Loss of the Inv(16) Oncogene *CBFB::MYH11* Eliminates Leukemia from the Blood and Spleen, but not the Bone Marrow

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Abstract

Inversion of chromosome 16 [inv(16)] is one of the most common chromosomal rearrangements in Acute Myeloid Leukemia (AML) and generates the fusion gene *CBFB::MYH11 (CM),* which initiates leukemogenesis. Patients with inv(16) at diagnosis invariably have the rearrangement at relapse, leading to the assumption that *CM* is required after leukemic transformation. However, this has yet to be shown experimentally. Using a knock-in mouse that allows for deletion of *CM* after leukemia development, we found that loss of the fusion gene increased apoptosis and decreased colony growth *in vitro*. Interestingly, 5-20% of the colonies had successfully deleted *CM*. To test the role of *CM in vivo*, we used an inducible shRNA knockdown (KD) construct against the fusion gene. We found that decreased *CM* expression eliminated leukemia cells from the peripheral blood and spleen, but not the bone marrow, despite these cells showing significant knockdown of *CM* at the mRNA and protein levels. Furthermore, with prolonged KD of *CM*, ~40% of mice re-established disease while maintaining KD of the fusion gene. Our work indicates that *CM* is required by leukemia cells in the spleen and blood, but that cells in the bone marrow can survive and re-establish disease independent of the fusion protein.

Introduction

Acute Myeloid Leukemia (AML) is a heterogeneous clonal malignancy arising from the immature cells in the bone marrow, resulting in a more than 20% increase in immature blast cells, reduced mature blood cell production and ultimately BM failure (1-3). It develops as a neoplastic disease, often with specific chromosomal rearrangements, including the inversion of chromosome 16(inv(16))(4, 5). Inv(16)] AML accounts for ~10% of human AML, making it one of the most common chromosomal abnormalities in AML (6-10). On the molecular level, the inv(16) generates a fusion between the transcription factor gene Core Binding Factor β (*CBFB*) and the gene for Smooth Muscle Myosin Heavy Chain (*MYH11*), generating the oncogene *CBFB::MYH11 (CM),* which encodes CBFβ::SMMHC (11, 12). Expression of *CM* induces a block in myeloid differentiation and is required but not sufficient for leukemogenesis (13). Additional cooperating mutations are required for transformation to a frank leukemia (14-16). However, it is not clear if this oncogenic fusion gene continues to be important during leukemia maintenance or if the cooperating mutations are sufficient to maintain leukemia. With current frontline treatments, inv(16) AML has a favorable prognosis due to high rates of initial response; however, ~40% of patients relapse, at which point there are limited curative options (10, 17, 18). Because *CM* is the initiating mutation in this subtype of AML, it is expressed in all leukemia cells but not in healthy cells, making it an attractive target for drug development. *CM* is assumed to be required for leukemia maintenance due to the near universal presence of Inv(16) at relapse. However, the knockdown of the fusion gene in the inv(16) AML cell line, ME-1, induces signs of differentiation but does not affect cell survival(19). Moreover, two different CBFβ-SMMHC inhibitors have been developed and in initial testing were able to decrease leukemic burden and increase survival in mouse models, but all mice eventually died of the disease (20, 21). For both compounds, it is unclear whether their limited pre-clinical effect was due to incomplete inhibition of CBFβ-SMMHC activity, or because the fusion protein is not strictly required after leukemic transformation. This issue has important implications for the further developed of targeted therapies for patients with inv(16) AML.

In this study, we address the role of *CM* in non-immortalized mouse leukemia cells. We demonstrate that *CM* loss eliminates leukemia from the peripheral blood and spleen, but not the bone marrow, indicating that loss of CBFβ::SMMHC activity is sufficient to control, but not cure, the disease.

Methods

<u>Mice</u>

All animal experiments were approved by the University of Nebraska Medical Center IACUC in accordance with NIH guidelines. 4-6-week-old mice of both sexes were used. To generate knock-in mice with the *flMYH11* allele, we started with a previously described pPNT vector containing the *CBFB Exon 5::MYH11* cassette, and added flanking *LoxP* sites by site directed mutagenesis (QuikChange, Agilent) (22). Mouse ES cells were electroporated, selected, screened for homologous recombination and injected into blastocysts, as described previously (22, 23). To confirm the $Cbfb^{flMYH11}$ allele functions as predicted, chimeric mice were bred to mice expressing *Cre* from the β-Actin promoter (*ActB* Cre^{+}), purchased from Jackson Laboratory (Bar Harbor, ME) and embryos harvested at embryonic day 12.5(24). To induce leukemia, chimeric $Cbfb^{+/flMYH11}$mice were treated with N-ethyl-N-nitrosourea (ENU) and monitored for leukemia development (13, 25, 26). Leukemia cells were transplanted into sub-lethally irradiated congenic recipient mice as described previously (27, 28). Knockdown studies were performed using leukemia cells from *Mx1-Cre,* $Cbfb^{+/56M}$ mice, which allows for inducible expression of *CM* (29). To induce shRNA expression, mice were given 5% sucrose supplemented drinking water with or without doxycycline at 2mg/ml. Mice were sacrificed on the indicated day of treatment and tissue collected.

qRT-PCR Analysis

RNA was extracted using Trizol method and cDNA synthesized using Luna RT Supermix (New England Biolabs, MA) and amplified using Luna Universal qPCR kit (New England Biolabs, MA). Primer sequences available upon request.

Western Blot Analysis

Cell lysates were prepared, subjected to SDS-PAGE and transferred to PVDF, as described previously (27, 28). Membranes were blocked in 5% milk and then probed using primary antibodies against MYH11 (Novus Biologicals, CO, and Origene, MD), GAPDH (Ambion, Texas), β-Actin (Santa Cruz Biotechnology, TX), MCL-1(Cell Signaling Technology, MA), BCL-2 (Cell Signaling Technology, MA), and GFP (Invitrogen, CA) and with secondary HRP-linked antibodies anti-rabbit or anti-mouse. Protein bands were visualized with SuperSignal West Pico PLUS (Thermo Scientific, MA). Protein levels were quantified using ImageJ.

