Tissue Specific Requirement for the Inv(16) Oncogene *CBFB::MYH11* in Acute Myeloid Leukemia

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Abstract

Inversion of chromosome 16 [inv(16)] generates the fusion gene *CBFB::MYH11 (CM)* and is one of the most common chromosomal rearrangements in Acute Myeloid Leukemia (AML). Expression of *CM* is required for leukemia initiation. Patients with inv(16) at diagnosis invariably have the rearrangement at relapse, leading to the assumption that *CM* is also required after leukemic transformation. However, a role for *CM* in leukemia maintenance has yet to be shown experimentally. To address this, we used an inducible *CM* knockdown (KD) mouse model and found that decreased *CM* eliminated leukemia cells from the peripheral blood and spleen, but not the bone marrow, despite all populations exhibiting significantly decreased *CM* mRNA and protein. The surviving *CM* KD cells in the bone marrow showed decreased apoptosis and proliferation, and increased expression of autophagy related genes. Surprisingly, with prolonged KD of *CM*, ~40% of mice re-established disease despite maintaining decreased *CM*. Our work indicates that *CM* is required for leukemia survival in the spleen and peripheral blood, but in the bone marrow *CM* KD leukemia cells can survive and re-establish disease independent of the fusion protein. These findings imply that targeting *CM* alone has potential to reduce leukemic burden but not cure the disease.

43 **Introduction**

44 Acute Myeloid Leukemia (AML) is a heterogeneous clonal malignancy arising from the
45 hematopoietic stem and progenitor cells of the bone marrow, as the result of a block in
46 differentiation that gives rise to a more than 20% increase in immature blast cells, reduced mature
47 blood cell production and ultimately BM failure (1-3). AML develops as a neoplastic disease, often
48 with specific chromosomal rearrangements, including the inversion of chromosome 16[inv(16)]
49 (4, 5). These rearrangements are considered the initiating mutation, and with the acquisition of
50 additional cooperating mutations, these abnormal myeloid cells can transform into a full-blown,
51 frank leukemia (6-8). Inv(16) accounts for ~10% of all AMLs, making it one of the most common
52 chromosomal abnormalities in AML (9-12). At the molecular level, inv(16) generates a fusion
53 between the genes encoding the transcription factor Core Binding Factor β (*CBFB*) and the
54 contractile protein Smooth Muscle Myosin Heavy Chain (*MYH11*). The resulting oncogene,
55 *CBFB::MYH11 (CM),* encodes the protein CBFβ::SMMHC (13-15). With current treatment
56 protocols, inv(16) AML has a favorable prognosis due to high rates of initial response to
57 chemotherapy. However, ~40% of patients will eventually relapse, at which point there are limited
58 curative options (12, 16, 17).

59

60 Because *CM* is the initiating mutation in this subtype of AML and is expressed in all leukemia cells
61 but not in healthy cells, it is an ideal drug target. However, the role and requirement for *CM* after
62 malignant transformation is poorly understood. In Chronic Myeloid Leukemia (CML), another
63 fusion protein driven leukemia, expression of BCR::ABL initiates the disease, but once
64 established, BCR::ABL activity is largely dispensable for maintenance of the leukemia stem cell
65 population. Currently, it is not known if this is a unique feature of BCR::ABL and CML, or if the
66 existence of a population of cells that no longer require the activity of the initiating mutation is a
67 more common phenomenon. Resolving this issue is critical to the development and eventual
68 clinical use of inhibitors targeting other leukemia fusion proteins like CBFβ::SMMHC in AML.

69

70 In this study, we investigate the role of *CM* in primary mouse leukemia cells after leukemia
71 initiation. We found that decreased *CM* expression eliminates leukemia from the peripheral blood
72 and spleen, but not the bone marrow, indicating that loss of CBFβ::SMMHC activity is sufficient
73 to control, but not cure the disease.

74

75 **Methods**

76 Mice

77 All animal experiments were approved by the University of Nebraska Medical Center IACUC in
78 accordance with NIH guidelines. 4-6-week-old mice of both sexes were used.

79

80 *Mx1-Cre, Cbfb$^{+/56M}$ mice*

81 To generate *CM* expressing leukemia cells, we used conditional knock-in mice expressing
82 *Cbfb::MYH11* from the endogenous promoter, (*Cbfb$^{+/56M}$*) paired with the inducible *Mx1-Cre*
83 transgene: *Mx1-Cre, Cbfb$^{+/56M}$* mice (18). To induce leukemia, *CM* mice were treated with 250μg
84 Polyinosine-polycytidylic acid (pI:pC) 3 times a week, for 3 rounds of treatment.

85

86 *Cbfb$^{+/flMYH11}$ mice*

87 To generate knock-in mice with the *flMYH11* allele, *LoxP* sites were added by site directed
88 mutagenesis (QuikChange, Agilent) flanking the *Cbfb Exon 5::MYH11* cassette in the *pPNT*
89 vector. Mouse embryonic stem (ES) cells were electroporated, selected, screened for
90 homologous recombination and injected into blastocysts, as described previously (19, 20). To
91 confirm the *Cbfb^{flMYH11}* allele functions as predicted, chimeric mice were bred to mice expressing
92 *Cre* from the *β-Actin* promoter (*ActB-Cre^{+}*), purchased from Jackson Laboratory (Bar Harbor, ME,
93 USA) and embryos harvested at embryonic day 12.5 (21). To induce leukemia, chimeric

94 *Cbfb*$^{+/flMYH11}$mice were treated with a single dose of N-ethyl-N-nitrosourea (ENU, 100mg/kg) and
95 monitored for leukemia development, as described previously (22-24).
96
97 *Transplantation of leukemia cells*
98 *CM* expressing leukemia cells were transplanted into sub-lethally irradiated congenic recipient
99 mice by retro-orbital injection, as described previously (25, 26).
100
101 Viral Transduction
102 To excise the *Cbfb* ^{flMYH11} allele, lentiviral plasmids co-expressing *Cre Recombinase* and either
103 GFP or DsRED were used. To knockdown *Cbfb::MYH11*, a doxycycline (Dox) inducible lentiviral
104 plasmid expressing GFP and a shRNA against *MYH11* (*shMYH11*) was kindly provided by Dr. J.
105 Martens, Nijmegen, NL (27). Virus was produced in 293KEK cells and used to transduce mouse
106 leukemia cells, as previously described (25, 26, 28). Transduced cells were sorted for GFP or
107 dsRED using Aria II Cell Sorter (BD Biosciences, San Diego, CA, USA) 48hrs post transduction
108 and transplanted into congenic mice as above. To induce *shMYH11* expression, mice were given
109 5% sucrose supplemented drinking water with or without (CTRL) Dox at 2mg/ml. Mice were
110 sacrificed on the indicated day of treatment and tissue collected.
111
112 qRT-PCR Analysis
113 RNA was extracted using Trizol (Thermo Scientific, Waltham, MA, USA) as described previously
114 (25, 26). cDNA was synthesized and amplified using Luna RT Supermix and Luna Universal
115 qPCR kit (New England Biolabs, Ipswich, MA, USA) per manufacturer's protocol. Primer
116 sequences available upon request.
117
118 Western Blot Analysis
119 Cell lysates were prepared, subjected to SDS-PAGE and transferred to PVDF, as described

120 previously (25, 26). Membranes were blocked in 5% milk in TBST and then probed using primary
121 antibodies and then with secondary HRP-linked antibodies as indicated in (Supplementary Table
122 1). Protein bands were visualized with SuperSignal West Pico PLUS (Thermo Scientific).
123 Quantification was performed using ImageJ software.
124
125 Flow Cytometry Analysis
126 Cells were stained with fluorophore coupled antibodies against B220 (RA3-6B2), CD3 (17A2),
127 Mac-1/CD11b (M1/70), GR-1 (RM4-5), CKIT (53-6.7), Csf2rb/CD131 (JORO50), IL1RL1 (U29-
128 93), CD41 (MWReg30), CD123 (5B11) (BD Bioscience) and analyzed by flow cytometry (BD,
129 LSR II). Apoptosis was assayed using Annexin V (BD). Proliferation was determined using the
130 BrdU Flow Kit (BD). Mice were injected with BrdU at dose of 200mg/kg body weight. After 1 hr,
131 mice were sacrificed and tissue harvested. All flow cytometry data was analyzed using FlowJo
132 10.0 (BD, Ashland, OR, USA).
133
134 Colony Assays
135 Equal numbers of cells were suspended in Methocult (m3434, StemCell Technologies,
136 Vancouver, BC) in triplicate and plated in 35mm dishes, according to manufacturer's protocol.
137 Colonies were counted after 14 days.
138
139 Whole Transcriptome sequencing
140 Total RNA was extracted as above. RNA was quantified using Qubit 2.0 Fluorometer (Thermo
141 Scientific) and integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo
142 Alto, CA, USA). Library preparation and sequencing was carried out by Azenta Life Sciences
143 (Burlington, MA, USA). Further details about analysis provided in (Supplementary Methods).
144
145 Single cell RNA sequencing

