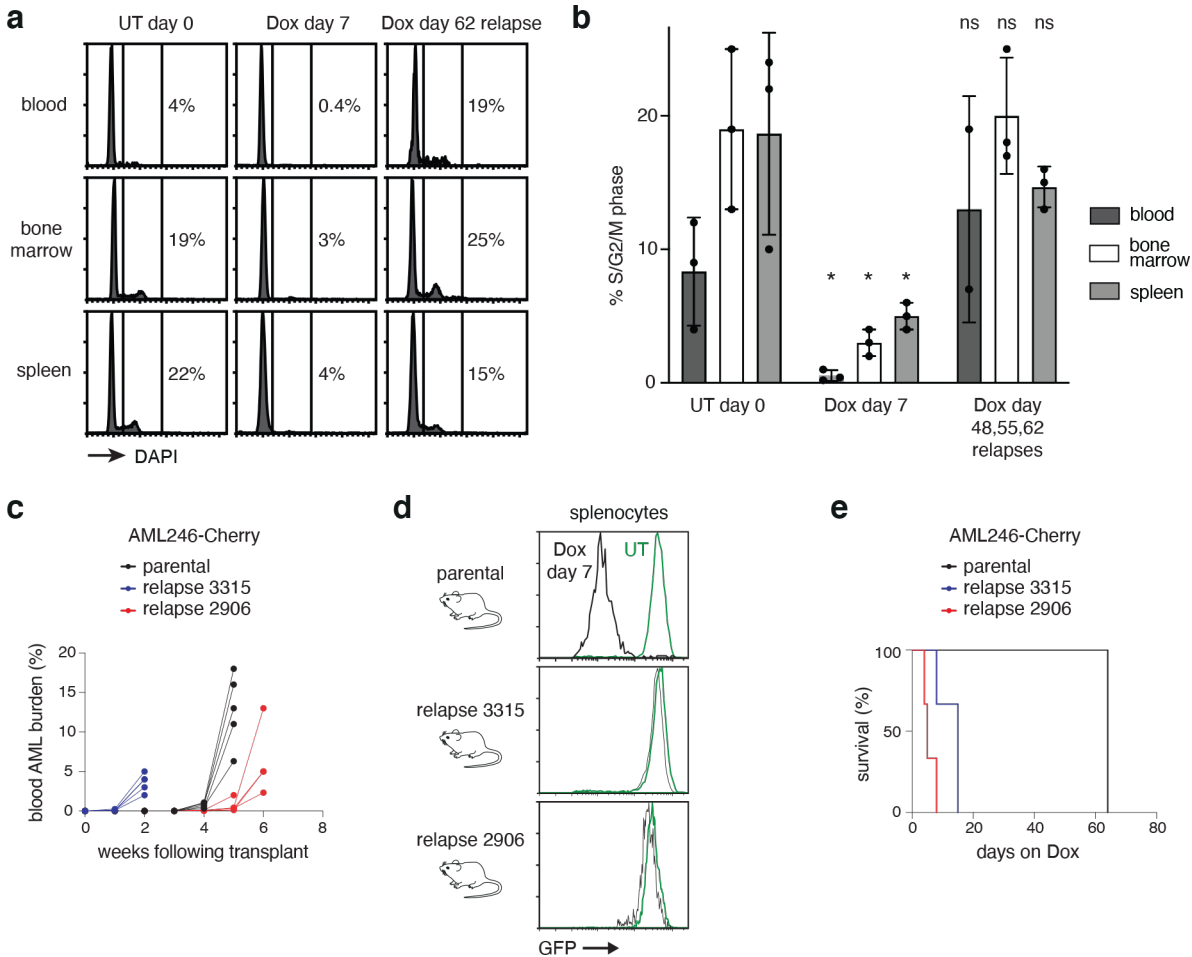
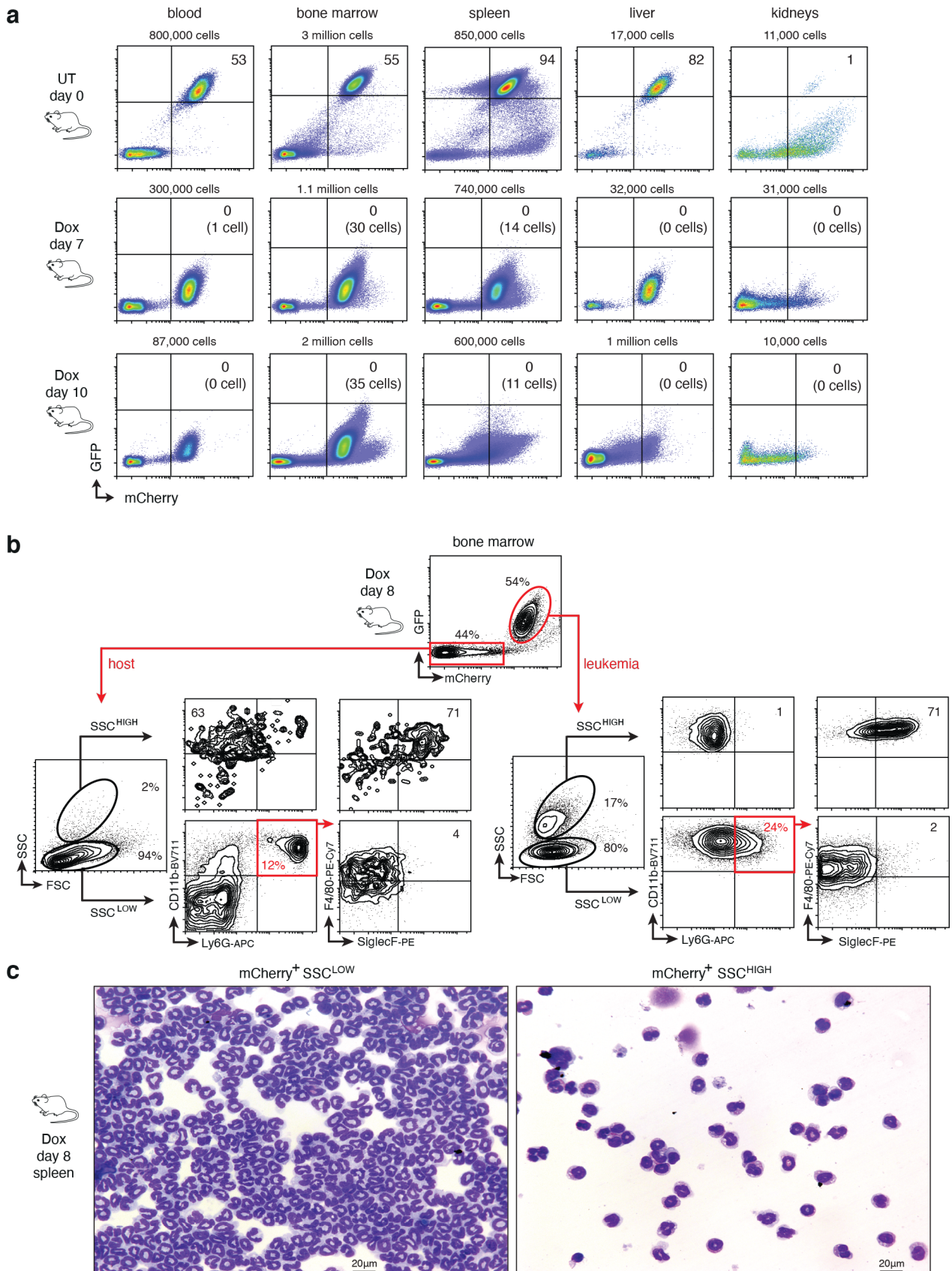