Flow Cytometry Analysis

Cells were stained with fluorophore coupled antibodies against B220 (RA3-6B2, Becton Dickinson, NJ), CD3 (17A2, BD Biosciences, NJ), Mac-1/CD11b (M1/70, BD Biosciences, NJ), GR-1 (RM4-5, BD Biosciences, NJ), CKIT (53-6.7, BD Biosciences, NJ), Csf2rb/CD131 (JORO50, BD Biosciences, NJ), IL1RL1 (U29-93, BD Biosciences, NJ), CD41 (MWReg30, BD Biosciences, NJ), CD123 (5B11, BD Biosciences, NJ) and analyzed by flow cytometry (BD LSR II). Apoptosis was assayed using Annexin V (BD Pharmigen, NJ). Proliferation was determined using the BrdU Flow Kit (BD Pharmigen, NJ). Mice were injected with BrdU at dose of 200mg/kg body weight. After 1 hr, mice were sacrificed and tissue harvested. All flow cytometry data was analyzed using FlowJo 10.0 (BD, OR).

Viral Transduction

To excise the *Cbfb* ^{flMYH11} allele, lentiviral plasmids co-expressing *Cre Recombinase* and either GFP or DsRED were used. To knockdown *Cbfb-MYH11*, a lentiviral plasmid expressing GFP and an shRNA against *MYH11* was kindly provided by Dr. J. Martens, Nijmegen, NL(19). Lentiviral plasmids were transfected into HEK293T cells. Mouse leukemia cells were incubated with viral supernatant, cytokines and 8ug/ml polybrene for transduction. Transduction was performed by spinfection centrifugation for 90 minutes at 2000 rpm, 30 °C, followed by a 6-hour incubation and a second spinfection. Transduced cells were sorted for GFP or dsREd 48hrs post transduction.

Colony Assay

Transduced cells were sorted, and equal numbers were suspended in 35mm dishes in Methocult 3434 (StemCell Technologies, Vancouver, BC) in triplicate, according to the manufacturer's protocol. Colonies were counted after 14 days in culture.

Statistical Analysis

Statistical significance was analyzed using either Student's T-Test or ANOVA, as appropriate (GraphPad Prism, version 10, Dotmatics, CA) with p-values ≤0.05 deemed significant.

Results

Generation and characterization of *Cbfb*^{floxMYH11} leukemia cells

To test the role of *Cbfb::MYH11* in leukemia maintenance, we generated and characterized a new knock-in mouse model, where the *Cbfb exon5::MYH11* fusion exon is flanked by *LoxP* sites and knocked into the endogenous *Cbfb* locus (*Cbfb*$^{+/flMYH11}$) (Fig. 1A). We identified embryonic stem cell (ES) clones with the knock-in allele by southern blot and confirmed CBFβ::SMMHC expression by western blot (Supplementary Fig. S1A, B). Three independent *Cbfb*$^{+/ floxMYH11}$ ES

clones were used to generate chimeric mice. To test that the *Cbfb* ^{flMYH11} allele can be fully excised, chimeric mice were crossed with mice expressing *Cre Recombinase* from the *Actin B* promoter (*ActB Cre*), which should allow for excision of the fusion gene in all cells of an embryo. We harvested embryos at day 12.5 in development. In *Cbfb*$^{+/\ flMYH11}$ embryos lacking the *ActB Cre* transgene, we observed central nervous system hemorrhaging, indicating expression of *Cbfb::MYH11*, as in previous knock-in models (Fig. 1B) (22). Importantly, this phenotype was not observed in *Cbfb*$^{+/flMYH11}$; *ActB Cre*$^{+}$, indicating loss of the fusion gene in the presence of *Cre Recombinase*. PCR using primers specific for the unexcised and excised alleles confirmed that the *Cbfb* ^{flMYH11} was efficiently excised in *Cbfb*$^{+/flMYH11}$; *ActB Cre*$^{+}$ mice. To generate leukemia cells expressing the *Cbfb* ^{flMYH11} allele, chimeric mice were treated with N-ethyl-N-nitrosourea and monitored for disease onset. Chimeric *Cbfb*$^{+/flMYH11}$ mice from each of the three ES clones developed transplantable leukemia with a similar immunophenotype and histological appearance as previous *CM knock-in* mouse models (Fig. 1C, D; Supplementary Fig. S1C, D) (29, 30). These results indicate that *Cbfb*$^{+/flMYH11}$ mice develop a frank leukemia with a similar phenotype to previous models and that the fusion gene can be deleted by *Cre Recombinase*.

Loss of *Cbfb::MYH11 (CM)* impairs the growth and survival of leukemia cells *in vitro,* but rare *Cbfb::MYH11* deleted cells form colonies

To test the effect of *CM* deletion *in vitro*, we transduced *Cbfb*$^{+/flMYH11}$ leukemia cells with lentivirus expressing *Cre* and GFP from an IRES (Fig. 2A). Transduced cells were sorted, and *CM* excision was confirmed by qPCR and western blot (Fig. 2B-C). We found that *CM* deletion caused a statistically significant increase in Annexin V, indicating induction of apoptosis (Fig. 1D). To test the effect of *CM* deletion on colony forming ability, we plated equal numbers of control and *Cre* transduced *Cbfb*$^{+/flMYH11}$ leukemia cells in methylcellulose. After 14 days, colonies were counted, resuspended as single cells, and equal numbers replated in methylcellulose**.** At both the first and 2nd plating, we found that *Cbfb*$^{+/flMYH11}$ cells transduced with *Cre* generated significantly fewer colonies (Fig. 1E). To determine if the colonies that were able to grow from *Cre*-infected cells had successfully deleted *CM*, individual colonies were picked and qPCR for the *Cbfb*^{flMYH11} allele was performed. We found that most colonies retained the unexcised *CM* allele; however, 5-20% of the colonies showed successful deletion of *CM* (Supplementary Fig. S2A, B). These findings imply that the majority of leukemia cells require continued expression of the fusion gene after leukemia initiation, but that some cells may be able to survive without *CM.*