146 Leukemia cells were enriched using the MagniSort Mouse Hematopoietic Lineage Depletion kit
147 (Invitrogen) then single cell RNA sequencing (scRNA-Seq) was performed using the Seq-well,
148 nanowell-based high throughput platform, as described previously (29, 30). Details provided in
149 the (Supplementary Methods).
150
151 Statistical Analysis
152 Statistical significance was determined using either Student's T-Test or ANOVA, as appropriate
153 (GraphPad Prism, version 10, Dotmatics, Boston, MA, USA) with p-values ≤ 0.05 deemed
154 significant. Sample size given in the figure legends.
155
156 **Results**
157 **Knockdown of *Cbfb::MYH11* (*CM*) decreases leukemic burden in the spleen and peripheral**
158 **blood, but not the bone marrow**
159 To test the role of *Cbfb::MYH11 (CM)* in leukemia maintenance *in vitro*, we used leukemia cells
160 from *Cbfb*$^{+/56M}$, *Mx1-Cre*$^{+}$ mice which conditionally express *CM* from the endogenous *Cbfb*
161 promoter and develop leukemia that faithfully recapitulates human inv(16) AML (18). We collected
162 and transduced these leukemia cells with a doxycycline (Dox) inducible shRNA against *MYH11*
163 (*shMYH11*) which also constitutively expresses GFP (27). We sorted GFP^{+} cells and cultured
164 them with or without Dox for 48 hours (hrs). As expected, we observed that Dox induced *CM* KD
165 resulted in a significant decrease of *CM* expression at both the mRNA and protein levels (Fig. 1A-
166 B). We also found that *CM* KD induced increased apoptosis and decreased colony formation (Fig.
167 1C, D). These findings indicate that *CM* is required for the growth and survival of frank leukemia
168 cells *in vitro*.
169
170 To determine the effect of decreased *CM* expression *in vivo*, we transplanted *shMYH11*
171 transduced cells into congenic mice and allowed them to engraft. Once mice showed at least 20%

172 GFP$^+$ cells in the peripheral blood (Supplementary Fig. S1A), we induced *CM* KD and determined
173 the percentage of GFP$^+$ leukemia cells after 4, 7, 14, 21, and 28 days. We observed that *CM* KD
174 induced a significant decrease in GFP$^+$ leukemia cells in the peripheral blood and spleen starting
175 at day 7, with near complete elimination by day 14 (Fig. 2A, B). In addition, we observed a notable
176 decrease in spleen size with *CM* KD (Supplementary Fig. S1B, C). In the bone marrow, there
177 was a significant decrease in leukemia cells starting at day 7 of Dox treatment. However, leukemia
178 cells continued to make up 20-30% of the bone marrow through day 28 (Fig. 2C). To determine if
179 the persistence of leukemia cells in the bone marrow was due to a failure of Dox to penetrate the
180 tissue, we sorted GFP$^+$ leukemia cells from the bone marrow of control and Dox treated mice at
181 days 7 and 21. Quantitative real time PCR (qRT-PCR) and western blot analysis of bone marrow
182 GFP$^+$ cells showed significant *CM* KD at both time points (Fig. 2D-F).
183
184 To confirm that the loss of leukemia cells in the Dox treated mice is due to decreased *CM*
185 expression and not the effect of Dox, we used *CM* expressing leukemia cells transduced with a
186 non-targeted shRNA construct. We observed no difference in the percentage of GFP$^+$ leukemia
187 cells between Dox and control treated mice (Supplementary Fig. S1D, E). These findings indicate
188 that cells with reduced *CM* can survive in bone marrow, but not the peripheral blood or spleen.
189
190 **Deletion of *Cbfb::MYH11* impairs the growth and survival, but does not eliminate colony**
191 **formation, of leukemia cells *in vitro***
192 To validate our finding that a population of leukemia cells can survive without the fusion gene, we
193 generated a new knock-in mouse model that allows deletion of the *Cbfb::MYH11* fusion gene by
194 Cre Recombinase. We used homologous recombination to knock-in the *Cbfb exon5::MYH11*
195 fusion flanked by *LoxP* sites into the endogenous *Cbfb* locus (*Cbfb*$^{+/flMYH11}$) (Fig. 3A). We identified
196 embryonic stem cell (ES) clones with the knock-in allele by southern blot and confirmed
197 CBFβ::SMMHC expression by western blot (Supplementary Fig. S2A, B). Three independent

198 *Cbfb*$^{+/floxMYH11}$ ES clones were used to generate chimeric mice. To test that the *Cbfb*^{flMYH11} allele
199 can be fully excised, chimeric mice were crossed with mice expressing *Cre Recombinase* from
200 the *Actin B* promoter (*ActB Cre*), which should allow for excision of the fusion gene in all cells of
201 an embryo. We harvested embryos at day 12.5 of development. In *Cbfb*$^{+/flMYH11}$ embryos lacking
202 the *ActB-Cre* transgene, we observed central nervous system hemorrhaging, indicating
203 expression of *Cbfb::MYH11*, as in previous knock-in models (Fig. 3B) (19). Importantly, this
204 phenotype was not observed in *Cbfb*$^{+/flMYH11}$; *ActB Cre*$^{+}$, indicating excision of the fusion gene in
205 the presence of Cre Recombinase. PCR using primers specific for the unexcised and excised
206 alleles confirmed that the *Cbfb*^{flMYH11} was efficiently excised in *Cbfb*$^{+/flMYH11}$; *ActB-Cre*$^{+}$ mice. To
207 generate leukemia cells expressing the *Cbfb*^{flMYH11} allele, chimeric mice were treated with N-ethyl-
208 N-nitrosourea and monitored for disease onset. Once leukemia was detected, cells were collected
209 and transplanted into congenic recipient mice and monitored for survival. Recipient mice
210 transplanted with leukemia cells from 3 different ES cell clones developed disease with a similar
211 immunophenotype and histological appearance as previous *CM* knock-in mouse models (Fig. 3C,
212 D; Supplementary Fig. S2C, D) (18, 31). These results indicate that *Cbfb*$^{+/flMYH11}$ mice develop a
213 frank leukemia with a similar phenotype to previous models and that the fusion gene can be
214 deleted by Cre Recombinase.
215
216 To test the effect of *CM* deletion *in vitro*, we transduced *Cbfb*$^{+/flMYH11}$ leukemia cells with a lentivirus
217 expressing *Cre* and GFP from an IRES (Fig. 4A). Transduced cells were sorted, and *CM* excision
218 was confirmed by qPCR and western blot (Fig. 4B-C). We found that *CM* deletion caused a
219 statistically significant increase in Annexin V, indicating induction of apoptosis (Fig. 4D). To test
220 the effect of *CM* deletion on colony forming ability, we plated equal numbers of control and *Cre*
221 transduced *Cbfb*$^{+/flMYH11}$ leukemia cells in methylcellulose. After 14 days, colonies were counted,
222 resuspended as single cells, and equal numbers of cells replated in methylcellulose**.** At both the
223 1st and 2nd plating, we found that *Cbfb*$^{+/flMYH11}$ cells transduced with *Cre* generated significantly

224 fewer colonies (Fig. 4E). To determine if the colonies that were able to grow from *Cre*-infected
225 cells had successfully deleted *CM*, individual colonies were picked and qPCR for the *Cbfb*^{flMYH11}
226 allele was performed. We found that most colonies retained the unexcised *CM* allele. However,
227 5-20% of the colonies showed successful deletion of *CM* (Supplementary Fig. S3A, B). These
228 findings are consistent with our *in vivo* knockdown data in which the majority of leukemia cells
229 require continued expression of the fusion gene after leukemia initiation, but that there is a
230 population of cells that are able to survive without *CM.*
231
232 **KD of *CM* alters apoptosis and proliferation**
233 To investigate potential mechanisms for the persistence of *CM* KD cells in the bone marrow, we
234 analyzed apoptosis and proliferation in the leukemic cells of Dox and control treated mice at Day
235 7 and Day 21. At Day 7, we found that Dox, but not control, treated mice had a significant increase
236 in apoptosis in the spleen, but not the bone marrow (Fig. 5A left). However, at day 21, a time point
237 in which control mice have high leukemic burden, we found high levels of apoptotic leukemia cells
238 in the bone marrow of control treated mice, but not Dox treated mice (Fig. 5A right). To determine
239 if the failure to induce apoptosis in the bone marrow with *CM* KD is related to the levels of anti-
240 apoptotic proteins, we sorted leukemia cells from the spleen and bone marrow of control and Dox
241 treated mice on day 7 and performed western blots. We found that KD of *CM* induced decreased
242 BCL-2 in the spleen but caused no change in MCL-1. In leukemia cells from the bone marrow of
243 both control and Dox treated mice, BCL-2 was barely detectable. In contrast, MCL-1 was higher
244 in leukemia cells from the bone marrow of both control and Dox treated mice, as compared to
245 cells from the spleen (Fig. 5B, C). These findings imply that MCL-1, but not BCL-2, may contribute
246 to the survival of *CM* KD cells in the bone marrow.
247
248 To determine if a difference in proliferation could also contribute to the persistence of *CM* KD cells
249 in the bone marrow, we used BrdU incorporation. Leukemia cells from both the spleen and bone

250 marrow of day 21 Dox treated mice had significantly lower proliferation compared to controls (Fig.
251 5D). To test if this decrease was due to a change in differentiation of the leukemia cells, we
252 examined markers of mature myeloid cells, Mac-1 and Gr-1. In the spleen of Dox treated mice,
253 we observed an increase in Mac1$^+$, GR-1$^+$ leukemia cells, consistent with differentiation. In
254 contrast, there was no difference in the presence of Mac1 or GR-1 on the bone marrow leukemia
255 cells (Fig. 5E). To test whether bone marrow *CM* KD cells were instead enriched for leukemia
256 stem cell (LSC) markers, we examined expression of KIT, CD59a, IL1RL1, and CD123. Work by
257 us and others previously showed that KIT, CD59a and IL1RL1 are enriched on the LSC population
258 driven by *CM* expression, and CD123 is an established LSC marker in human AML (25, 32, 33).
259 We observed a small yet statistically significant increase in CD123 and decrease in IL1RL1 in the
260 leukemia cells in the bone marrow of Dox treated mice (Fig. 5F). These results indicate that the
261 decreased proliferation observed in the bone marrow *CM* KD cells is not caused by differentiation
262 to a post-mitotic mature myeloid cell or enrichment for quiescent LSCs.
263

264 **KD of *CM* is associated with distinct changes in gene expression in the spleen and bone**
265 **marrow**