SUPPLEMENTARY INFORMATION

“Acute myeloid leukemia maturation lineage influences residual disease and relapse following differentiation therapy”

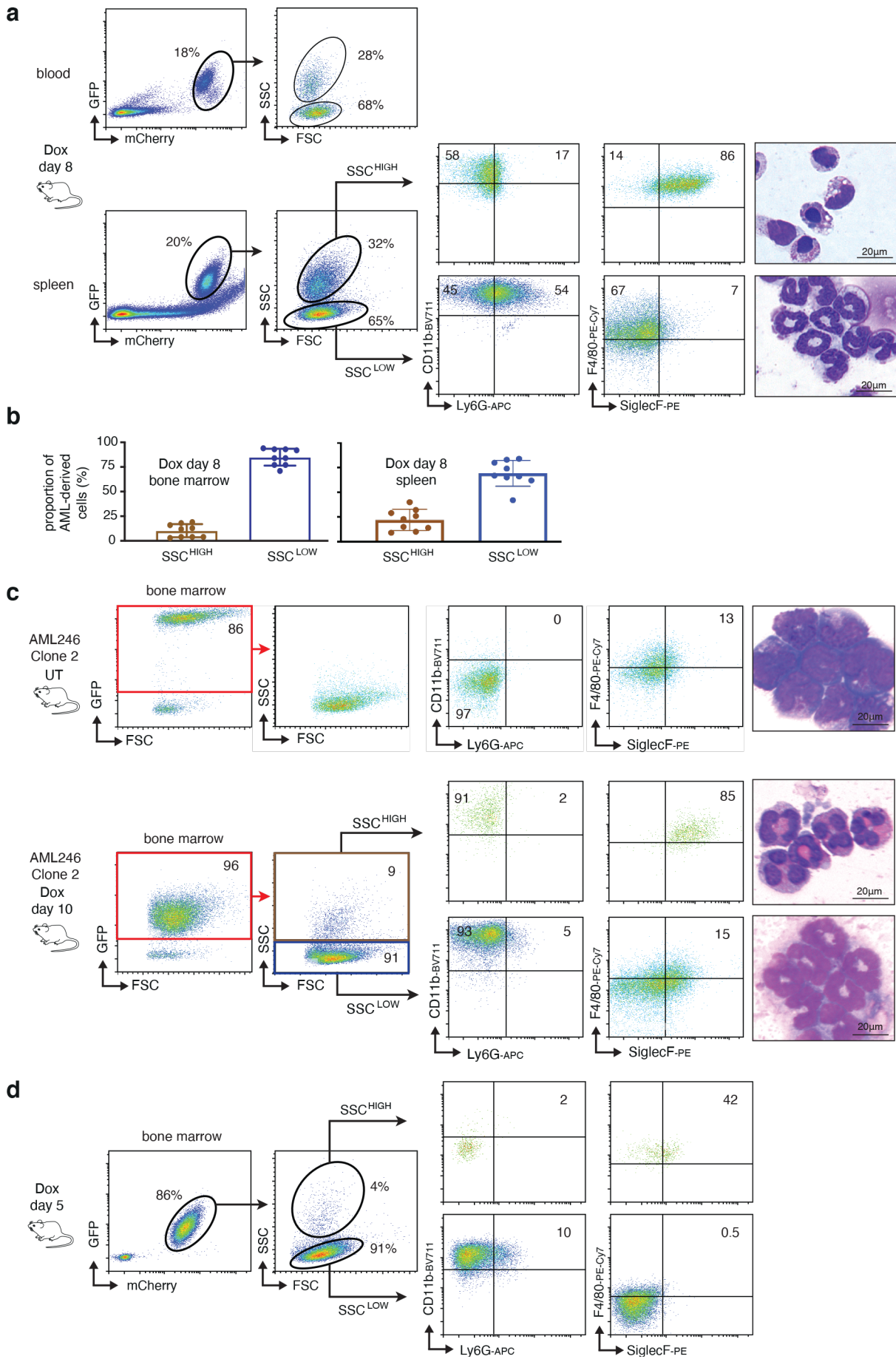


Supplementary Figure 1. Cell cycle and GFP analysis upon Dox-induced AML246 differentiation and relapse. (a) DAPI cell cycle analysis indicating the proportion of mCherry⁺ cells in S/G2/M phases of the cell cycle in the blood, bone marrow, and spleen of representative untreated, Dox day 7 treated and Dox-treated relapsed mice. (b) Summary of the proportion of AML derived mCherry⁺ cells in S/G2/M phase for each organ and time point. Mean \pm SD, n=3 mice per condition. Relative to untreated p=0.01 for bone marrow, 0.03 for blood and 0.04 for spleen, unpaired two-sided Student’s t-test. (c) Proportion of mCherry⁺ AML cells in the peripheral blood of untreated mice re-transplanted with AML cells from relapse mouse 3315 (blue), relapse mouse 2906 (red) or AML246-Cherry parental control cells (black) showing the rate of disease establishment. (d) GFP profile of AML cells in the blood of representative mice from each subgroup at the commencement of Dox treatment (untreated) and after 7 days of Dox treatment. (e) Kaplan-Meier survival analysis of mice from c over 2 months of sustained Dox treatment initiated upon disease establishment at day 0. p=0.017 for relapse 2906 or relapse 3315 versus parental control, Mantel-Cox test. Source data are provided as a Source Data file.