Knockdown of *Cbfb::MYH11* impairs leukemia survival *in vivo*

Due to the high rate of apoptosis seen *in vitro* with genetic deletion of *Cbfb*^{flMYH11} allele, we used a different mouse model and a shRNA knockdown (KD) construct to test the requirement for *Cbfb::MYH11* in leukemia maintenance *in vivo*. We generated leukemia cells from mice with the *Cbfb*^{56M} allele, which allows inducible expression of *Cbfb::MYH11* and the *Mx1-Cre* transgene, which is inducibly expressed in nearly all hematopoietic cells (29). Leukemia cells from *Cbfb*$^{+/56M}$, *Mx1-Cre*$^{+}$ were transduced with a doxycycline (Dox) inducible shRNA against *MYH11* (*shMYH11*) that also expresses GFP constitutively (19). To test the feasibility of this approach *in vitro*, we sorted GFP^{+} cells and cultured them with Dox for 48 hours. After 48 hrs, *CM* expression was determined by qRT-PCR and western blot. We observed that KD resulted in 70-95% decrease in expression at mRNA levels and 51-98% decrease of the fusion protein *in vitro* (Supplementary Fig. S3A-C). We also found that *CM* KD induced a similar phenotype to the genetic deletion of the fusion gene, with increased apoptosis and decreased colony formation *in vitro* (Supplementary Fig. S3D, E).

197 To determine the effect of decreased *CM* expression *in vivo*, we transplanted *shMYH11*
198 transduced cells into congenic mice, allowing them to engraft. Once mice showed at least 20%
199 GFP$^+$ leukemia cells in the peripheral blood, mice were treated with control (5% sucrose) or Dox
200 (5% sucrose and Dox) drinking water (Supplementary Fig. S4A). Mice were sacrificed after 4, 7,
201 14, 21, and 28 days, and the presence of GFP$^+$ leukemia cells was determined. We observed that
202 *CM* KD induced a significant decrease in GFP$^+$ leukemic cells in the peripheral blood and spleen
203 starting at day 7, with near complete elimination by day 14 (Fig. 3A, 3B). In addition, we observed
204 a notable decrease in spleen size with *CM* KD (Supplementary Fig. S4B, C). In the bone marrow,
205 there was a significant decrease in leukemia cells starting at day 14 of Dox treatment. However,
206 20-30% of leukemia cells persisted through day 28 (Fig. 3C). To determine if the persistence of
207 leukemia cells in the bone marrow was due to failure of Dox to penetrate the tissue and adequately
208 KD the fusion gene, we sorted GFP$^+$ leukemia cells from the bone marrow of control and Dox
209 treated mice at days 7 and 21. qRT-PCR and western blot of bone marrow GFP$^+$ cells showed
210 significant *CM* KD at both time points (Fig. 3D-F). These findings indicate that cells with reduced
211 *CM* can survive in the bone marrow, but not the peripheral blood and spleen.
212
213 To investigate potential mechanisms for the persistence of *CM* KD cells in the bone marrow, we
214 analyzed apoptosis and proliferation in the leukemic cells of Dox and control treated mice at Day
215 21. We found a significantly lower percentage of leukemia cells undergoing apoptosis in the bone
216 marrow of Dox treated mice, as compared to control (Fig. 4A). To determine if the decrease in
217 apoptosis is related to expression of anti-apoptotic proteins, we sorted leukemia cells from the
218 spleen and bone marrow of control and Dox treated mice and performed western blots. We found
219 that KD of *CM* induced decreased BCL-2 protein in the spleen but caused no change in MCL-1.
220 In leukemic cells from the bone marrow of both control and Dox treated mice, BCL-2 was barely
221 detectable. In contrast, MCL-1 expression was higher in leukemia cells from the bone marrow of
222 both control and dox treated mice, as compared to cells from the spleen (Fig. 4B, C). These
223 findings imply that MCL-1, but not BCL-2, may contribute to the survival of CM knockdown cells
224 in the bone marrow.
225
226 We used BrdU incorporation to determine if a difference in proliferation also contributed to the
227 persistence of *CM* KD cells in the bone marrow. Leukemia cells from both the spleen and bone
228 marrow of day 21 Dox treated mice had significantly lower proliferation compared to the control
229 (Fig. 4D). To test if the decrease in proliferation was due to a change in the differentiation state
230 of the leukemia cells, we examined the expression of Mac-1 and Gr-1, markers of mature myeloid
231 cells. In the spleen of Dox treated mice, we observed an increase in Mac1$^+$, GR-1$^+$ cells,
232 consistent with differentiation. In contrast, there was no difference in the expression of Mac1 or
233 GR-1 on the leukemia cells in the bone marrow (Fig. 4E). To test whether the *CM* KD cells in the
234 bone marrow were instead enriched for Leukemia Stem Cell (LSC) markers, we examined
235 expression of KIT, CD59a, IL1RL1, CD123. Work by us and others previously showed that KIT,
236 CD59a and IL1RL1 are enriched on the LSC population driven by *CM* expression, and CD123 is
237 an established LSC marker in human AML (27, 31, 32). We observed a small yet statistically
238 significant increase in CD123 and decrease in IL1RL1 in the leukemia cells in the bone marrow
239 of Dox treated mice (Fig. 4F). These results indicate that the decreased proliferation observed in
240 the *CM* KD cells in the bone marrow is likely not due to differentiation to a post-mitotic mature
241 myeloid cell or enrichment for quiescent LSCs.
242
243 The persistence of a *CM* KD leukemic population in the bone marrow raises the possibility that
244 these cells may be able to cause relapse. To test this possibility, we maintained mice transplanted
245 with *shMYH11* transduced leukemia cells on Dox or control drinking water until they showed signs
246 of lethal disease. Five out of 12 Dox treated mice showed re-emergence of GFP$^+$ cells in the
247 blood (Fig. 5A). To determine if the relapse cells in the blood expressed *CM*, we sorted for GFP