266 To investigate the transcriptomic differences associated with the survival of *CM* KD leukemic cells
267 in the bone marrow, but not the spleen, we performed bulk RNA sequencing (RNA-Seq) on GFP$^+$
268 leukemia cells from both populations on day 7 of control or Dox treated mice. We identified a total
269 of 348 differentially expressed genes (DEGs); (221 upregulated and 127 downregulated) in
270 leukemia cells from the spleen of Dox treated animals, as compared to control. In leukemia cells
271 from the bone marrow, we identified a total of 4650 DEGs (2328 upregulated and 2322
272 downregulated) in cells from Dox treated mice, as compared with control. (Supplementary Table
273 2). Hierarchical clustering shows the bone marrow replicates from control and Dox treated mice
274 have similar DEG patterns (Supplementary Fig. S4A). While spleen control replicates cluster
275 together, one Dox treated sample clustered more closely with the control spleen samples,

276 suggesting an intermediate level of transcriptional changes, perhaps related to inefficient KD of
277 the fusion protein (Supplementary Fig. S4B, C). In the bone marrow, several known targets of the
278 CM fusion protein, including *Cebpe, Cebpd, Runx1 and Il1rl1*, are downregulated with *CM* KD. In
279 addition, genes associated with an abnormal myeloid progenitor (AMP) phenotype, including
280 *Rhag, Cldn13, Add2, Gata2* were found to be upregulated (32). Overall, these changes in genes
281 expression are consistent with loss of CM activity but imply that *CM* KD cells retain transcriptional
282 changes associated with leukemic blasts (Fig. 6A).
283
284 To identify biological functions that promote survival of bone marrow *CM* KD cells, we compared
285 DEGs in spleen and bone marrow leukemia cells and performed pathway analysis on only those
286 genes altered in the bone marrow. Of the 2162 unique DEGs upregulated in bone marrow
287 leukemia cells we identified autophagy, a known promoter of AML survival, as well as mTOR
288 signaling, AMPK signaling, and mitophagy which have been implicated in promoting autophagy,
289 survival, and stress adaptation in AML (Fig 6C) (34-38). Analysis of individual autophagy related
290 genes showed significantly increased expression of *Atf6*, *Atg14*, *Atg2A*, *Becn1* and *mTor* (Fig
291 6D). These results imply a role for autophagy in the survival of *CM* KD cells in the bone marrow.
292 Pathway analysis of leukemia cells from the spleen indicated that the top deregulated pathway
293 with KD of *CM* is apoptosis, consistent with the Annexin V staining and western blot analysis in
294 Figure 5 (Supplementary Fig. 4C).
295
296 To determine if *CM* KD induces distinct gene expression changes in different subpopulations of
297 leukemia cells, we performed single-cell RNA sequencing (scRNA-Seq). We harvested cells from
298 the bone marrow and spleen of control and dox treated mice at day 7 of treatment. Single cell
299 suspensions were lineage depleted and analyzed by flow cytometry to confirm enrichment of
300 leukemia cells. All samples showed >60% leukemia cells, with the bone marrow approaching 80%

301 in both control and Dox treated samples (Supplementary Fig. 5A). Single-cell transcript libraries
302 were prepared and sequenced.
303
304 Unbiased clustering analysis revealed multiple subpopulations of leukemia cells in both the spleen
305 and bone marrow. Interestingly, *CM* KD affected the relative proportion of these clusters, with the
306 number of cells increased in some clusters while others decreased in samples from Dox treated
307 mice (Fig 7A, B). In the spleen, Clusters 2 and 4 appeared to be the most sensitive to *CM* KD
308 (Supplementary Fig. 5B,C). In the bone marrow, Clusters 3, 4, 5, 6, 9 and 10 showed marked
309 reduction with *CM* KD while the number of cells in Clusters 0, 1, 2, 7 and 8 increased, indicating
310 these clusters don't require *CM* expression for survival (Fig. 7B). This analysis shows that there
311 is dramatic cellular heterogeneity among the leukemia cells in both the bone marrow and spleen,
312 with different subpopulations differentially dependent on *CM* expression.
313
314 To test if there are inherent transcriptional programs promoting the survival of the *CM*-
315 independent cells in the bone marrow, we compared the transcripts expressed in the clusters that
316 don't require CM expression. We identified 456 genes expressed in all *CM*-independent clusters
317 (Fig 7C). Among these, several genes including *Ccl5, Ccr1, S100a4, CD74, CD27, Ccnd2* and
318 *Cdkn1a* are all associated with leukemia survival and proliferation (39-43). Consistent with this
319 finding, pathway analysis indicates that these genes are significantly enriched for molecular
320 functions involving cytokine and chemokine activity (Fig. 7D). In contrast, the common genes
321 expressed in *CM*-dependent clusters were associated with non-immune molecular functions
322 (Supplementary Fig. 5D). Collectively these results imply that cytokine and chemokine signaling
323 within the bone marrow microenvironment may be a critical mechanism promoting survival of *CM*-
324 independent leukemia cells.
325
326 ***CM* KD leukemia cells can re-establish disease**

327 The persistence of a *CM* KD leukemic population in the bone marrow raises the possibility that
328 these cells may be able to cause relapsed disease. To test this possibility, we maintained mice
329 transplanted with *shMYH11* transduced leukemia cells on Dox or control drinking water until they
330 showed signs of lethal disease. 11 out of 18 Dox treated mice showed re-emergence of GFP^+
331 cells in the blood (Fig. 8A). To determine if these cells expressed *CM*, we sorted for GFP^+ cells
332 and performed qRT-PCR and western blot (Fig. 8 B-D). We found that GFP^+ relapse cells had
333 reduced *CM* at both the mRNA and protein levels, as compared to parental leukemia cells from
334 control treated mice. In addition, we found that the immunophenotype of relapsed *CM* KD
335 leukemia cells was similar to parental leukemia cells from control mice (Supplementary Fig. S6A
336 and B). To understand the molecular mechanism allowing *CM* KD cells to re-emerge into the
337 blood, we performed whole transcriptome sequencing on relapse and parental leukemia cells.
338 The leukemic cells from relapsed mice were harvested from the spleen and compared with either
339 spleen or bone marrow from control mice. Principal component analysis (PCA) showed that
340 relapsed samples did not cluster with parental leukemia cells harvested from either the bone
341 marrow or spleen indicating that relapse is accompanied by widespread alterations in transcription
342 (Fig. 8E). In addition, only 3 of the 4 relapsed samples clustered together, implying that there may
343 be multiple transcriptional programs that promote re-emergence of *CM* KD cells from the bone
344 marrow. Comparing the transcriptome of relapsed samples to control, we observed a total of 930
345 DEGs (359 upregulated and 571 downregulated) in relapsed samples compared to parental
346 leukemia cells (Supplementary Table 3). Further, pathway analysis indicates that the DEGs are
347 associated with multiple pathways implicated in leukemia survival and relapse, including
348 interferon regulator factor (IRF) signaling (Fig. 8F). Specifically, *Irf1*, *Irf7*, and *Irf9* are decreased
349 in relapse samples compared to controls (Fig. 8G). Notably, loss of these genes is associated
350 with relapse in AML patients (46-48). This implies that suppression of IRF signaling allows *CM*
351 KD leukemia cells to survive outside the bone marrow.
352

353 Collectively, this study indicates that while *CM* is required for the survival of most frank leukemia
354 cells, a population of leukemia cells in the bone marrow do not strictly require *CM* activity and can
355 re-establish fatal disease.
356
357 **Discussion**
358 Given that CBFβ::SMMHC is expressed in all leukemia cells of inv(16) AML patients, but not in
359 healthy cells it is a tempting target for drug development. However, most studies have focused
360 on the role of the fusion protein during leukemia initiation and less is known about its role in frank
361 leukemia cells in the context of cooperating mutations. Previous work in the inv(16) immortalized
362 cell line, ME-1, reported that decreased expression of *CM* caused changes in gene expression
363 consistent with differentiation, but did not cause any changes in viability at the time point analyzed,
364 raising the possibility that continued *CM* expression may not be strictly required for leukemia
365 maintenance (27). To our knowledge, ours is the first study to test whether *CM* activity is required
366 for leukemia cell survival using non-immortalized leukemia cells. We found that KD of *CM* induced
367 apoptosis and decreased colony forming ability *in vitro*. *CM* KD *in vivo* caused a near complete
368 elimination of leukemia cells in the blood and spleen. Surprisingly, the leukemic burden in the
369 bone marrow did not decrease below 20-30% despite almost complete loss of the CBFβ::SMMHC
370 protein, implying that the bone marrow microenvironment can sustain leukemia cells despite the
371 loss of *CM* expression.
372
373 Our observation that bone marrow *CM* KD cells , but not spleen, had decreased apoptosis as
374 compared to controls suggests tissue specific effects on the survival of leukemic cells *in vivo*.
375 Thus, to identify potential factors promoting the survival of bone marrow *CM* KD cells, we analyzed
376 the anti-apoptotic proteins BCL-2 and MCL-1 (44, 45). However, we observed that both control
377 and *CM* KD leukemic cells had robust expression of MCL-1 but nearly undetectable levels of BCL-
378 2, indicating that differential expression of anti-apoptotic proteins does not explain their survival.