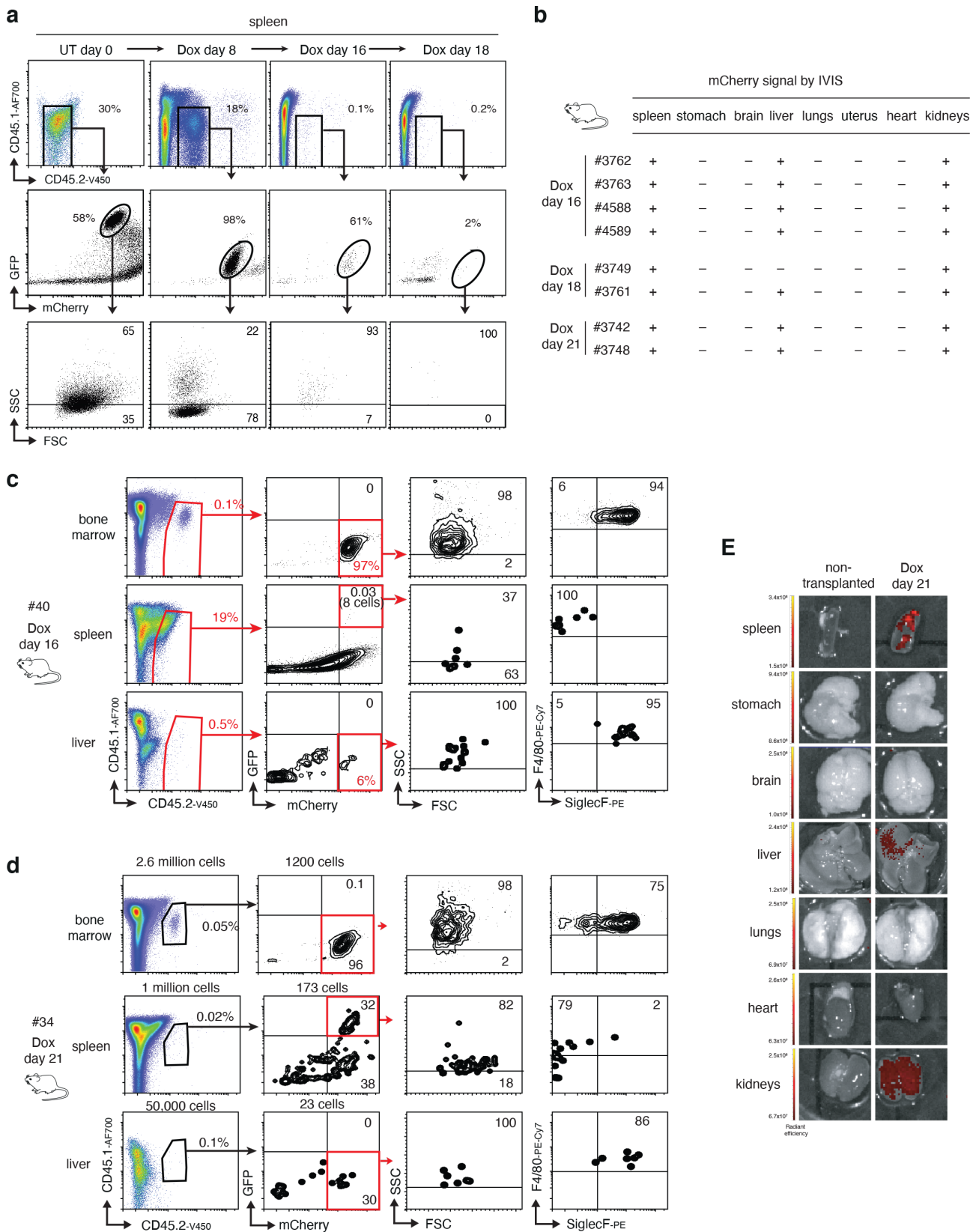
Supplementary Figure 2. Bifurcated AML differentiation in multiple organs following Dox treatment. (a) Flow cytometry analysis of mCherry and GFP in the blood, bone marrow, spleen, liver and kidneys of *Rag1*^{-/-} mice with established AML246 that were either untreated or Dox treated as indicated. (b) Flow cytometry analysis of the bone marrow of the 8 day Dox treated mouse shown in Fig. 2b, comparing the immunophenotype of AML-derived (mCherry⁺) SSC^{LOW}

and SSC^{HIGH} populations to host (mCherry⁻) SSC^{LOW} and SSC^{HIGH} populations containing normal CD11b⁺Ly6G⁺ neutrophils and F4/80^{HIGH}SiglecF⁺ eosinophils respectively. (c) Wide field cytopins of sorted mCherry⁺SSC^{LOW} and mCherry⁺SSC^{HIGH} cells from the spleen of the 8 day Dox treated mouse shown in Fig. 2b. Data are representative of 2 independent experiments with similar results.