248 and performed qRT-PCR (Fig. 5B) and western blot (Fig. 5C, D). We found that relapsed samples
249 had reduced expression of *CM* at both the mRNA and protein level, as compared to control treated
250 mice. In addition, we found that the immunophenotype of the *CM* KD relapse leukemia cells was
251 similar to leukemia cells from control mice (Supplementary Fig. S5A and B). These results imply
252 that despite the reduction in *CM* expression, relapse cells acquired additional mutations that
253 promote survival outside the bone marrow microenvironment, and re-establishment of fatal
254 disease.
255
256 **Discussion**
257 CBFβ::SMMHC is a tempting target for drug development, as it expressed in all leukemia cells in
258 patients with inv(16) AML, but not in healthy cells. However, work in the immortalized cell line ME-
259 1 raises the possibility that the fusion protein may not be strictly required after leukemic
260 transformation (19). To our knowledge, this is the first study to test this possibility using non-
261 immortalized leukemia cells. We found that genetic deletion of *CM* induced significant apoptosis
262 and impaired colony growth. However, a small number of colonies were able to grow after *CM*
263 deletion, implying that there is a population of leukemia cells that don't strictly require the fusion
264 gene. *In vivo*, we found that KD of *CM* induced a near complete elimination of leukemic cells in
265 the blood and spleen. Surprisingly, the leukemic burden in the bone marrow did not decrease
266 below 20-30% despite almost complete loss of CBFβ-SMMHC protein, implying that the bone
267 marrow microenvironment can sustain leukemia cells despite the loss of *CM* expression.
268
269 Our results concerning the role of CBFβ::SMMHC during leukemia maintenance are strikingly
270 similar to what has been demonstrated with the BCR::ABL fusion protein in Chronic Myeloid
271 Leukemia (CML). Like CBFβ::SMMHC, BCR::ABL is the product of a chromosomal
272 rearrangement characteristic of the disease, and its expression is the initiating event.
273 Pharmacological inhibitors of BCR::ABL, such as imatinib, are remarkably successful at inducing
274 long-term, stable cytogenetic remission (33, 34). However, these kinase inhibitors generally do
275 not eliminate the LSC population in the bone marrow. Similarly, near complete knockdown of
276 *BCR::ABL* expression reduces the LSC population but does not eliminate it, and the remaining
277 LSCs are able to re-establish disease when BCR::ABL activity is restored (35). In our model, we
278 also observed a population of LSC-like cells that can survive in the bone marrow and re-establish
279 disease, despite near complete loss of the CBFβ::SMMHC protein. In our case, relapse did not
280 require re-expression of the fusion protein. However, given the period of latency before relapse,
281 it is likely that additional mutations developed to compensate for the lack of CBFβ::SMMHC
282 activity.
283
284 Our observation that *CM* knockdown cells can persist only in the bone marrow implies that there
285 are factors present in the microenvironment critical for their survival. This is consistent with other
286 work demonstrating that the bone marrow microenvironment is unique in promoting leukemia cell
287 survival and preventing apoptosis (35-37). In fact, we observed that *CM* KD cells in the bone
288 marrow, but not the spleen, had decreased rates of apoptosis as compared to control. Thus, to
289 identify potential therapeutically targetable factors promoting the survival of *CM KD* cells, we
290 performed westerns for the anti-apoptotic proteins BCL-2 and MCL-1 (38, 39). We observed that
291 both control and *CM KD* had had robust expression of MCL-1 but nearly undetectable levels of
292 BCL-2. This finding is consistent with recent reports indicating that the BCL-2 inhibitor venetoclax
293 is less effective in patients with French-American-British (FAB) M4 and M5 monocytic
294 differentiation AML, which includes inv(16) AML (40).
295
296 Collectively, this study supports the development of inhibitors targeting the *CM* fusion protein for
297 the treatment of inv(16) AML. To date, drugs targeting CBFβ-SMMHC show the feasibility of such
298 an approach, even if the current inhibitors are not suitable for use in humans (20, 21). Our data

shows that as the next generation of such inhibitors are developed, they would likely be effective at managing leukemia burden, and when used in combination with other drugs such as MCL-1 inhibitors, have the potential to cure the disease.

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Author Contributions

S.P. designed and performed experiments, analyzed data and wrote the paper. Y.W. designed and performed experiments and analyzed data. M.B., C.L., A.D., and S.S. performed experiments. C.R., L.A., and L.G. generated, bred, and mutagenized *Cbfb*$^{+/floxMYH11}$ knock-in mice. R.K.H designed experiments, analyzed data and wrote the paper.

Disclosure of Conflicts of Interest

The authors have no conflicts of interest to disclose.

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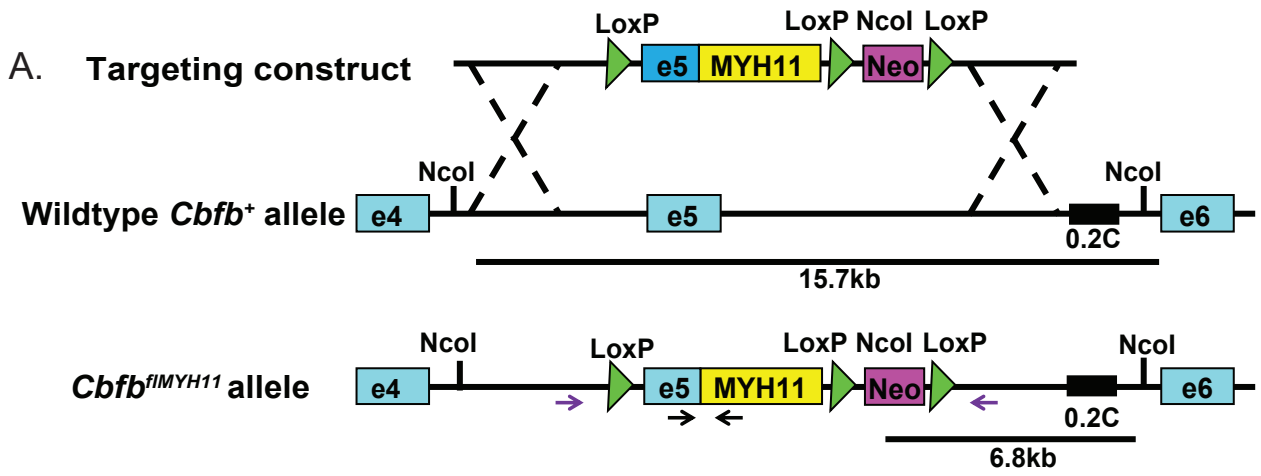
445