379 Rather, our bulk RNA-seq indicates that the upregulation of autophagy may contribute to the
380 survival of bone marrow *CM* KD cells. These findings are consistent with recent work showing a
381 role for autophagy in leukemia progression and that overexpression of the autophagy genes
382 *BECLIN1* and *ATG* are associated with shorter overall survival in AML patients (46, 47).
383
384 To understand the cellular heterogeneity in *CM* sufficient and KD cells, we performed scRNA-
385 Seq. We found that there are clusters of leukemia cells with distinct transcriptional profiles and
386 different requirements for *CM* expression. Some clusters had decreased cellularity while others
387 were increased, implying the presence of *CM*-dependent and *CM*-independent leukemic
388 populations in the bone marrow. This is consistent with our *in vivo* results where we observed an
389 initial decrease in bone marrow leukemic burden of dox treated mice, but eventual stabilization of
390 the leukemia population at 20-30%. Interestingly, the population of *CM*-independent leukemia
391 cells did not continue to increase over time, implying that there is a maximum number of *CM*-
392 independent leukemia cells that the bone marrow can support. Of note, the *CM*-independent
393 clusters in the bone marrow shared a transcriptional profile associated with cytokine and
394 chemokine activity, which is consistent with a model in which the bone marrow microenvironment
395 provides a limited amount of survival factors, keeping the *CM*-independent population from
396 expanding indefinitely in the bone marrow.
397
398 Our results concerning the role of CBFβ::SMMHC during leukemia maintenance are strikingly
399 similar to what has been demonstrated with the BCR::ABL fusion protein in CML. Pharmacological
400 inhibitors of BCR::ABL, such as imatinib, are remarkably successful at inducing long-term, stable
401 cytogenetic remission in CML (48, 49). However, these kinase inhibitors generally do not eliminate
402 the LSC population in the bone marrow. Further, transgenic models have demonstrated that with
403 near complete knockdown of *BCR::ABL* expression, LSCs remain in the bone marrow and are
404 able to re-establish disease when BCR::ABL activity is restored (50, 51). In our model, we also

405 observed a similar population of LSC-like cells that can survive in the bone marrow and re-
406 establish disease, despite near complete loss of the CBFβ::SMMHC protein. In our case, relapse
407 did not require re-expression of the fusion protein. However, given the period of latency before
408 relapse, it is likely that additional mutations developed to compensate for the lack of
409 CBFβ::SMMHC activity.
410
411 Collectively, this study demonstrates that *CM* expression continues to be required after leukemic
412 transformation and supports the development of inhibitors targeting the fusion protein for the
413 treatment of inv(16) AML. Our data indicates such inhibitors would be effective at managing
414 leukemic burden and, when used in combination with other therapeutics, have the potential to
415 cure the disease.
416
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430

431 **Author Contributions**

432 S.P. designed and performed experiments, analyzed data and wrote the paper. Y.W. designed
433 and performed experiments and analyzed data. M.B., C.L., A.D., and S.A.S. performed
434 experiments. R.D. and P.W analyzed single cell and bulk RNA-Seq data, respectively. C.R., L.A.,
435 and L.G. generated, bred, and mutagenized *Cbfb*$^{+/floxMYH11}$ knock-in mice. S.A.S., K.J.H. and R.K.H
436 designed experiments, analyzed data and wrote the paper.

437

438 **Disclosure of Conflicts of Interest**

439 The authors have no conflicts of interest to disclose.

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635 **Figure Legends**

636

637 **Figure 1: Inducible knockdown of *CM* causes apoptosis and impairs colony formation *in***
638 ***vitro*. A. Bar** graph showing quantitative reverse transcription PCR (qRT-PCR) of *CBFB::MYH11*
639 in *Cbfb*$^{+/56M}$ leukemia cells transduced with an inducible shRNA against *MYH11* (shMYH11)
640 treated *in vitro* with either control (CTRL) or doxycycline (Dox) for 48 hrs **B.** Representative
641 western blot (left) and bar graph of relative CBFβ::SMMHC protein levels (right) in *shMYH11*
642 transduced leukemia cells treated as in A. **C**. Bar graph showing the percentage (%) of *shMYH11*
643 transduced leukemia cells treated with control or Dox that are positive for Annexin V. **D.** Bar graph
644 showing relative colony numbers generated by cells treated as in A. N ≥ 3 biological and technical
645 replicates, * = p ≤ 0.05 compared to CTRL.

646

647 **Figure 2: *CM* knockdown decreases leukemic burden *in vivo.* A.** Line graph showing % GFP^{+}
648 *shMYH11* transduced leukemia cells (left) and representative flow cytometry plots (right) in the
649 peripheral blood (PB), **B.** spleen (SP) and **C.** bone marrow (BM) of mice treated with Dox or CTRL
650 for 7, 14, 21, or 28 days. **D.** Bar graph showing relative expression of *CM* in sorted GFP^{+} leukemia
651 cells from the BM of mice treated with Dox or CTRL for 7 and **E.** 21 days. **F.** Representative
652 western blot (left) and bar graph (right) of relative CBFβ::SMMHC protein levels in sorted GFP^{+}
653 cells from the BM of mice treated with either CTRL or Dox for 7 days. N ≥ 3 biological replicates,
654 ns = not significant, * = p ≤ 0.05.

655

656 **Figure 3: Generation of flox*Cbfb::MYH11* mice*.* A.** Schematic representation of the embryonic
657 stem (ES) cell targeting strategy. The probe (0.2C) and restriction enzyme sites (NcoI) used for
658 screening are indicated. The black arrows represent PCR primers for the excised allele, and the
659 purple arrows represent primers for the unexcised allele. **B.** Pictures of representative embryos
660 harvested at embryonic day 12.5 generated by crossing *Cbfb*$^{+/floxMYH11}$ chimeras with transgenic

661 mice expressing *Cre recombinase* (Cre) from the *β–Actin* promoter (*ActB Cre*). The right panel
662 shows a representative PCR for the excised and unexcised *Cbfb*$^{+/floxMYH11}$ allele in DNA from
663 embryos of the indicated genotype. **C.** Kaplan-Meyer survival curve of recipient mice transplanted
664 with *Cbfb*$^{+/flMYH11}$ leukemic cells from three different chimeric donor mice. **D.** Representative
665 Wright-Giemsa-stained peripheral blood smear and hematoxylin & eosin-stained spleen and liver
666 section from a leukemic *Cbfb*$^{+/flMYH11}$ recipient mouse.
667
668 **Figure 4. Deletion of *Cbfb::MYH11* induces apoptosis and reduces colony formation *in***
669 ***vitro* A.** Schematic representation of the experimental design to test the effect of *Cbfb::MYH11*
670 deletion *in vitro*. **B.** Bar graph of qRT-PCR of the unexcised or excised *Cbfb*$^{+/floxMYH11}$ alleles in
671 leukemia cells transduced with a lentivirus expressing Cre Recombinase (Cre) or Control (CTRL)
672 and sorted for GFP. **C.** Representative western blot of CBFβ::SMMHC in *Cbfb*$^{+/flMYH11}$ leukemia
673 cells infected with CTRL or Cre virus. **D.** Bar graph of % Annexin V in CTRL or Cre transduced
674 *Cbfb*^{flMYH11} leukemia cells. **E.** Bar graph showing the relative number of colonies from equal
675 numbers of CTRL or Cre transduced *Cbfb*$^{+/flMYH11}$ leukemia cells. Colonies were scored on day
676 14. N = 3 biological and technical replicates, * = $p \leq 0.05$, *** = $p \leq 0.001$ as compared to CTRL.
677
678 **Figure 5: *CM* Knockdown induces apoptosis in leukemia cells in the spleen, but not the**
679 **bone marrow. A.** Bar graph showing the % Annexin V^{+}, GFP^{+} cells from the SP and BM of mice
680 treated with CTRL or Dox for 7 (left) or 21 days (right). **B.** Representative western blot and **C.** Bar
681 graph of quantification for the indicated proteins from leukemia cells sorted for GFP from the SP
682 and BM after 7 days of treatment with CTRL or Dox **D.** Bar graph of the % BrdU^{+}, GFP^{+} leukemia
683 cells harvested from the SP and BM of mice after 21 days of treatment with CTRL or Dox. **E.** Bar
684 graph showing % GFP^{+} cells expressing the mature myeloid markers Mac-1 and Gr-1 from the
685 SP and BM of mice treated as in D. **F.** Bar graph showing the % GFP^{+} cells expressing the

686 indicated leukemia stem cell markers in the BM from of mice treated as in D. N ≥ 3 biological
687 replicates, ns = not significant, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001 as compared to CTRL.
688
689 **Figure 6: Increased expression of autophagy related genes in *CM* knockdown leukemia**
690 **cells from the bone marrow. A.** Volcano plot showing distribution of the statistically significant
691 differentially expressed genes (DEGs) in the GFP^+ sorted leukemia cells from the BM of mice
692 treated for 7 days with CTRL or Dox. A few of the key genes with a log_2 fold change ≥ 0.5 and
693 adjusted p value ≤ 0.05 are highlighted. **B**. Venn diagram showing overlap of significantly
694 upregulated DEGs in *CM* KD leukemia cells from the SP and BM, as compared to CTRL. **C.**
695 Pathway analysis of upregulated DEGs in *CM* KD cells from the BM showing the top KEGG
696 pathways affected. **D.** Box plot depicting relative expression of indicated autophagy associated
697 genes in control and *CM* KD leukemia cells from the BM. N = 3 biological replicates, * = padj ≤
698 0.05 as compared to CTRL.
699
700 **Figure 7: Single cell transcriptomics reveals distinct *CM*-dependent and *CM*-independent**
701 **subpopulations in the bone marrow. A.** Umap projection of leukemia cells from the BM of CTRL
702 or Dox treated mice. Each dot represents a single cell and is colored according to the assigned
703 transcriptional cluster. **B.** Bar graph of the number of cells in each cluster in BM samples from
704 CTRL or Dox treated mice **C.** Venn diagram representing the shared genes expressed by cells in
705 the *CM* independent clusters in BM samples from CTRL mice with 456 genes common among
706 the five groups. **D.** Top 5 molecular functions associated with the 456 shared genes from C.
707
708 **Figure 8: The surviving *CM* knockdown cells in the bone marrow can give rise to relapse.**
709 **A.** Line graph showing the % GFP^+, *shMYH11* transduced cells in PB of mice treated for up to 42
710 days with either CTRL or Dox**. B.** Bar graph of relative *CM* expression in GFP^+ sorted leukemia
711 cells from Dox treated mice after relapse. **C.** Representative western blot and **D.** Bar graph of

712 relative CBFβ::SMMHC levels from sorted GFP^+ cells harvested from CTRL or Dox treated mice.
713 Quantification to GFP. **E.** Principal Component Analysis (PCA) plots of whole transcriptome
714 sequencing data from GFP sorted relapse or CTRL leukemic cells harvested from the SP or BM.
715 **F.** Pathway analysis of the differentially expressed genes in relapse samples compared to the
716 combined SP and BM CTRL leukemia samples. **G.** Bar graph depicting the *Irf* genes deregulated
717 in Dox treated mice after relapse. N ≥ 3 biological replicates, * = $p \leq 0.05$ as compared to CTRL.