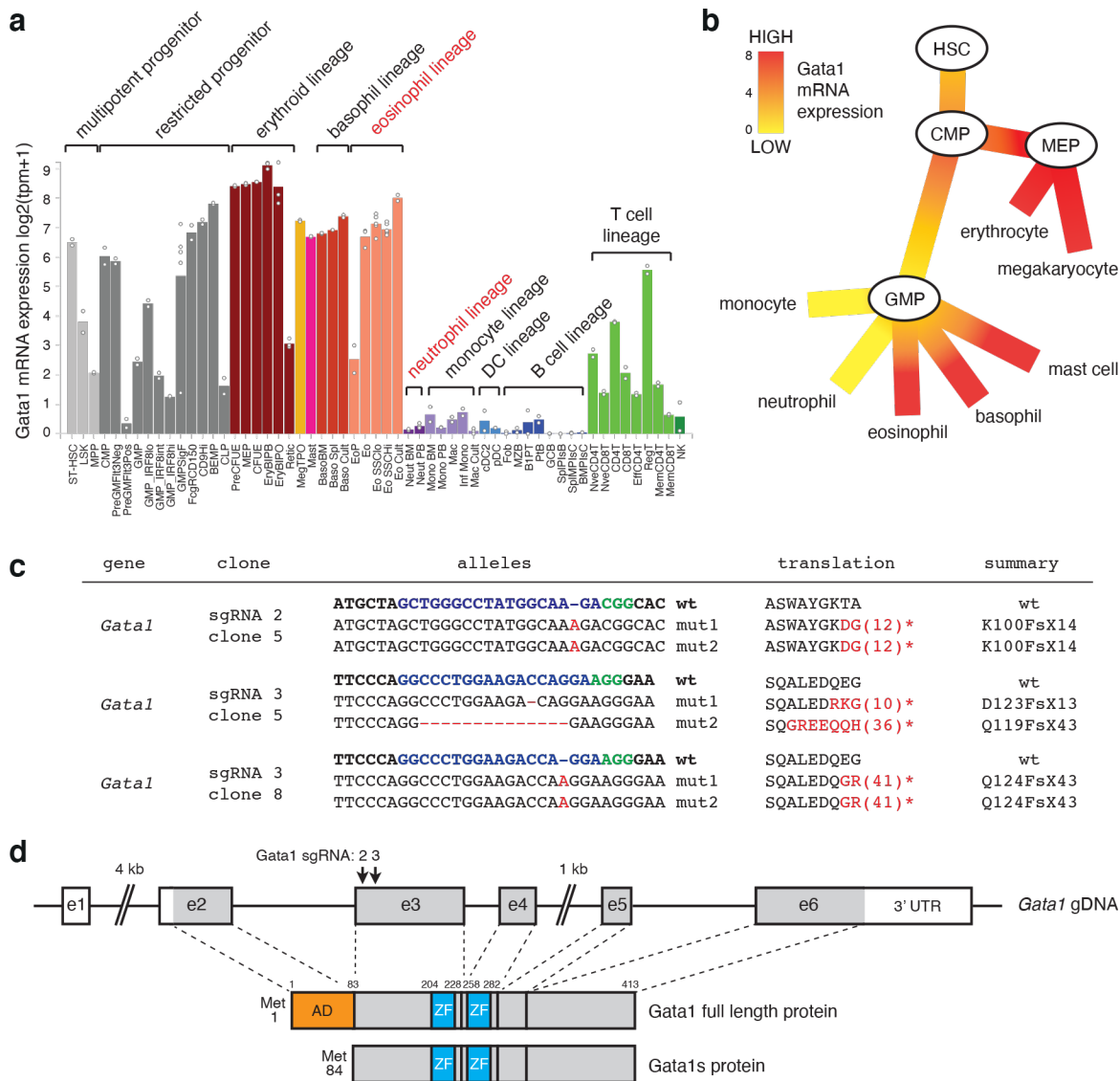
Supplementary Figure 3. Dox-induced AML246 bifurcation in several *in vivo* contexts. (a) Flow cytometry analysis of blood and spleen from the 8 day Dox treated mouse shown in Fig. 2b.

Data are representative of 3 independent experiments with similar results. (b) Quantitative analysis showing reproducible AML246 bifurcation in the bone marrow and spleen. Mean \pm SD, n=9 mice over 3 independent experiments. (c) Analysis of bone marrow from mice transplanted with AML246 Clone 2 (noting that AML246-Cherry was derived from AML246 Clone 1¹). Data are representative of 2 independent mice with similar results from a single experiment. (d) Flow cytometry analysis of *Rag1*^{-/-} mice with established AML246 treated with Dox for 5 days, showing early bifurcation of AML-derived cells into SSC^{HIGH} and SSC^{LOW} populations.

