Myc-Miz-1 signaling promotes self-renewal of leukemia stem cells by repressing Cebpα and Cebpδ

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Materials and methods (Additional)

Plasmid generation and virus production. High-titer viruses expressing specific genes of interest were generated by co-transfection of HEK293T cells with packaging plasmids and retroviral/lentiviral plasmids containing the genes, respectively, using the *Calphos* mammalian transfection kit (Clontech). Retroviral/lentiviral supernatants were harvested 24 and 48 hours after transfection. After filtration by passage through a 0.45μM filter, retroviral supernatants were aliquoted and frozen at -80°C. Retroviral/lentiviral titers were determined by infecting 3T3 cells. *pCDNA3-human c-Myc^{V394D}* and *pCDNA3-HA-HA-human c-Myc* plasmids were purchased from Addgene and were sub-cloned into *MSCV-IRES-GFP or MSCV-IRES-mCherry* to generate *MSCV-Myc^{V394D}-IRES-GFP*, *MSCV-Myc-IRES-GFP*, *Myc-mCherry* and *Myc^{V394D}-mCherry* plasmids. *MSCV-mCEBPα-IRES-GFP* and *MSCV-mCEBPδ-IRES-GFP* viral plasmids were generated in our laboratory as described.[1] *Cebpα*-mcherry set shRNA Lentivector (mouse) and *Cebpδ*-mcherry set shRNA Lentivector (mouse) were purchased from Applied Biological Materials (ABM). *MSCV-MLL-AF9-Puro* plasmid was described previously. *MSCV-Runx1-Eto9a-GFP* plasmid (MigR1-AE9a, #12433) was purchased from Addgene. *MSCV-PML-RARa-GFP* plasmid was kindly provided by Dr. Jian Zhu of Tongji University of Shanghai.

Assessment of self-renewal and frequency of LSCs. *Myc-GFP*- and *Myc^{V394D}-GFP* AML cells were collected from the spleens of leukemic mice. After endogenous *Myc* deletion was confirmed by PCR assay, serial transplantations were conducted to assess the self-renewal capacity of the LSCs. In this study, each recipient mouse received 10^6 AML cells without pre-conditioning and each was then monitored for leukemia development. Serial dilution/competitive transplantation assays were conducted to examine the frequency of LSCs. Briefly, AML cells were diluted serially and mixed with support BM cells and transplanted into lethally-irradiated recipient mice. Each

mouse received 100, 300, 1,000, 10,000 or 50,000 AML cells plus 2×10^5/ml support BM cells. The frequency of LSCs was analyzed by ELDA software.
(http://bioinf.wehi.edu.au/software/elda/).

Study the role of the* Myc V394D *mutation in the pathogenesis of other leukemic fusion gene-induced AML. We co-transduced *Cre^{ERT}Myc$^{fx/fx}$* HSPCs with either *MSCV*-mcherry, *Myc*-mCherry, or *Myc^{V394D}*-mcherry plus *PML-RARa-GFP* and *RUNX1-ETO9a-GFP*, respectively. The transduced HSPCs were purified and seeded for serial replating CFU assay with or without 4-OHT treatment. We found that without 4-OHT treatment, *Myc* and *Myc^{V394D}* transductions significantly enhanced the CFC for both *PML-RARa*- and *RUNX1-ETO9a*-HSPCs in the 1st plating when compared to *MSCV*-transduction. However only *Myc* transduction maintained the high CFC during serial plating, while the CFC in both *MSCV and Myc^{V394D}* transductions were reduced in the 2nd and 3rd replatings. In the 4-OHT treated groups, the *MSCV*-transduced cells failed to generate CFC, while the CFC in *the Myc^{V394D}*-transduction condition was significantly reduced in the 2nd and 3rd replatings when compared to the *Myc*-transduced group.

RNAseq: *Myc/Cre^{ERT}Myc$^{fx/fx}$* and *Myc^{V394D}/Cre^{ERT}Myc$^{fx/fx}$* AML cells were treated with 4-OHT to induce the deletion of the endogenous *Myc* gene. On day 4, after complete *Myc* deletion was confirmed, AML cells were collected and mRNA was extracted. RNAseq was conducted by Novogene Corporation Inc. Raw data FASTQ files were aligned to the mouse genome (mm9) using Tophat (version 2.0) and Bowtie2[2]. Gene expression profile for the individual samples was calculated as FPKM (paired-end fragments per kilobase of exon model per million mapped reads) values. The Gene Ontology analysis was carried out with the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (https://david.ncifcrf.gov/, Version 6.8).[3] The differential expression cluster for the heatmap was generated using JavaTreeview.[4] The RNAseq dataset was deposited to the Gene Expression Omnibus archive (accession no. GSE70899) and GEO number (GSE139143).