Figure 1

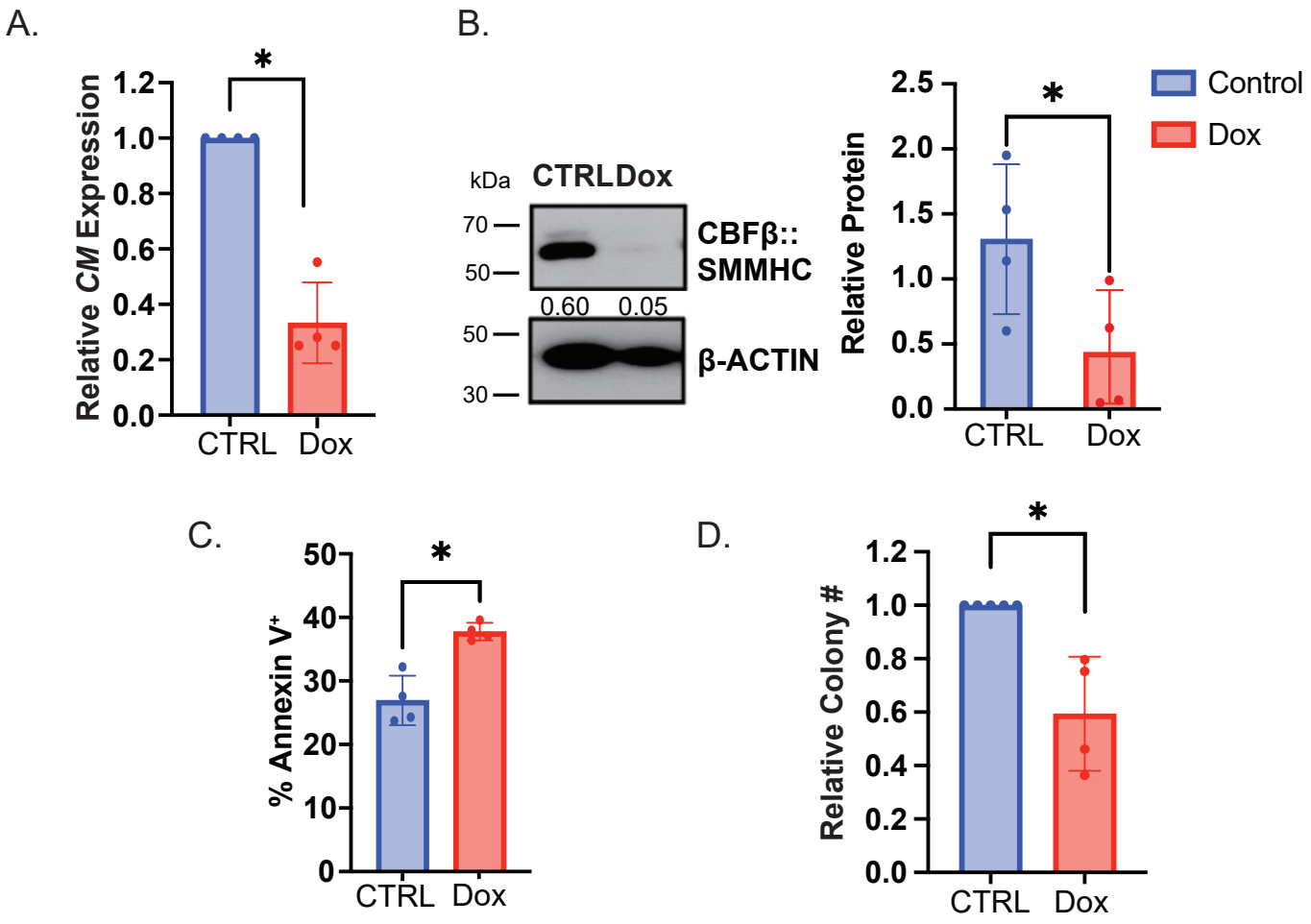

A.
Relative CM Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
CTRL
Dox
B.
kDa
CTRL
Dox
70
50
CBFβ::
SMMHC
0.60
0.05
50
β-ACTIN
30
Relative Protein
2.5
2.0
1.5
1.0
0.5
0.0
*
CTRL
Dox
Control
Dox
C.
% Annexin V+
50
40
30
20
10
0
*
CTRL
Dox
D.
Relative Colony #
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
CTRL
Dox

Figure 2

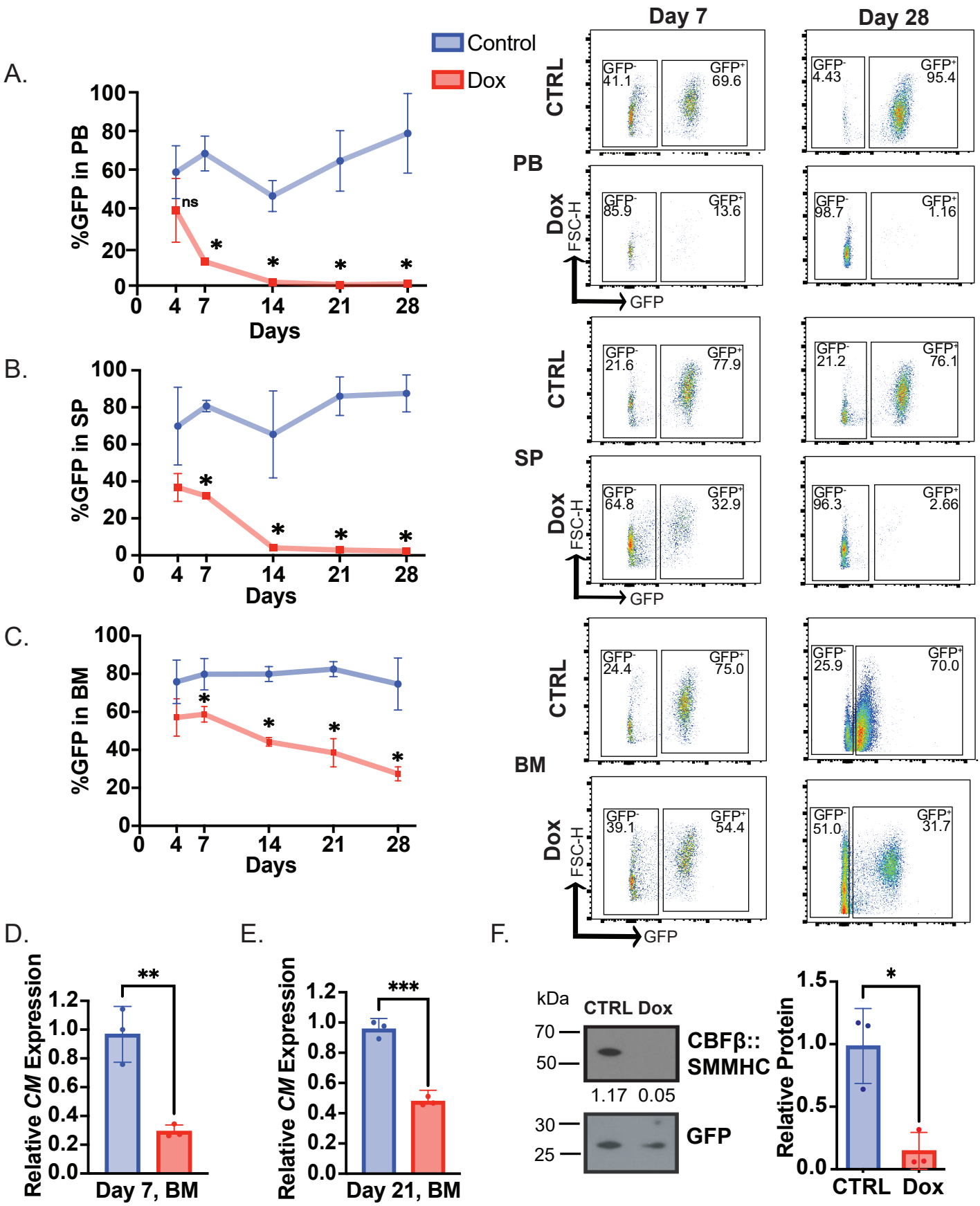
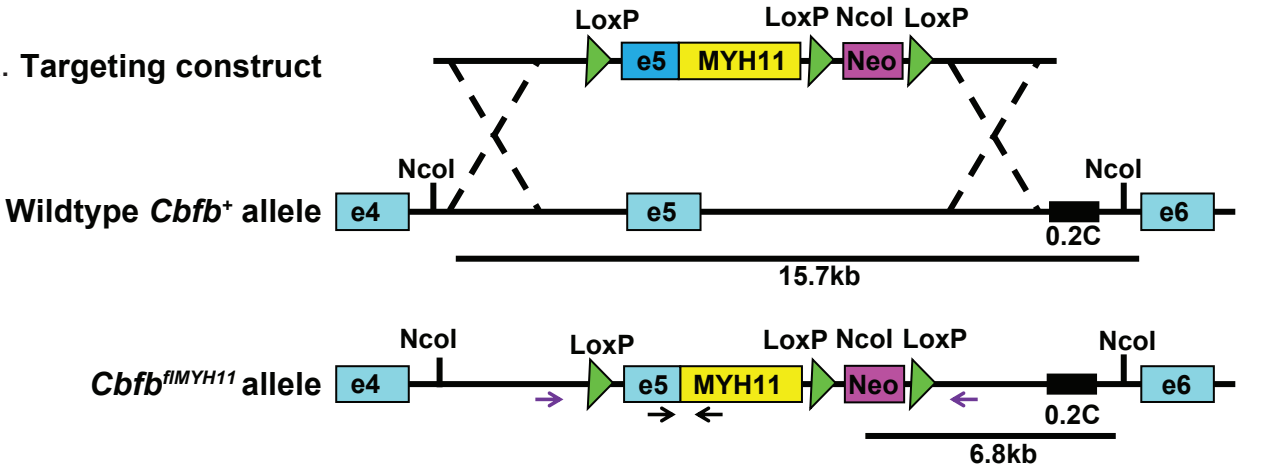
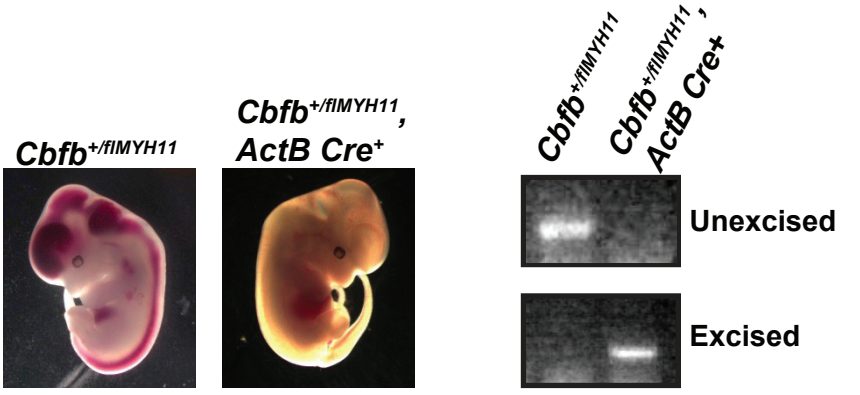