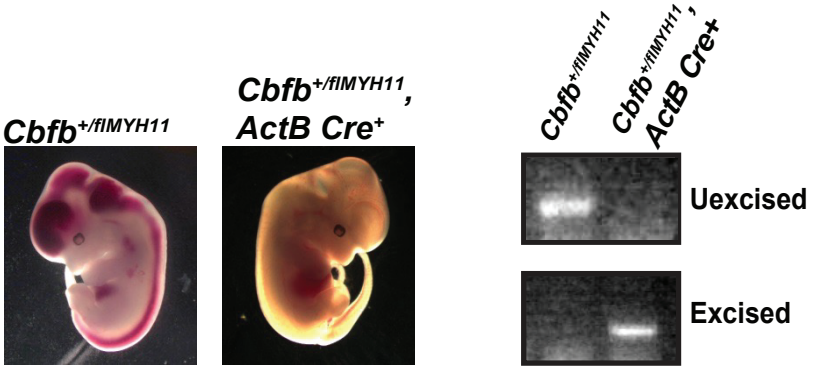
446 **Figure Legends**
447
448 **Figure 1: Generation of the novel model and *in vitro* analysis. A.** Schematic representation
449 of the ES-cell targeting strategy. The probe (0.2C) and restriction enzyme sites (NcoI) used for
450 screening are indicated. The black arrows represent PCR primers for the excised allele, and the
451 purple arrows represent primers for the unexcised allele. **B.** Pictures of representative embryos
452 harvested at embryonic day 12.5 generated by crossing *Cbfb*$^{+/floxMYH11}$ chimeras with transgenic
453 mice expressing *Cre recombinase* (Cre) from the *β–Actin* promoter (*ActB Cre*). The right panel
454 shows a representative PCR for the excised and unexcised *Cbfb*$^{+/floxMYH11}$ allele in DNA from
455 embryos of the indicated genotype. **C.** Kaplan-Meyer survival curve of recipient mice transplanted
456 with *Cbfb*$^{+/flMYH11}$ leukemic cells from the chimeric donor mice. **D.** Representative Wright-Giemsa-
457 stained peripheral blood smear and hematoxylin and eosin-stained spleen and liver section from
458 a leukemic *Cbfb*$^{+/flMYH11}$ recipient mouse.
459
460 **Figure 2: Deletion of *Cbfb::MYH11* induces apoptosis and decreased colony formation *in***
461 ***vitro* A.** Schematic representation of the experimental design to test the role of *Cbfb::MYH11* in
462 leukemia maintenance *in vitro*. **B.** Quantitative Reverse Transcription PCR (qRT-PCR) analysis
463 for the unexcised and excised *Cbfb*$^{+/floxMYH11}$ alleles in leukemia cells transduced with a lentivirus
464 expressing Cre or Control (CTRL) and sorted for GFP expression. **C.** Western blot analysis of
465 CBFβ::SMMHC in *Cbfb*$^{+/flMYH11}$ leukemia cells infected with Cre and CTRL virus. **D.** Graph of the
466 percent (%) Annexin V^{+} Cre and CTRL transduced *Cbfb*^{flMYH11} leukemia cells from 3 independent
467 mice. **E.** Bar graph showing the relative number of colonies from equal numbers of Cre and control
468 transduced *Cbfb*$^{+/flMYH11}$ leukemia cells from 3 independent mice, each plated in triplicate plated
469 in methylcellulose. Colonies were scored on day 14. N=3. ***p <0.001 as compared to CTRL.
470
471 **Figure 3: *CM* knockdown decreases leukemia burden in *vivo.* A.** Line graph showing percent
472 (%) GFP^{+} *shCM* transduced leukemia cells (left) and representative flow cytometry plots (right) in
473 the peripheral blood (PB), **B.** spleen (SP) and **C.** bone marrow (BM) of mice treated with Dox or
474 control for 7, 14, 21, or 28 days. **D.** Bar graph showing relative expression of *CM* in sorted GFP^{+}
475 leukemic cells from the BM of mice treated with Dox or control for 7 and **E.** 2 days. **F.**
476 Representative western blot (left) and graph of relative CBFB::MYH11 protein expression in
477 sorted GFP^{+} cells from the BM of mice treated with either control or Dox for 7 days. For all
478 experiments N≥ 3 biological replicates, ns = not significant, *p=<0.05.
479
480 **Figure 4: Leukemia cells in the bone marrow with decreased *CM* have a similar**
481 **immunophenotype to control leukemia cells.**
482 **A.** Bar graph showing the percentage (%) of Annexin V^{+}, GFP^{+} from the spleen (SP) and bone
483 marrow (BM) of mice treated with Dox or control for 21 days. **B.** Representative western blot for
484 the indicated proteins in leukemic cells sorted from SP and BM after 7 days of treatment with
485 control or Dox **C.** Graph of the relative protein expression from the westerns blots in B. **D.** Graph
486 of the % of BrdU^{+}, GFP^{+} leukemia cells harvested from the SP and BM of mice treated with Dox
487 or control for 21 days. **E.** Bar graph showing % of GFP^{+} cells expressing the mature myeloid
488 markers Mac-1 and Gr-1. Leukemic cells were harvested from the BM of mice treated as in A. **F.**
489 Bar graph showing the % of GFP^{+} cells expressing the indicated leukemia stem cell markers in
490 the BM from of mice treated for 21 days with Dox or vehicle. For all experiments N≥ 3 biological
491 replicates, *p=<0.05, ** = p<0.01, *** = p<0.001
492
493 **Figure 5: The remaining *CM* knockdown cells in the bone marrow can give rise to relapse.**
494 **A.** Line graph showing the percentage (%) of GFP^{+}, *shCM* transduced cells in peripheral blood
495 (PB) of mice treated for up to 42 days with either control or Dox**. B.** Graph of relative expression
496 of *CM* mRNA in GFP^{+} sorted leukemia cells from dox treated mice after relapse. **C.**

497 Representative western blot of the indicated proteins in sorted GFP^+ cells harvested from control
498 and Dox treated mice. **D.** Graph of relative CBFβ::SMMHC protein expression in samples treated
499 as in C. N≥ 3 biological replicates, *p=<0.05
500
501

Figure 1



B.