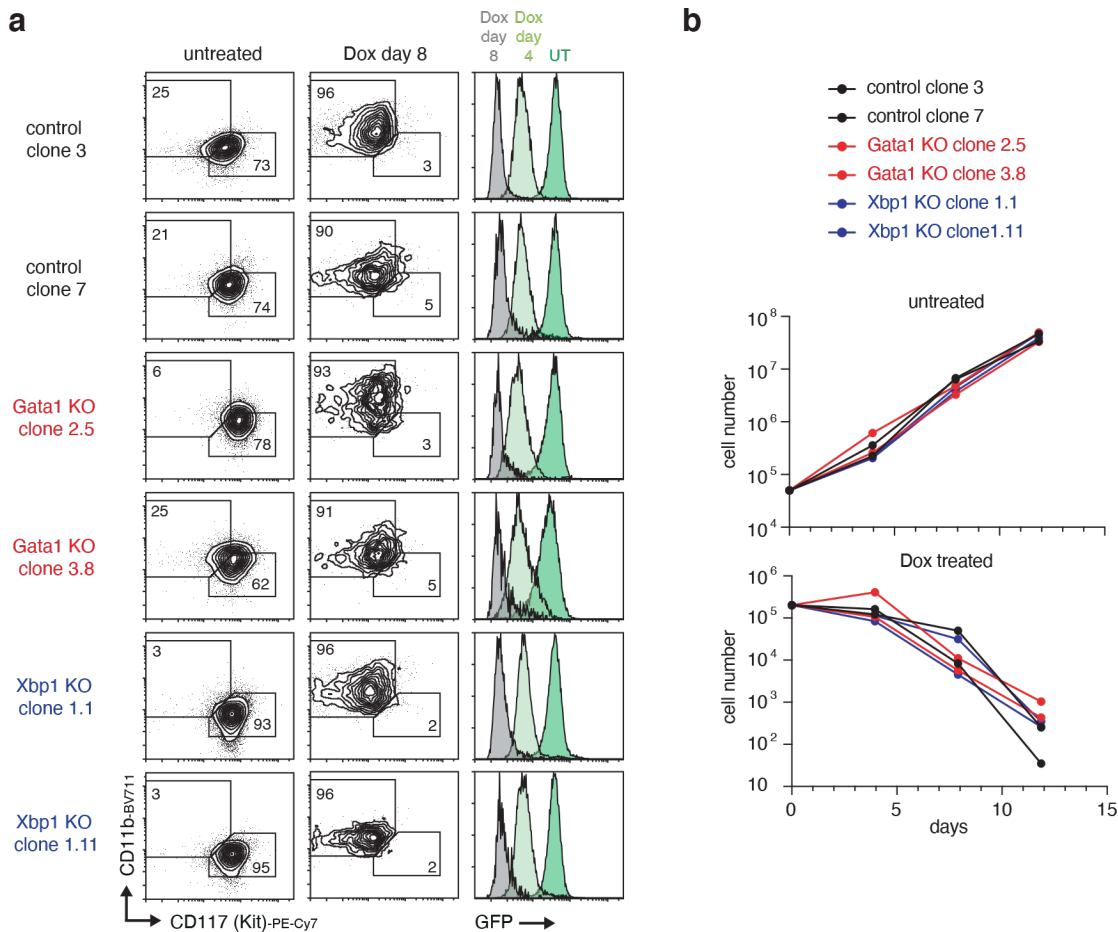


Supplementary Figure 4. Analysis of 16-21 day Dox treated mice in remission. (a) FSC/SSC profile of AML-derived cells harvested from the spleen of representative AML246-Cherry leukemic mice during an 18 day Dox treatment time course (same mice shown in Fig. 3a), showing viable AML-derived cells identified based on surface CD45.2 and mCherry. (b) Summary of IVIS organ imaging of 16-21 day Dox treated mice. (c) Flow cytometry analysis of AML-derived cells identified based on surface CD45.2 and mCherry in the bone marrow, spleen, and liver of mouse #40, showing early relapsing mCherry⁺GFP^{HIGH}SiglecF⁻ cells specifically in the spleen. Note that

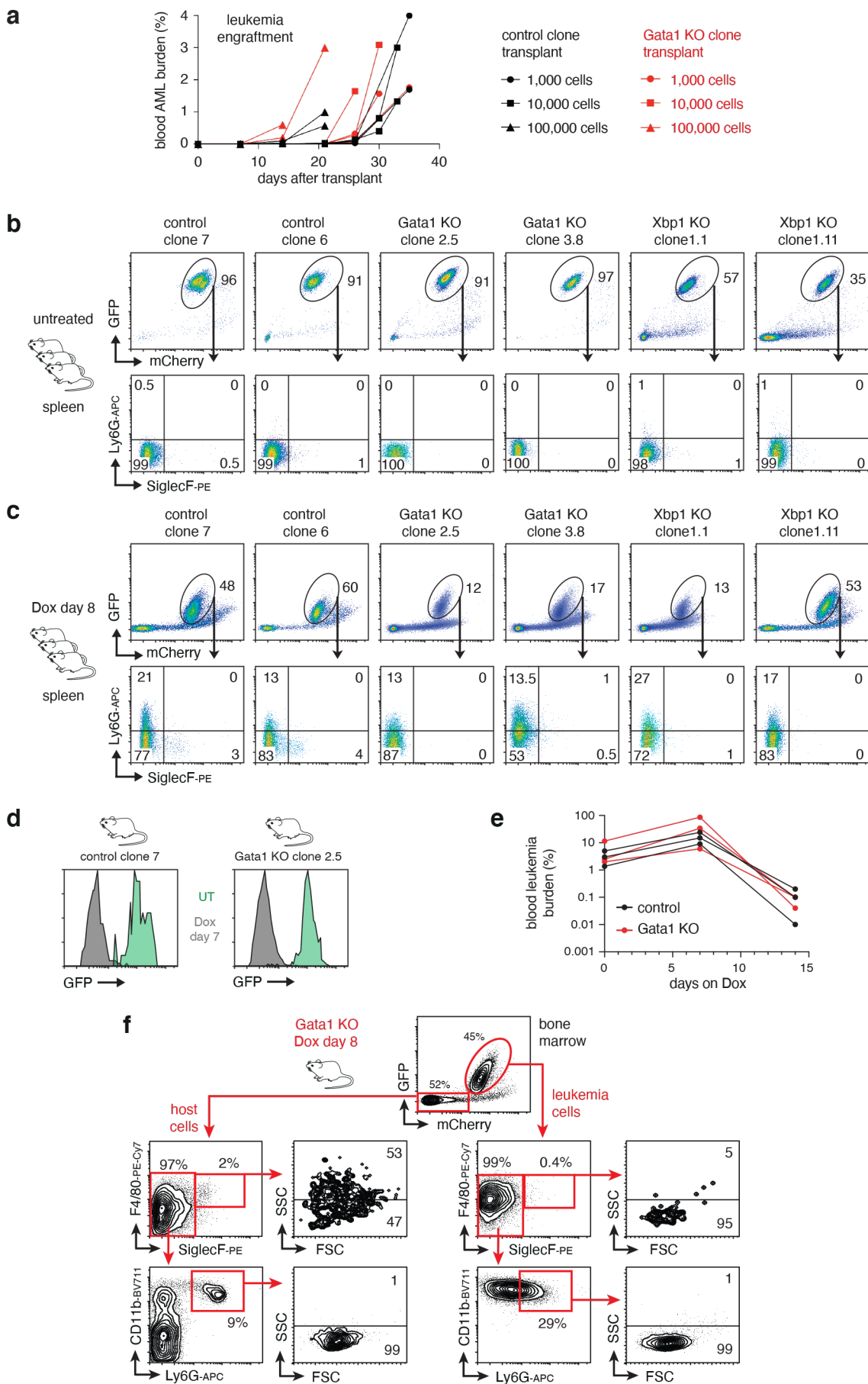
bone marrow and liver are gated on mCherry⁺GFP⁻ cells, whereas spleen is gated on mCherry⁺GFP⁺ early relapse cells. (d) Flow cytometry analysis of bone marrow, liver, and spleen from day 21 Dox treated mouse #34 with AML-derived cells identified based on surface CD45.2 and mCherry. Note that bone marrow and liver are gated on mCherry⁺GFP⁻ cells, whereas spleen is gated on mCherry⁺GFP⁺ early relapse cells. (e) IVIS mCherry imaging of organs from day 21 Dox treated mouse #3742 where GFP⁺mCherry⁺ early relapse cells were detected in the spleen by flow cytometry, showing mCherry signal restricted to the spleen, kidneys and liver. At each time point leukemic mouse organs were imaged alongside matched control organs from a non-transplanted *Rag1*^{-/-} mouse for signal calibration.



Supplementary Figure 5. Characterization of *Gata1* knockout AML246-Cherry clones. (a) Mouse *Gata1* expression across hematopoietic cell types from Haemosphere, associated with the Haemopedia database ². (b) Schematic representation of mouse *Gata1* expression across myeloid lineages based on data from a. (c) Sequence analysis of three independent AML246-Cherry *Gata1* KO clones showing biallelic genomic DNA mutations (mut1 and mut2) and their predicted protein products. sgRNA target sites are blue and PAM sequences are green. (d) Schematic of the *Gata1* gene and its encoded full length *Gata1* and *Gata1s* proteins.