A.
B.
C.
Control
Dox
%GFP in PB
%GFP in SP
%GFP in BM
Days
ns
Day 7
Day 28
CTRL
Dox
PB
SP
BM
FSC-H
GFP
GFP⁻ 41.1
GFP⁺ 69.6
GFP⁻ 4.43
GFP⁺ 95.4
GFP⁻ 85.9
GFP⁺ 13.6
GFP⁻ 98.7
GFP⁺ 1.16
GFP⁻ 21.6
GFP⁺ 77.9
GFP⁻ 21.2
GFP⁺ 76.1
GFP⁻ 64.8
GFP⁺ 32.9
GFP⁻ 96.3
GFP⁺ 2.66
GFP⁻ 24.4
GFP⁺ 75.0
GFP⁻ 25.9
GFP⁺ 70.0
GFP⁻ 39.1
GFP⁺ 54.4
GFP⁻ 51.0
GFP⁺ 31.7
D.
E.
F.
Relative CM Expression
Day 7, BM
Day 21, BM
kDa
CTRL Dox
CBFβ::SMMHC
GFP
1.17 0.05
Relative Protein
CTRL Dox

Figure 3

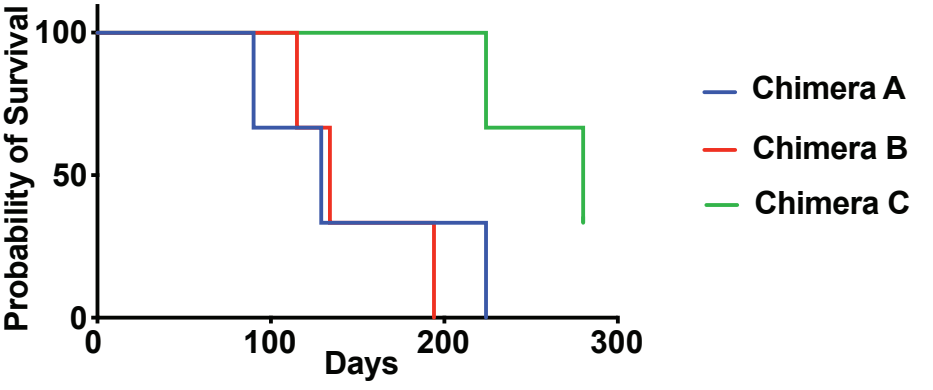
A. Targeting construct



B.



C.



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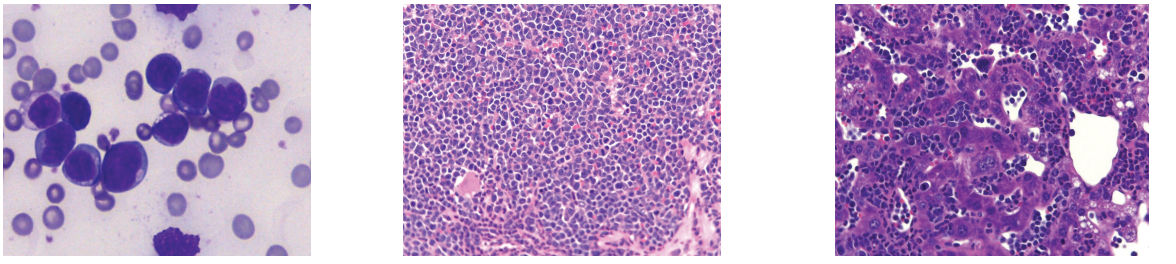
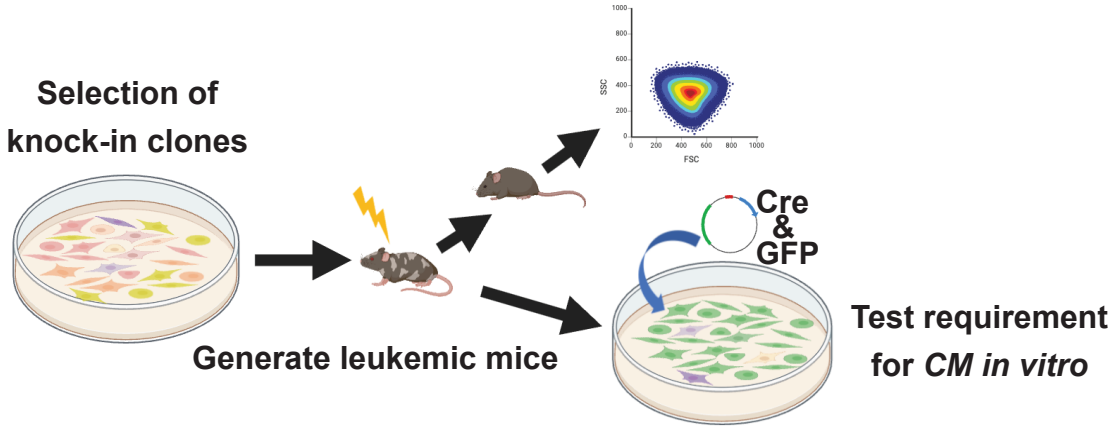


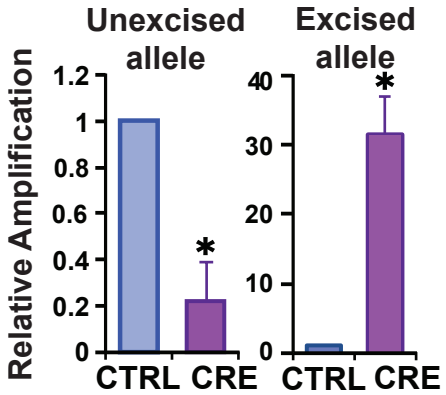
Peripheral Blood | Spleen | Liver

Figure 4

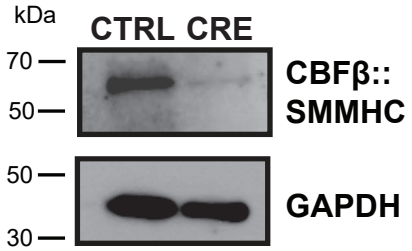
A.



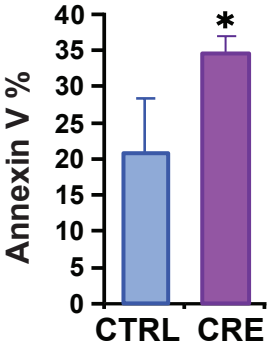
B.



C.



D.



E.