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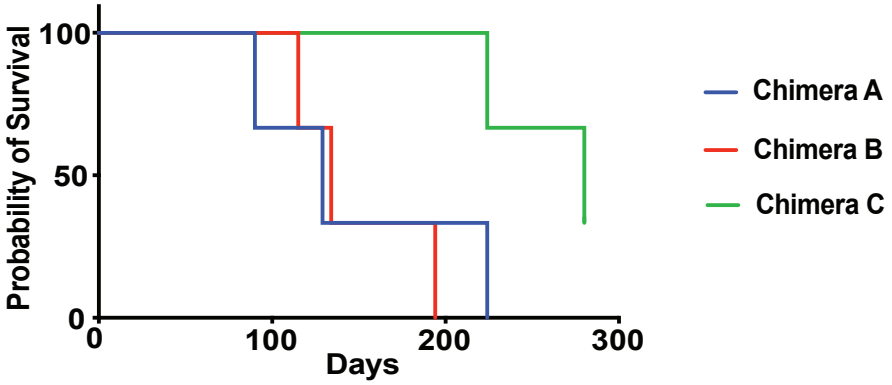

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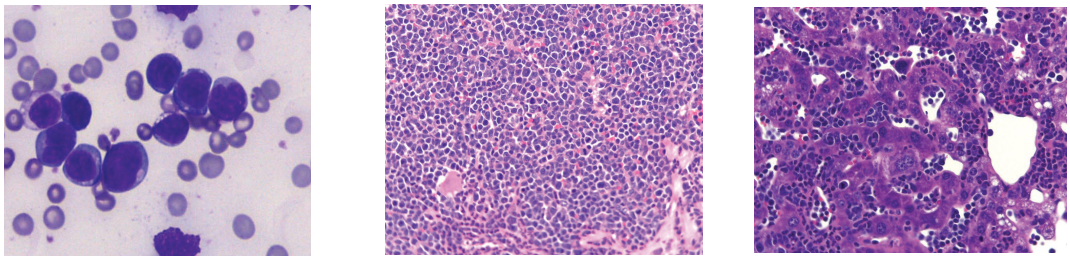
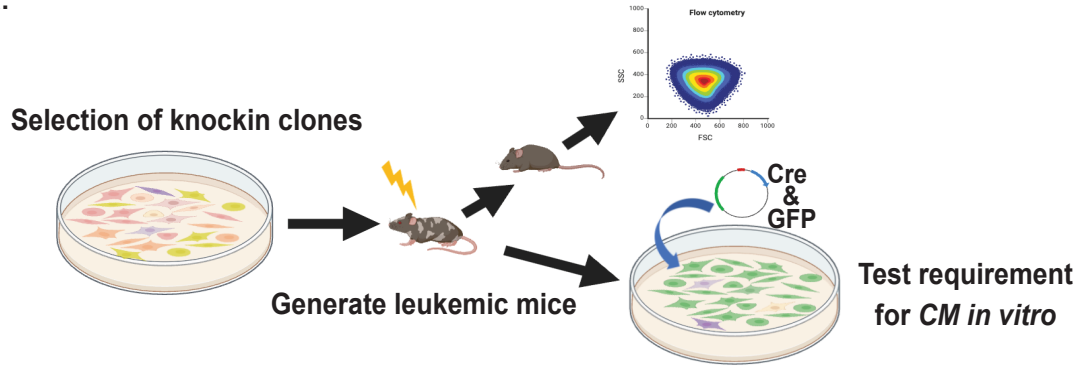


Peripheral Blood Spleen Liver

Figure 2

A.



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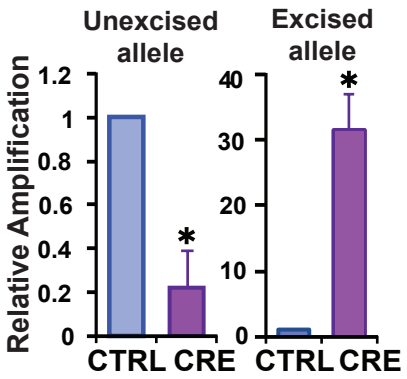

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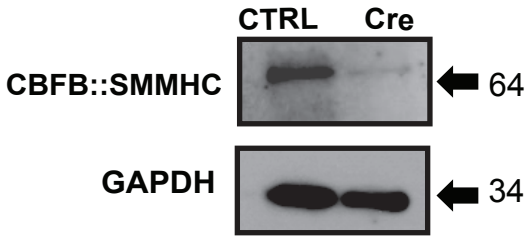
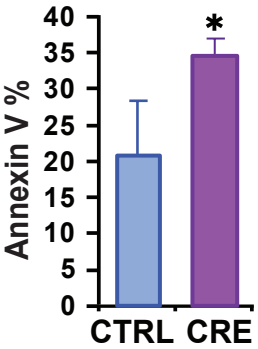


D.



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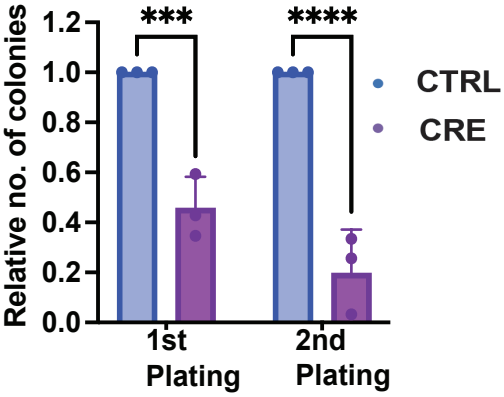