Supplementary Figure 6. Gata1 and Xbp1 knockout clone analysis *in vitro*. (a) Flow cytometry analysis of the Dox response of clones in culture, showing similar CD11b induction, CD117 (Kit) repression, and GFP repression for control, Gata1, and Xbp1 knockout AML246 cells. (b) Time course showing proliferation of untreated and Dox-treated clones in culture. Average of 3 technical replicates per clone.

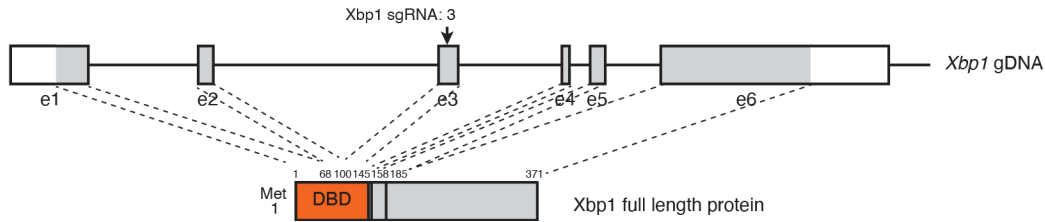


Supplementary Figure 7. Gata1 and Xbp1 knockout clone analysis *in vivo*. (a) Blood leukemia burden indicating engraftment following transplant of a dilution series of control and Gata1 knockout AML246 cells as indicated. Two mice were transplanted for each condition. (b-c) Flow

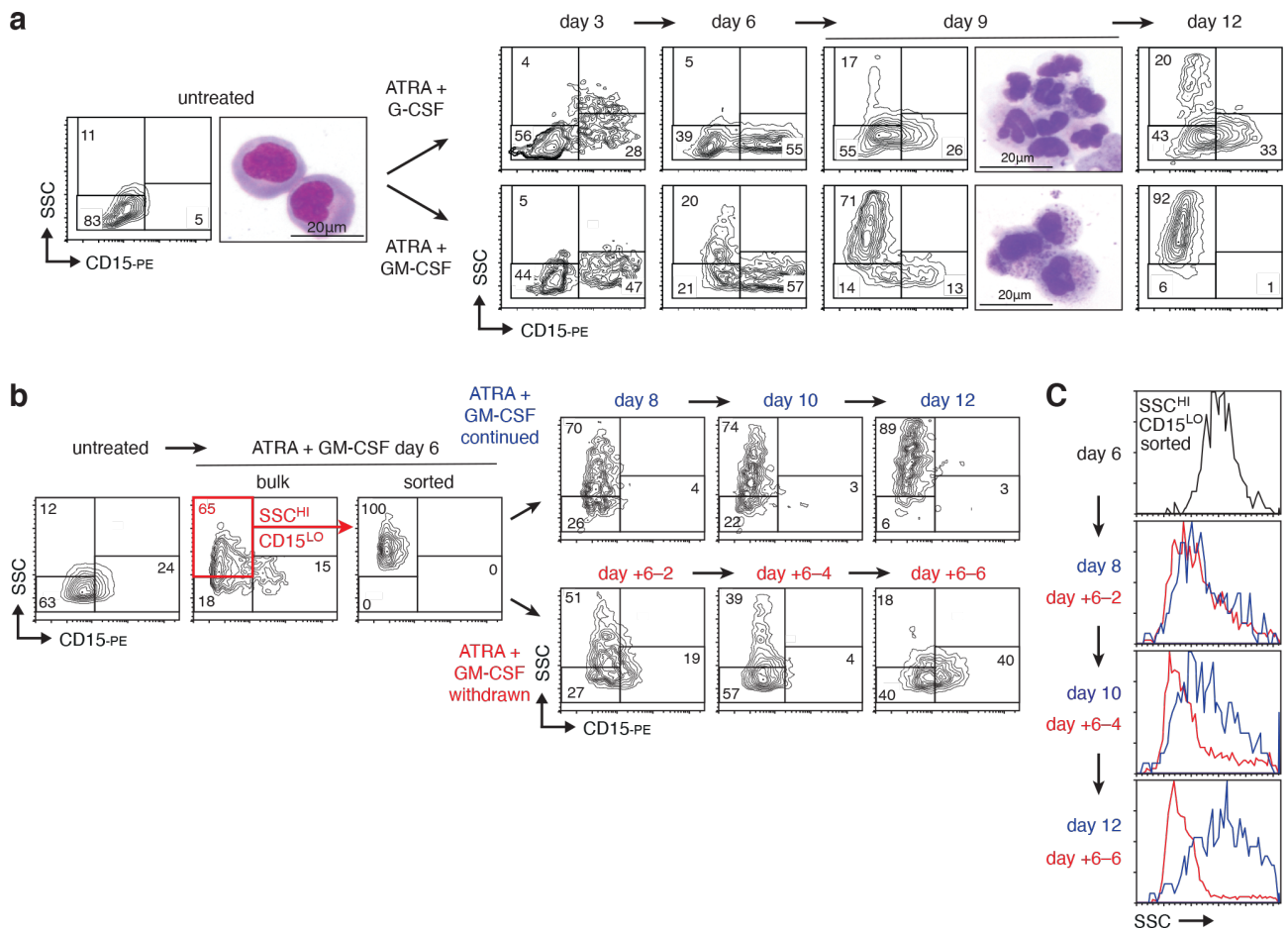
cytometry analysis of splenocytes from mice with established disease following transplant of the indicated control or knockout clones, then either untreated (b) or following 8 days Dox (c). Leukemia gates are indicated in the upper plots. (d) Peripheral blood GFP profile of mice transplanted with the indicated clones and left untreated (UT; green) or Dox treated for 7 days (grey). (e) Leukemia burden in the blood of mice with established disease following transplant of control or Gata1 knockout clones then treated with Dox over a 14 day time course. (F) Flow cytometry analysis of a representative *Rag1*^{-/-} mouse with established Gata1 KO AML246-Cherry after 8 days Dox treatment, comparing the immunophenotype of AML-derived (mCherry⁺) populations to host (mCherry⁻) neutrophils (SSC^{LOW}Ly6G⁺) and eosinophils (SSC^{HIGH}SiglecF⁺).