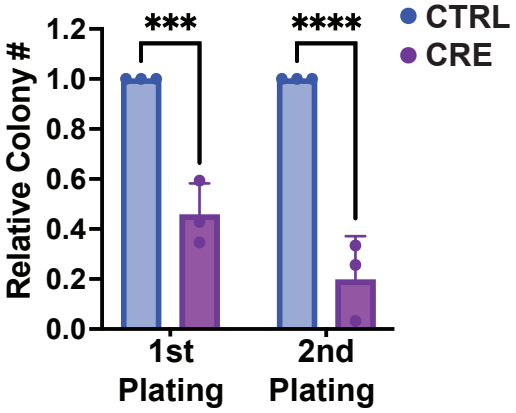


Figure 5

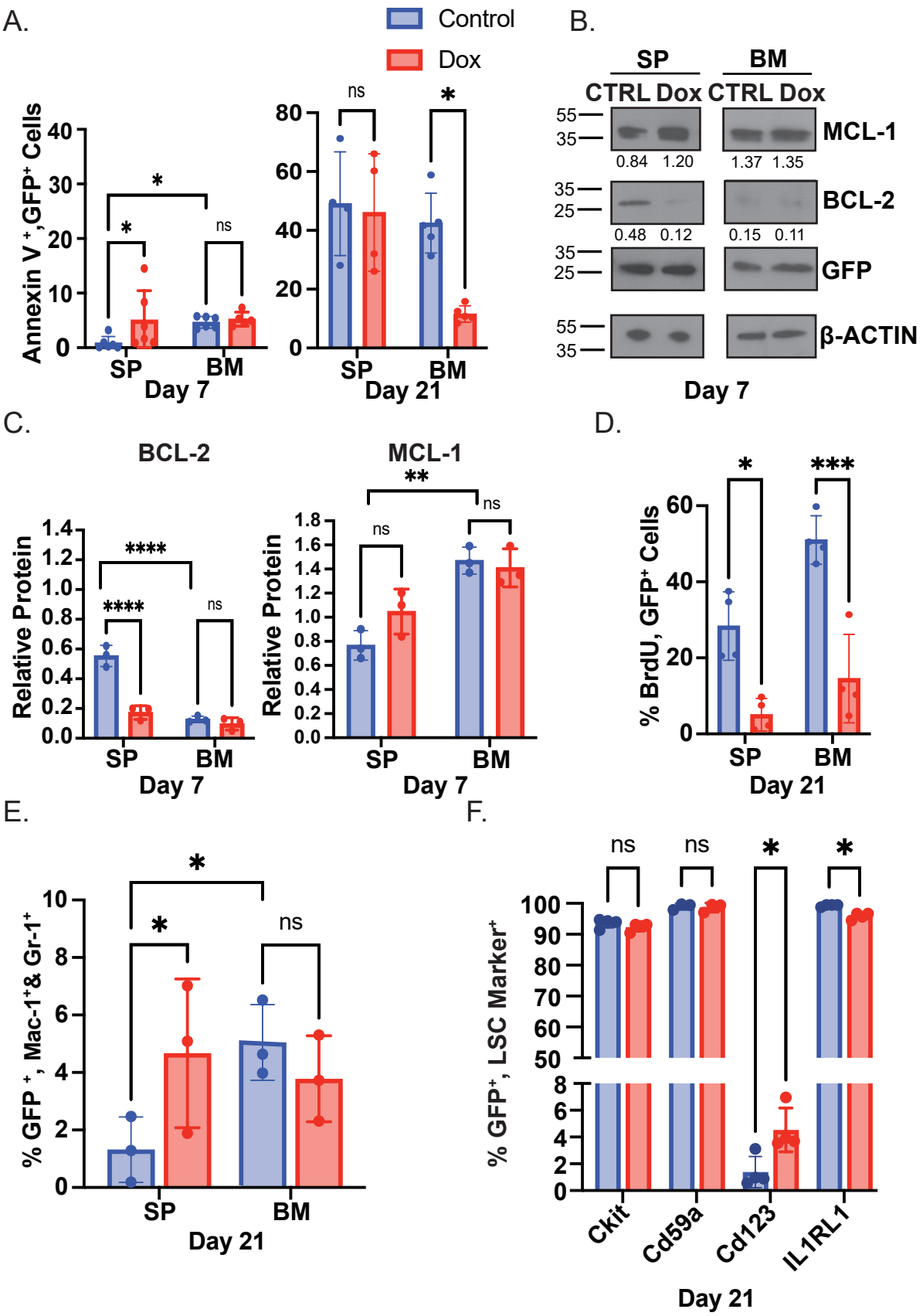

A.
Control
Dox
Annexin V+,GFP+ Cells
SP
BM
Day 7
Day 21
ns
*
B.
SP
BM
CTRL Dox CTRL Dox
MCL-1
BCL-2
GFP
β-ACTIN
0.84 1.20
1.37 1.35
0.48 0.12
0.15 0.11
Day 7
C.
BCL-2
MCL-1
Relative Protein
****
**
D.
% BrdU, GFP+ Cells
***
Day 21
E.
% GFP+, Mac-1+& Gr-1+
F.
% GFP+, LSC Marker+
Ckit
Cd59a
Cd123
IL1RL1

Figure 6

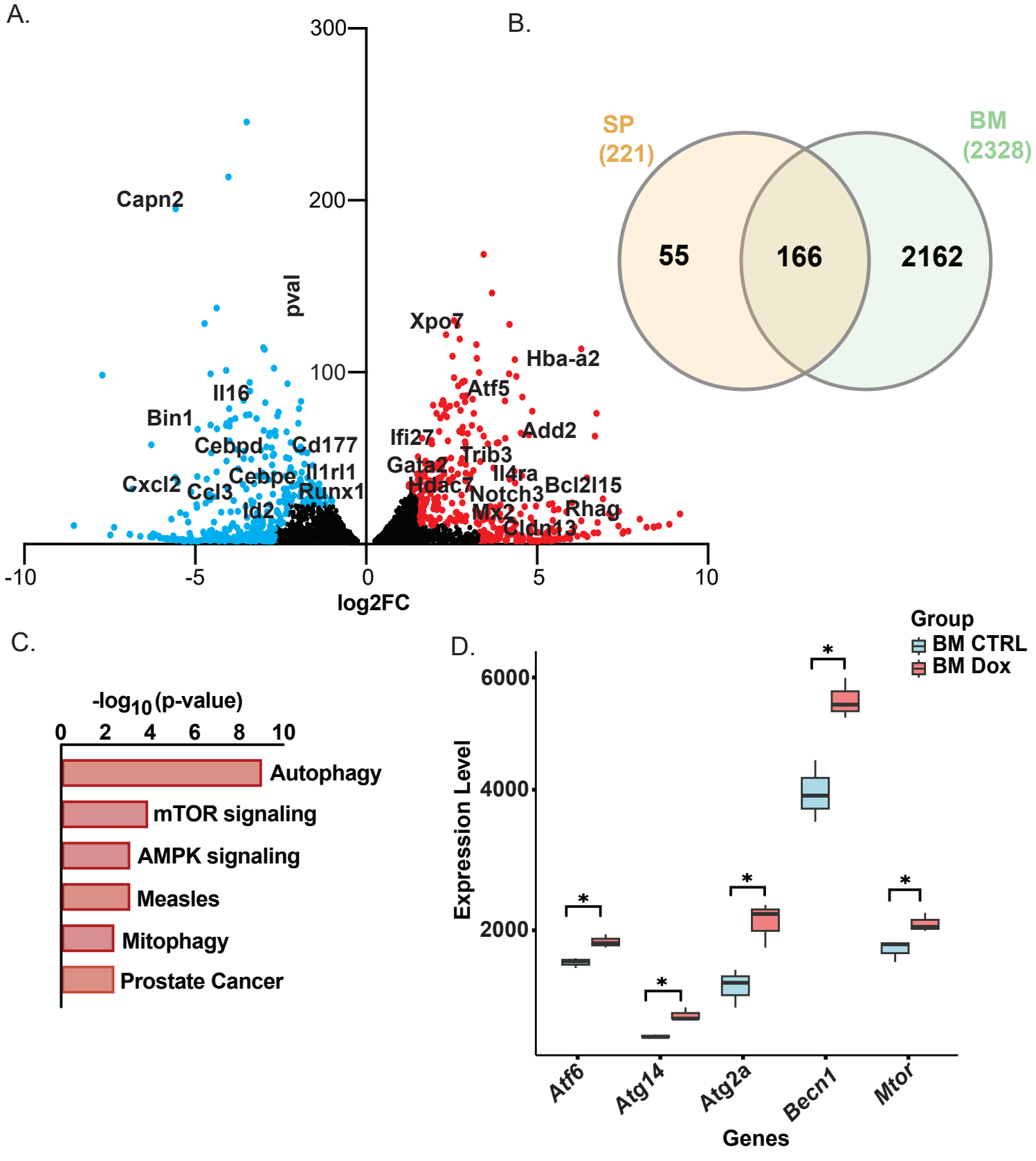
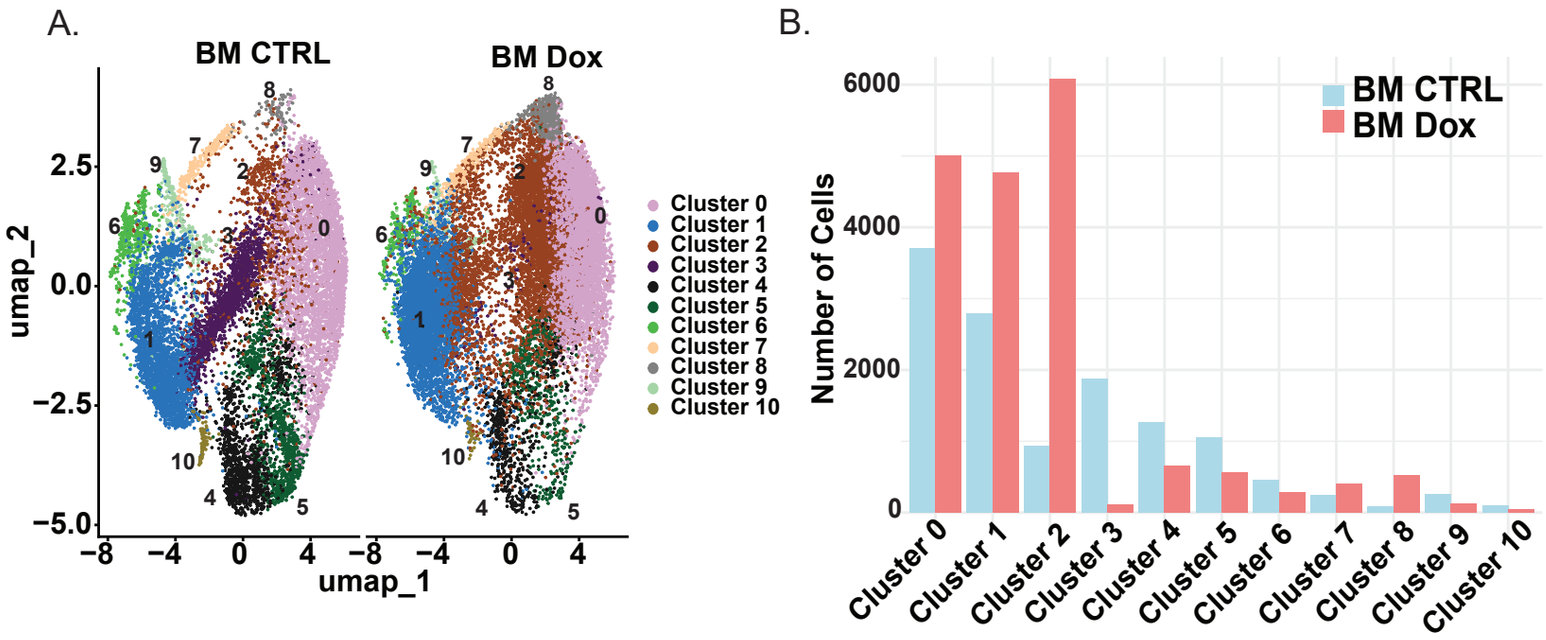
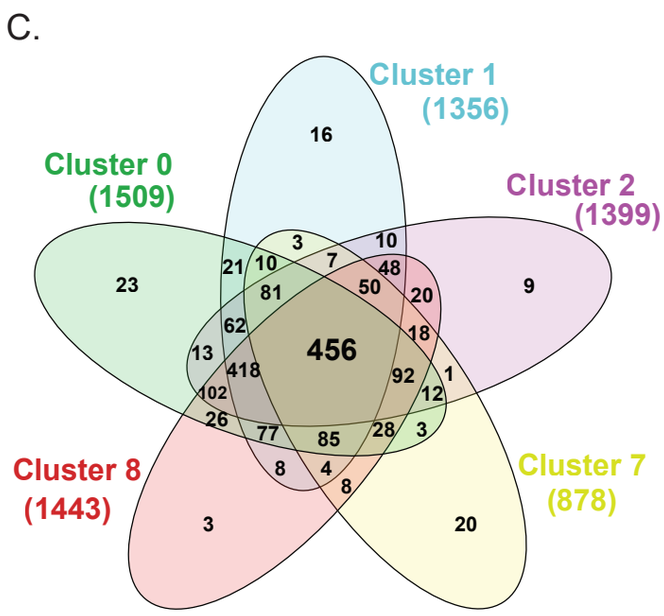