Chromatin immunoprecipitation (ChIP): Twenty thousand **c**ells were fixed with 0.75% formaldehyde and incubated with glycine (50mM final concentration) for 10 min. After washing with cold PBS, the cells were resuspended into ChIP lysis buffer and incubated for 10 min. on ice

followed by sonication to shear chromatin into an average fragment size of 200–1000 bp. After centrifugation for 10 min. at 4°C., 8,000 × g, cell supernatant was transferred to a new tube and the protein/DNA complexes were collected by immunoprecipitation (IP) with antibodies listed in **Table 2**. The *CEBPα* and *CEBPδ* promotors in the protein/DNA complexes were detected by q-PCR assay using the primers listed in **Table 3.**

Table 1. Primers used for mouse genotyping

c-myc 1		GCCCCTGAATTGCTAGGAAGACTG
c-myc 2	500bp	CCGACCGGGTCCGAGTCCCTATT
c-myc 3		TCGCGCCCCTGAATTGCTAGGAA
c-myc 4	379bp	TGCCCAGATAGGGAGCTGTGATACTT

Table 2. Antibodies for ChIP and Western blotting:

Miz1 (10E2, Santa Cruz),

Myc (N262, Santa Cruz),

$H3K9^{Me3}$ (D4W1U) Rabbit mAb #13969,

$H3K9^{Ac}$ (C5B11) Rabbit polyclonal (ab4441)

Anti-EHMT2/G9A antibody (ab229455)

Control normal mouse IgG.

Control normal Rabbit IgG.

Table 3. Primers for *Cebpα* and *δ* promoters.

***Cebpδ* proximal promoter** (region **-226 to -24)**

5'-GCGTGTCGGGGCCAAATCCA-3' (forward primer)

5'-TTTCTAGCCCCAGCTGACGCGC-3' (reverse primer);

***Cebpδ* distal promoter (**P1.8K region, **-1856 to -1676)**:

5'-TGCTTCTATGGCATCCAG-3' (forward primer)

5'-GAGGGGCTGTGGAATATT-3' (reverse primer).

***Cebpα* proximal promoter (**region **-144 to -322):**

5′-CTG GAA GTG GGT GAC TTA GAG G-3′(forward primer)

5′-GAG TGG GGA GCA TAG TGC TAG-3’. (reverse primer).

Cebpα **distal promoter: (region -1361 to -1200)**

(−1422 to −1121 upstream from transcription start site)

5’-GACAAATGGAG TTCGAGAGGACG-3’ (forward primer)

5’-GTAGGAGTTTGCGTGCATCGG-3’ (reverse primer).

Table 4. PCR primers and probes for Taqman qRT-PCR were purchased from ThermoFisher scientific

Cdkn1a (p21): Mm00432448_m1

Cdkn1b (p27): Mm00438168_m1

Cdkn2a (p16)：Mm00494449_m1

Cdkn2b (p15): Mm00483241_m1

Cdkn2d (p19): Mm00486943_m1

Flt3: Mm00439016_m1

c-Kit: Mm00445212_m1

Il13ra1: Mm01302068_m1

IL1r1: Mm00434237_m1

IL3ra: Mm00434273_m1

Il1rap: Mm00492638_m1

Evi (Mecom): Mm00491303_m1

Bcl2: Mm00477631_m1

Gata2: Mm00492301_m1

Pbx3: Mm00479413_m1

Gpr56 (Adgrg1): Mm00817704_m1

Abcg1: Mm00437390_m1

Gapdh: Mm99999915_g1

actb (beta-actin): Mm02619580_g1

Supplementary data:

Figure S1. Schematic diagram of the experimental procedures for the studies in Figures 1 and 2. **a**. BM c-Kit$^+$ HSPCs were isolated from wild type C57Bl6/J mice and transduced with *MSCV*-GFP, *Myc-GFP* or *Myc^{V394D}-GFP*, respectively. The transduced Kit$^+$ HSPCs were purified by FACS 2 days post-transduction and used for *in vitro* studies in **Figure 1** and *in vivo* studies in **Figure 2**. **b.** The expression of Myc in the transduced HSPCs was examined on both days 5 and 7 post transduction by Western blotting. **c.** HSPCs were transduced with *Flag-Myc* or *Flag-Myc^{V394D}* and purified. The interaction of Miz1 with Myc or Myc^{V394D} was examined by immunoprecipitation assay.