a

gene	clone	alleles		predicted translation	summary
<i>Xbp1</i>	3.1	ACCAGGAGTTAAGAACACGCTTGGGAATGGACACGCTGG	wt	QELRTRLGMDTLDP	wt
		ACCA-----AATGGACACGCTGG	mut 1	QDTLDPDEVPEVEA (227)	Q124delX241
		ACCAGGA-----ATGGACACGCTGG	mut 2	QEWTRWILTRFQR (7)	E125FsX19
<i>Xbp1</i>	3.11	ACCAGGAGTTAAGAACACGCTTGGGAATGGACACGCTGG		QELRTRLGMDTLDP	wt
		ACCAGGAGTTAAG-----GAATGGACACGCTGG	mut 1	QELRNGHAGS-	R127FsX7
		ACCAGGAGTTAAGAACACGC-----TGG	mut 2	QELRTRWILTRFQR (8)	R129FsX16

b

Supplementary Figure 8. Characterization of Xbp1 knockout AML246-Cherry clones. (a) Sequence analysis of two independent AML246-Cherry *Xbp1* KO clones showing biallelic (mut1 and mut2) genomic DNA mutations and their predicted protein products. sgRNA target sites are blue and PAM sequences are green. (b) Schematic of the *Xbp1* gene and its encoded protein.



Supplementary Figure 9. Reversion of mature human HT93-derived eosinophils to an immature proliferative state. (a) SSC/CD15 profiles (matching the SSC/CD11B profiles in Fig. 8a) of HT93 cells during ATRA treatment combined with G-CSF (upper panels) or GM-CSF (lower panels). Representative cytopspins are shown for untreated (UT) and ATRA day 9 treatments. This experiment was performed once. (b) SSC/CD15 profiles (matching the SSC/CD11B profiles in Fig. 8b) of SSC^{HIGH}CD11B⁺CD15⁻ cells sorted from ATRA+GM-CSF cultures then either maintained on treatment (upper panels) or following ATRA/GM-CSF withdrawal (lower panels). (c) SSC profile of cells from b, showing progressive return to an immature state following ATRA/GM-CSF withdrawal.

SUPPLEMENTARY REFERENCES

1. McKenzie MD, *et al.* Interconversion between Tumorigenic and Differentiated States in Acute Myeloid Leukemia. *Cell Stem Cell* **25**, 258-272 e259 (2019).
2. Choi J, *et al.* Haemopedia RNA-seq: a database of gene expression during haematopoiesis in mice and humans. *Nucleic Acids Res* **47**, D780-D785 (2019).

Supplementary Table 1. Mice transplanted with AML246-Cherry used in this study.

mouse ID	Figures	time point	mouse ID	Figures	time point
3393	1B	Dox day -14	4588	4A, 4B, S4B	Dox day 16
77	1B	UT	4589	4B,S4B	Dox day 16
3391	1B, S1B	Dox day 7	39	4B,4C	Dox day 16
3410	1B	Dox day 14	40	4B,4C,S4C	Dox day 16
3316	1B	Dox day 35	3749	3A,3B,4B,4C,S4A, S4B	Dox day 18
3315	1B-1D, S1B	relapse	3761	3B,3D,4A,4B S4B	Dox day 18
3341	1C, 1D,S1A, S1B	relapse	3742	4B,S4B	Dox day 21
3158	1C, 1D, S1B	relapse	3748	4B,S4B	Dox day 21
3589	1C	relapse	1 (sn23)	4B	Dox day 21
3327	1C, 4A	relapse	2 (sn23)	4B	Dox day 21
3302	1C	relapse	5 (sn23)	4B	Dox day 21
3324	1C	relapse	45	4A,4B	Dox day 21
3568	1C	relapse	34	4B,S4D	Dox day 21
3592	1C	relapse	41	4B	Dox day 21
3591	1C	relapse	3404	S1A, S1B	UT
3440	1C	relapse	3501	S1B	UT
8 (sn25)	1C	relapse	3298	S1B	UT
3584	1C	relapse	3403	S1A, S1B	Dox day 7
3687	2B, S2B, S2C, S3A, S3B	Dox day 8	3406	S1B	Dox day 7
4443	2C, S2A	Dox day 10	4446	S1C	UT
82	2A	UT	4444	S1C	Dox day 7
31	2D	UT	1 (sn25)	S1C, S1E	UT and KM
12	2D	UT	2 (sn25)	S1C, S1E	UT and KM
20	2D	Dox day 5	3 (sn25)	S1C, S1E	UT and KM
23	2D	Dox day 5	4 (sn25)	S1C, S1D	UT and Dox day 7
7	3D, 4A	Dox day 10	5 (sn25)	S1C,S1D	UT and KM
3920	3A-3D, 4A	UT	6 (sn25)	S1C	UT
1	3B	UT	7 (sn25)	S1C, S1D	UT and Dox day 7
3918	3B, S4A	UT	8 (sn25)	S1C, S1E	UT and KM
3692	3A-3B, S3B,S4A	Dox day 8	9 (sn25)	S1C, S1D	UT and Dox day 7
3693	3B, 4A, S3B	Dox day 8	10 (sn25)	S1C, S1E	UT and KM
3762	3B,S4B	Dox day 16	11 (sn25)	S1C, S1D	UT and Dox day 7
3763	3A,3B,4B, S4A,S4B	Dox day 16	12 (sn25)	S1C, S1D	UT and Dox day 7
3768	S3B	Dox day 8	13 (sn25)	S1C, S1E	UT and KM
30	S3B	Dox day 8	14 (sn25)	S1C, S1E	UT and KM
3784	S3B	Dox day 8	15 (sn25)	S1C, S1E	UT and KM
3	S3B	Dox day 8	4446	S2A	UT
27	S3B	Dox day 8	4444	S2A	Dox day 7
0	S3B	Dox day 8			
4151	S3C	UT			
2	S3C	Dox day 10			
4	S3D	Dox day 5			

Supplementary Table 2. Detection of early relapse mCherry+GFP+ cells following 7-14 day Dox treatment. The number of GFP+ cells per total cells assessed by flow cytometry within each organ is shown. The GFP+ threshold was set using a GFP-HIGH control sample harvested from an untreated mouse on the same day, adjusted so that 96% of these cells were in the GFP+ gate. Dox-treated mice where mCherry+GFP+ cells were part of a GFP continuum at the tail end of the bulk leukemia profile (prevalent at Dox day 7 where bulk GFP repression is incomplete) in at least one of the tissues analyzed are indicated in blue. Dox-treated mice where mCherry+GFP+ cells were a discrete population clearly distinct from the bulk leukemia (prevalent at Dox days 10-14 where the bulk leukemia is GFP-LOW) in at least one of the tissues analyzed are indicated in red.