Figure 3

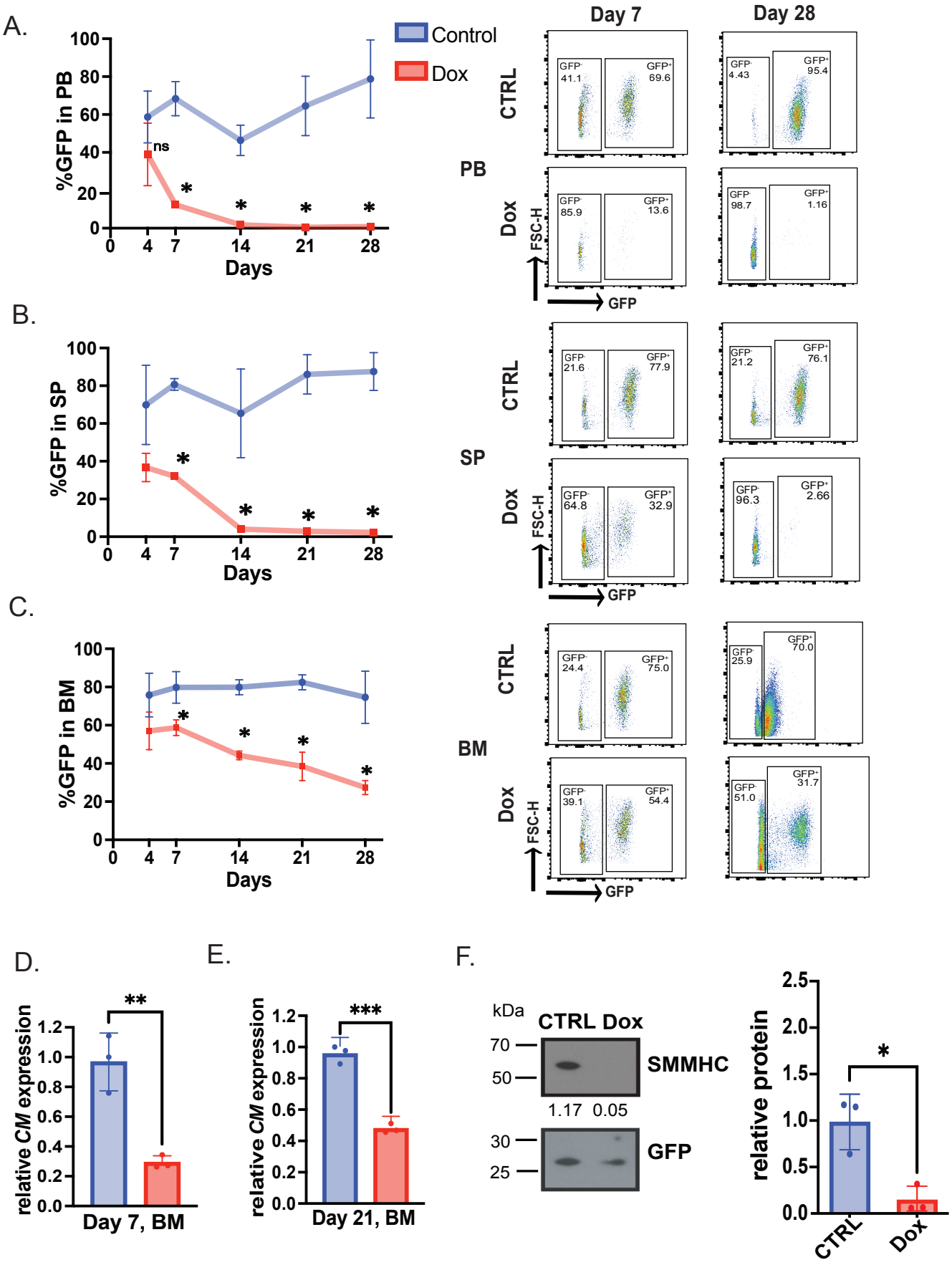

A.
Control
Dox
%GFP in PB
Days
ns
Day 7
Day 28
CTRL
PB
Dox
FSC-H
GFP
GFP- 41.1
GFP+ 69.6
GFP- 4.43
GFP+ 95.4
GFP- 85.9
GFP+ 13.6
GFP- 98.7
GFP+ 1.16
B.
%GFP in SP
SP
GFP- 21.6
GFP+ 77.9
GFP- 21.2
GFP+ 76.1
GFP- 64.8
GFP+ 32.9
GFP- 96.3
GFP+ 2.66
C.
%GFP in BM
BM
GFP- 24.4
GFP+ 75.0
GFP- 25.9
GFP+ 70.0
GFP- 39.1
GFP+ 54.4
GFP- 51.0
GFP+ 31.7
D.
relative CM expression
Day 7, BM
E.
Day 21, BM
F.
kDa
CTRL Dox
70
50
SMMHC
1.17 0.05
30
25
GFP
relative protein
CTRL
Dox