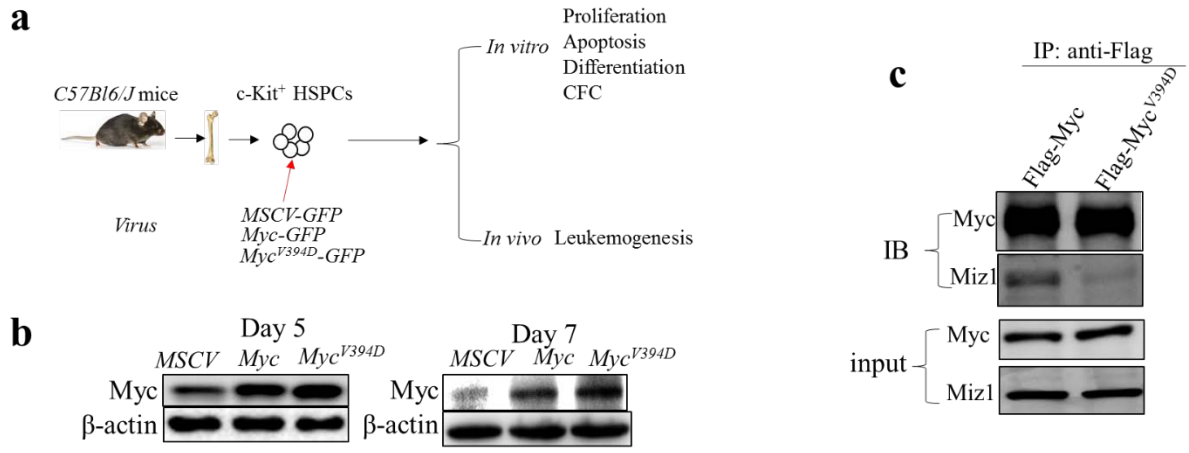


Figure S2 (associated with Figure 1). Proliferation and differentiation analysis of transduced HSPCs. c-Kit^{+} HSPCs were transduced with *MSCV*-GFP, *Myc-GFP* or *Myc^{V394D}-GFP*, respectively. The transduced Kit^{+} HSPCs were purified by FACS 2 days post-transduction and incubated in HSPC culture medium with medium change every other day. Proliferation of the transduced HSPCs was examined by BrdU pulse-labelling (**a**), Ki67 staining (**b**), and cell cycle analysis (**c**) at the indicated time points. Differentiation was evaluated by morphology (**d**). In **e**, the proliferation of Kit^{+} and Kit^{-} HSPCs was examined separately on day 5 by both BrdU pulse-labelling and Ki67 staining.