A.
300
200
100
pval
Capn2
Il16
Bin1
Cebpd
Cd177
Cxcl2
Ccl3
Cebpe
Il1rl1
Id2
Runx1
Xpo7
Hba-a2
Atf5
Ifi27
Add2
Trib3
Gata2
Il4ra
Hdac7
Notch3
Bcl2l15
Mx2
Rhag
Cldn13
-10
-5
0
5
10
log2FC
B.
SP
(221)
BM
(2328)
55
166
2162
C.
-log10(p-value)
0
2
4
6
8
10
Autophagy
mTOR signaling
AMPK signaling
Measles
Mitophagy
Prostate Cancer
D.
Group
BM CTRL
BM Dox
6000
4000
2000
Expression Level
*
Atf6
Atg14
Atg2a
Becn1
Mtor
Genes

Figure 7


A.
BM CTRL
BM Dox
umap_2
umap_1
Cluster 0
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10
B.
BM CTRL
BM Dox
Number of Cells
Cluster 0
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10


C.
Cluster 0
(1509)
Cluster 1
(1356)
Cluster 2
(1399)
Cluster 7
(878)
Cluster 8
(1443)
456

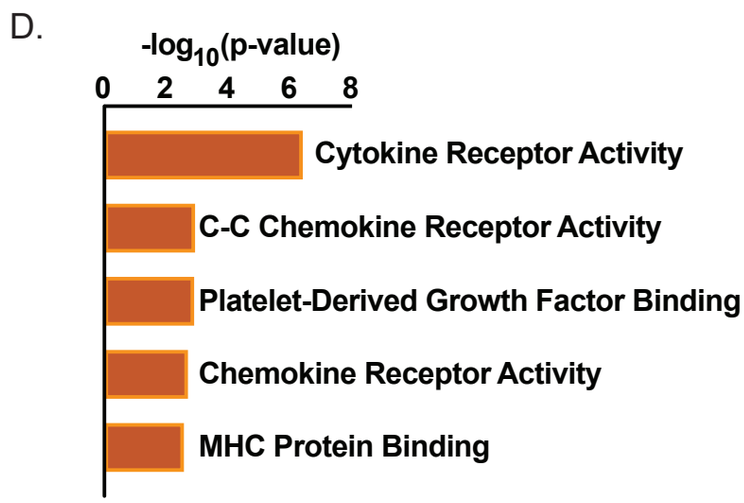
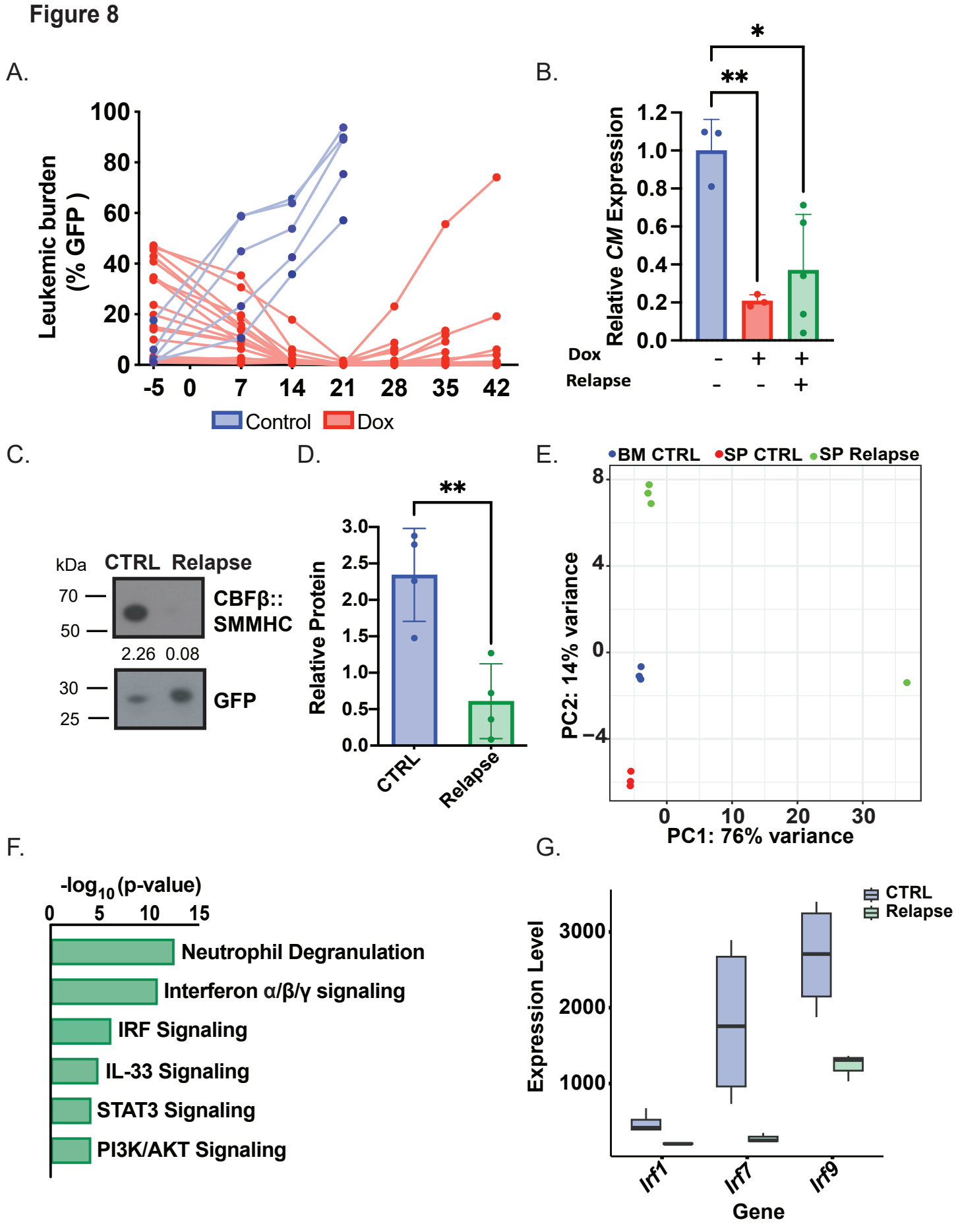

D.
-log10(p-value)
Cytokine Receptor Activity
C-C Chemokine Receptor Activity
Platelet-Derived Growth Factor Binding
Chemokine Receptor Activity
MHC Protein Binding

Figure 8


A.
Leukemic burden (% GFP)
100
80
60
40
20
0
-5
0
7
14
21
28
35
42
Control
Dox
B.
Relative CM Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Dox
Relapse
C.
kDa
CTRL
Relapse
70
50
CBFβ::SMMHC
2.26
0.08
30
25
GFP
D.
Relative Protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
CTRL
Relapse
E.
BM CTRL
SP CTRL
SP Relapse
PC2: 14% variance
8
4
0
−4
0
10
20
30
PC1: 76% variance
F.
-log10(p-value)
0
5
10
15
Neutrophil Degranulation
Interferon α/β/γ signaling
IRF Signaling
IL-33 Signaling
STAT3 Signaling
PI3K/AKT Signaling
G.
CTRL
Relapse
Expression Level
3000
2000
1000
Irf1
Irf7
Irf9
Gene