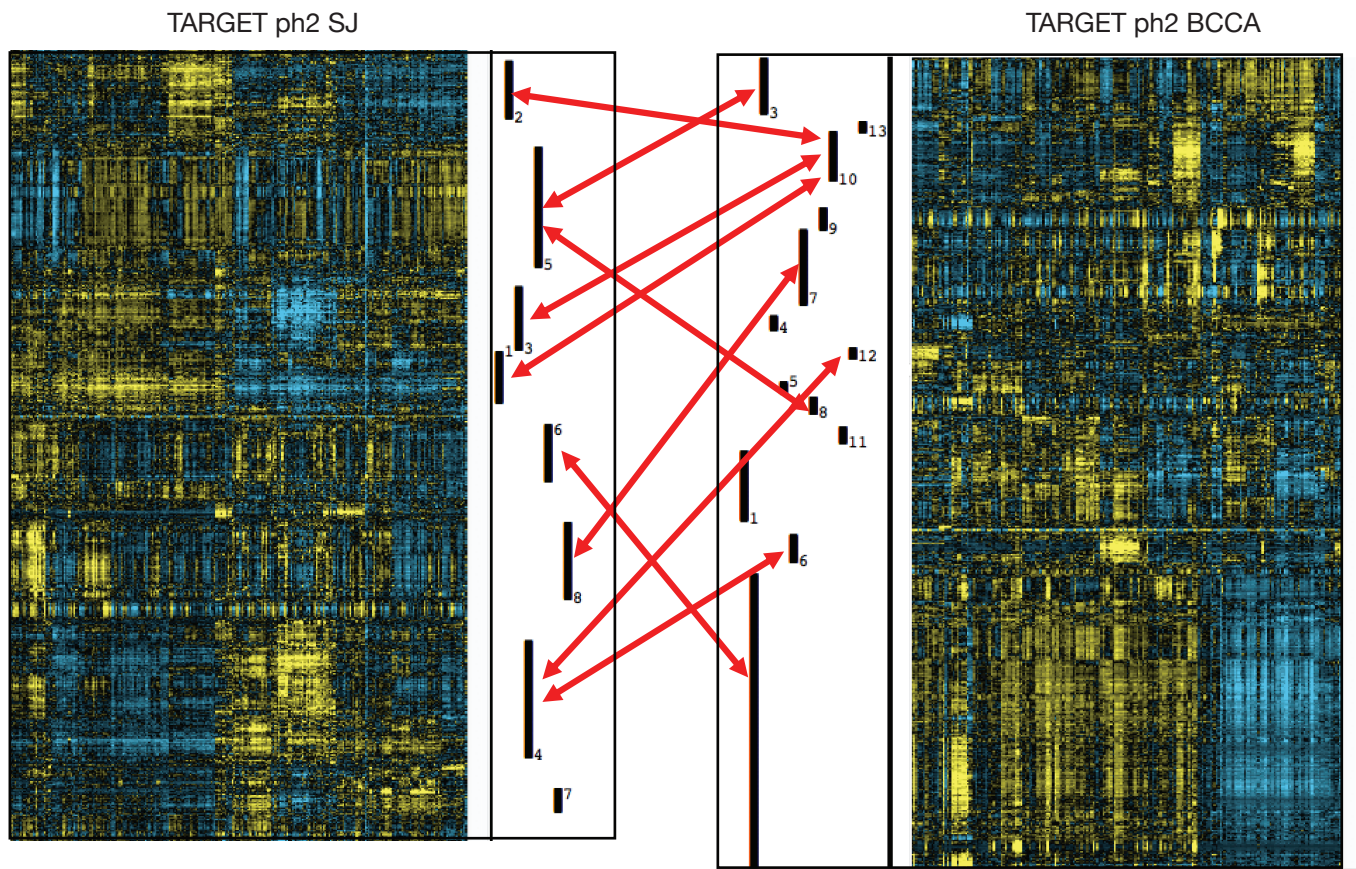


Figure 8

A



B