Dox day 7	number of mCherry+GFP+ cells		
mouse ID	blood	bone marrow	spleen
3406	0/10,000	0/11,000	0/13,000
3391	0/9000	1/11,000	0/14,000
3903	ND	1/10,000	1/12,000
3693	0/40,000	0/60,000	0/210,000
3692	0/21,000	1/51,000	1/215,000
3687	0/26,000	0/55,000	0/175,000
10	1/11,000	0/10,000	0/10,000
3907	0/10,000	15/230,000	27/30,000
3900	0/10,000	19/130,000	35/200,000
3899	0/11,000	34/220,000	18/64,000
3932	ND	0/90,000	0/13,000
3924	ND	1/20,000	1/16,000
4444	0/200,000	30/1,000,000	14/740,000
Dox day 10	number of mCherry+GFP+ cells		
mouse ID	blood	bone marrow	spleen
20	0/76,000	2/22,000	2/200,000
3793	0/150,000	0/370,000	0/150,000
24	ND	1/470,000	0/72000
5	0/15,000	0/350,000	0/350,000
6	0/25,000	0/276,000	1/161,000
4	0/55,000	2/755,000	1/150,000
13	0/8,000	1/8,000	0/13,000
18	0/8,000	5/9,000	0/8,000
3503	0/64,000	0/60,000	0/20,000
3390	0/5,000	0/20,000	0/40,000
3380	0/8,000	0/11,000	0/21,000
3405	0/5,000	0/21,000	0/26,000
4569	ND	5/719,000	0/132,000
4443	0/130,000	35/2,000,000	11/600,000
4603	0/300,000	5/160,000	10/3,750,000
Dox day 14	number of mCherry+GFP+ cells		
mouse ID	blood	bone marrow	spleen
3410	0/9,000	0/11,000	0/17,000
3289	0/11,000	0/5,000	1/13,000
3395	0/55,000	0/11,000	1/20,000
3764	1/400,000	239/1,000,000	60/300,000
3757	0/340,000	20/1,000,000	30/500,000
4567	2/23,000	6/1,000,000	130/300,000
4568	0/8,000	3/1,000,000	170/800,000

Supplementary Table 4. Primer and sgRNA sequences. All sequences are 5' to 3'.

Description	Sequence
Control sgRNA	Forward: caccTAGCGAACGTGTCCGGCG Reverse: aaacCGCCGGACACGTTTCGCTA
Gata1 sgRNA 2	Forward: caccGCTGGGCCTATGGCAAGA Reverse: aaacTCTTGCCATAGGCCAGC
Gata1 sgRNA 3	Forward: caccGGCCCTGGAAGACCAGGA Reverse: aaacTCTGGTCTTCCAGGGCC
Gata1 gDNA PCR	Forward: AACTCCGAAGTCACCCAAGCAG Reverse: AGGATGGGAGAACATGGGTT
Gata1 cDNA PCR	Forward: GTTCAACCCCAGTGTTCCA Reverse: AAGGGGCCAATGGCAGG
Gata1 RT-qPCR	Forward: GGGATCACCTGAACTCGTC Reverse: GGTTGAACCTGGGCTTGTTG
Rn18s RT-qPCR	Forward: GTAACCCGTTGAACCCATT Reverse: CCATCCAATCGGTAGTAGCG
SiglecF RT-qPCR	Forward: GGTCTCACAGGTGAAGGTCC Reverse: GGCAAGATGGTTGCCTTTCG
Prg3 RT-qPCR	Forward: CCCTTGGGTAGTGAGTGCTG Reverse: GTCCAACGCAGCTTCTATGC
Epx RT-qPCR	Forward: CTGCTTAGCTGTAGTGGGGG Reverse: TGTGAGCACATCAGTGGCAT
Ly6G RT-qPCR	Forward: AGAGGAAGTTTTATCTGTGCAGCC Reverse:TCAGGTGGGACCCCAATACA
Coro1a RT-qPCR	Forward: GGGCTGAGTCCCCATTAAG Reverse: GAGACGCGCACATCCTCATA
Spi1 RT-qPCR	Forward: CTGGAGCTCAGCTGGATGTTAC Reverse: GCCATCAGCTTCTCCATCAGA
Xbp1 sgRNA 3	Forward: caccGGAGTTAAGAACACGCTT Reverse: aaacAAGCGTGTTCTTAACTCC
Xbp1 gDNA PCR	Forward: CTCTGTCCCATTAGCCACCG Reverse:TGAATTTTCCTGTTTCCTTGA

Supplementary Table 5. shRNA oligonucleotide sequences. All sequences are 5' to 3'.	
shRNA	Oligonucleotide sequence
Renilla.713	Forward: TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGC CACAGATGTATAGATAAGCATTATAATTCCTATGCCTACTGCCTCGGACTTCAAGGGGCTAG
	Reverse: AATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCATAGGAATTATAATGCTTATCTATACATCTGT GGCTTCACTATAGATAAGCATTATAATTCCTGCGCTCACTGTCAACAGCAATATACCTTC
Gata1.1377	Forward: TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCTGTAGCTATGTAGCTATGAATAGTGAAGC CACAGATGTATTCATAGCTACATAGCTACAGATGCCTACTGCCTCGGACTTCAAGGGGCTAG
	Reverse: AATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCATCTGTAGCTATGTAGCTATGAATACATCTG TGGCTTCACTATTCATAGCTACATAGCTACAGGCGCTCACTGTCAACAGCAATATACCTTC
Gata1.1388	Forward: TCGAGAAGGTATATTGCTGTTGACAGTGAGCGATAGCTATGAACTATGTAGATATAGTGAAGC CACAGATGTATATCTACATAGTTCATAGCTACTGCCTACTGCCTCGGACTTCAAGGGGCTAG
	Reverse: AATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCAGTAGCTATGAACTATGTAGATATACATCTG TGGCTTCACTATATCTACATAGTTCATAGCTATCGCTCACTGTCAACAGCAATATACCTTC