Figure 4

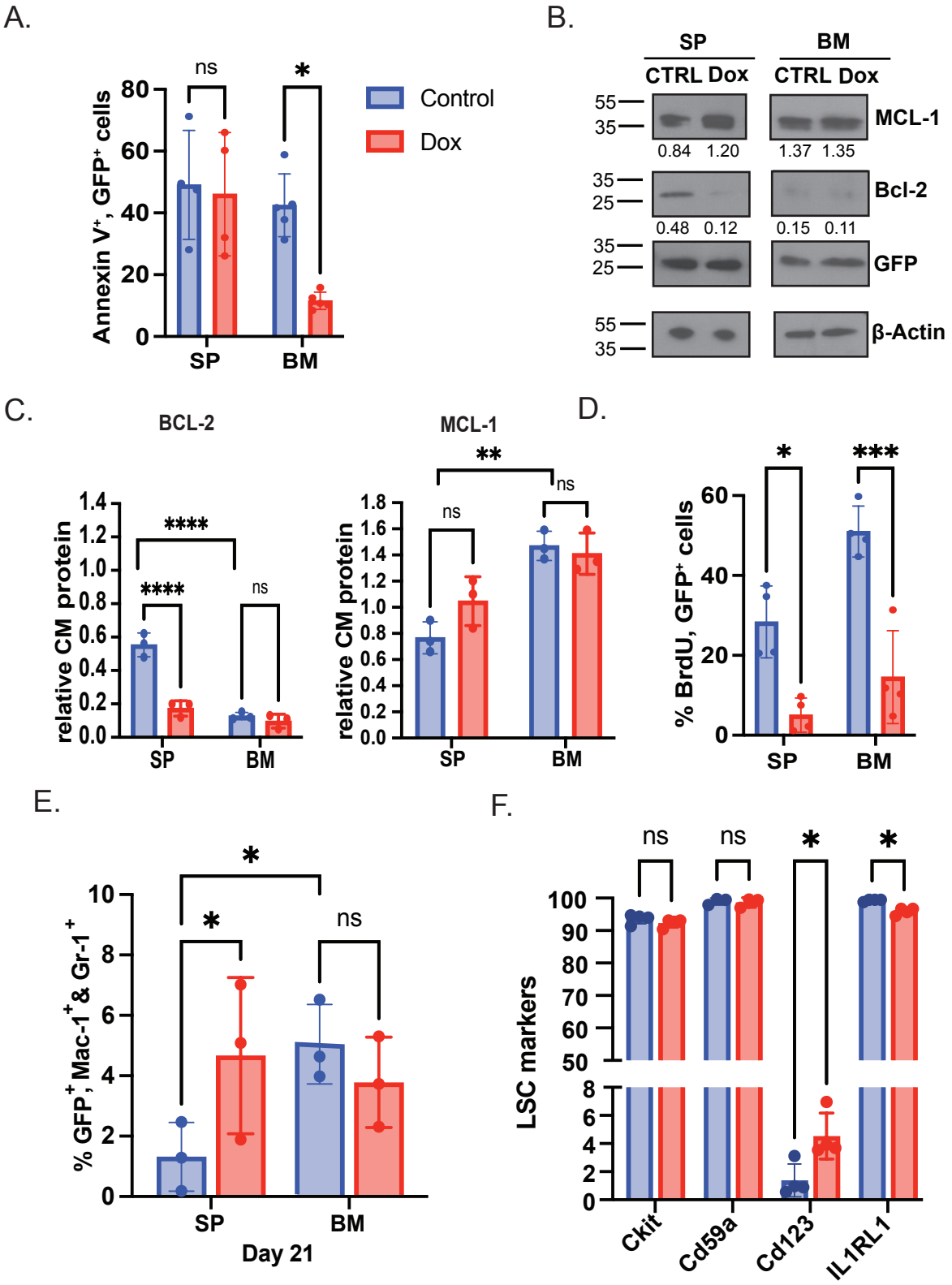
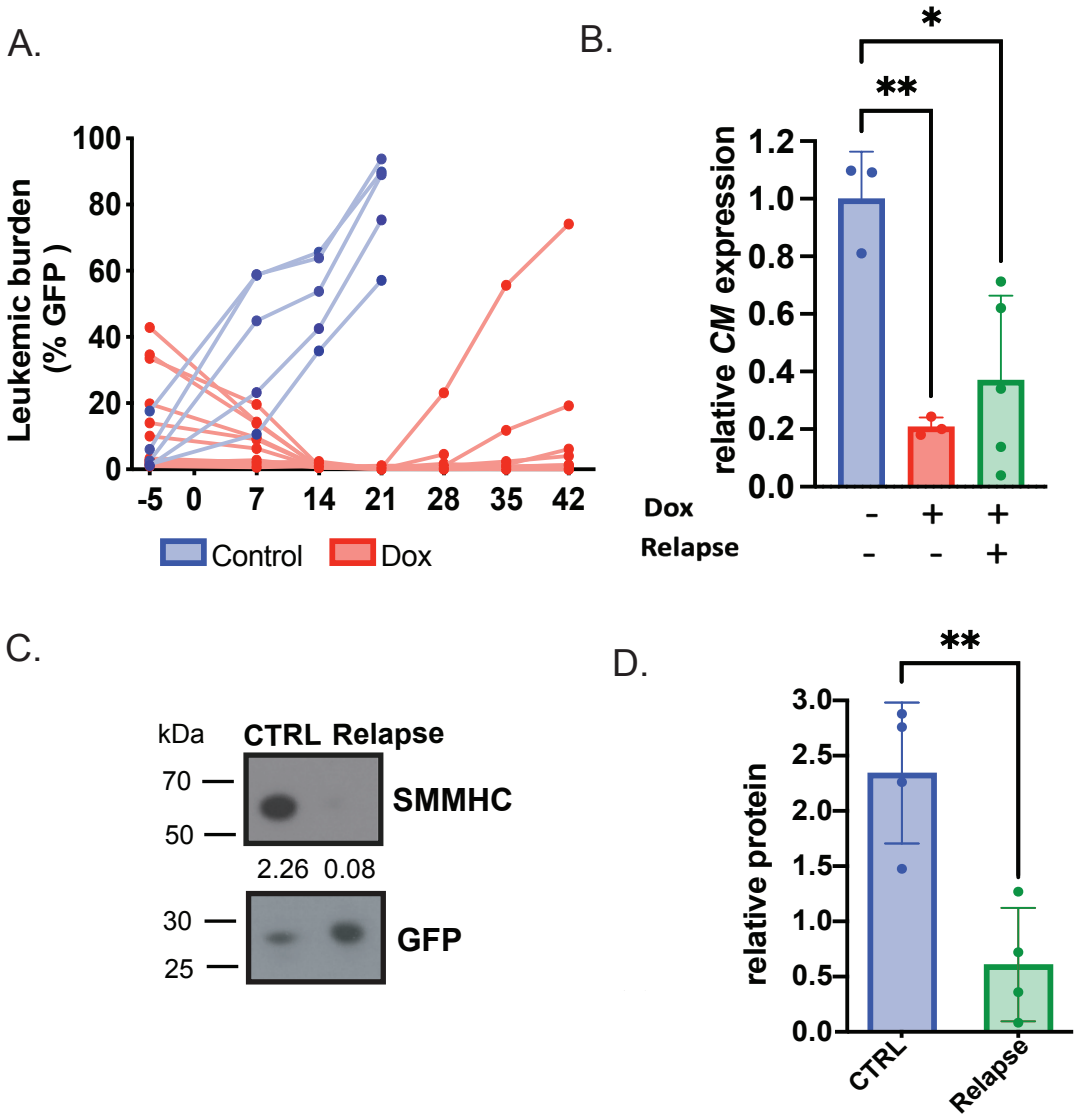

A.
Annexin V+, GFP+ cells
ns
*
Control
Dox
SP
BM
B.
SP
BM
CTRL Dox
CTRL Dox
MCL-1
0.84 1.20
1.37 1.35
Bcl-2
0.48 0.12
0.15 0.11
GFP
β-Actin
C.
BCL-2
MCL-1
relative CM protein
****
ns
**
D.
% BrdU, GFP+ cells
***
E.
% GFP+, Mac-1+ & Gr-1+
Day 21
F.
LSC markers
Ckit
Cd59a
Cd123
IL1RL1

Figure 5


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
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