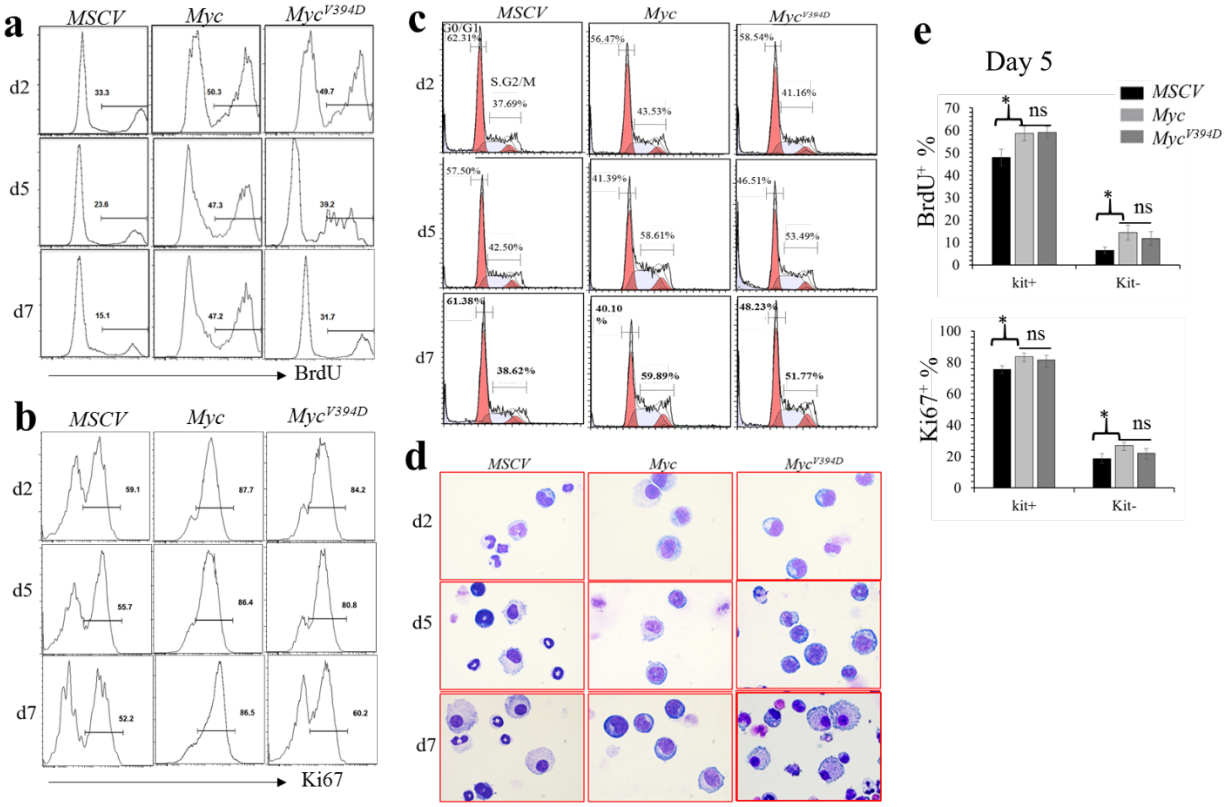


Figure S3 (associated with Figure 1). Apoptosis analysis of transduced HSPCs. c-Kit^{+} HSPCs were transduced with *MSCV*-GFP, *Myc-GF*P or *Myc*^{V394D}*-GFP*, respectively. The transduced Kit^{+} HSPCs were purified by FACS 2 days post-transduction and incubated in HSPC culture medium with medium change every other day. **a-b**. Apoptosis of the transduced HSPCs was examined by Annexin-V staining, P.I. staining and flow cytometry (**a**). The percentages of apoptotic cells are summarized (**b**). **c-d**. Bcl2 expression in the transduced HSPCs was examined by Western blotting on day 5 of culturing (**c**) and quantitated (**d**).

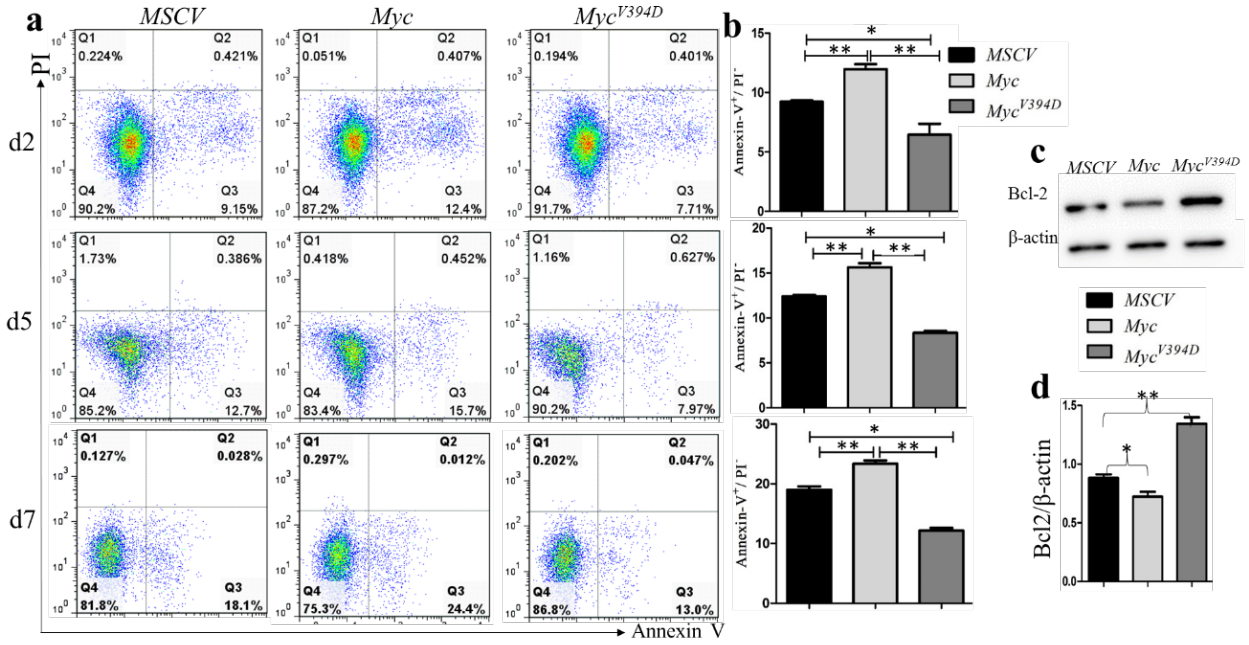


Figure S4 (associated with Figure 1). Analysis of the CFC in the transduced HSPCs. c-Kit^{+} HSPCs were transduced with *MSCV*-GFP, *Myc-GF*P or *Myc*^{V394D}*-GFP*, respectively. The transduced Kit^{+} HSPCs were purified 2 days post-transduction and seeded into methylcellulose medium for serial replating CFU assay. **a.** Cells were collected from CFUs on day 7 of each plating and differentiation of the cells was examined by flow cytometry for Kit^{+}Gr1^{low} and Gr1^{+} phenotypes. **b.** The percentages of BFU-E, CFU-GM and CFU-GEMM were quantitated for the 1st plating. **c.** CFUs from the 2rd plating were imaged.

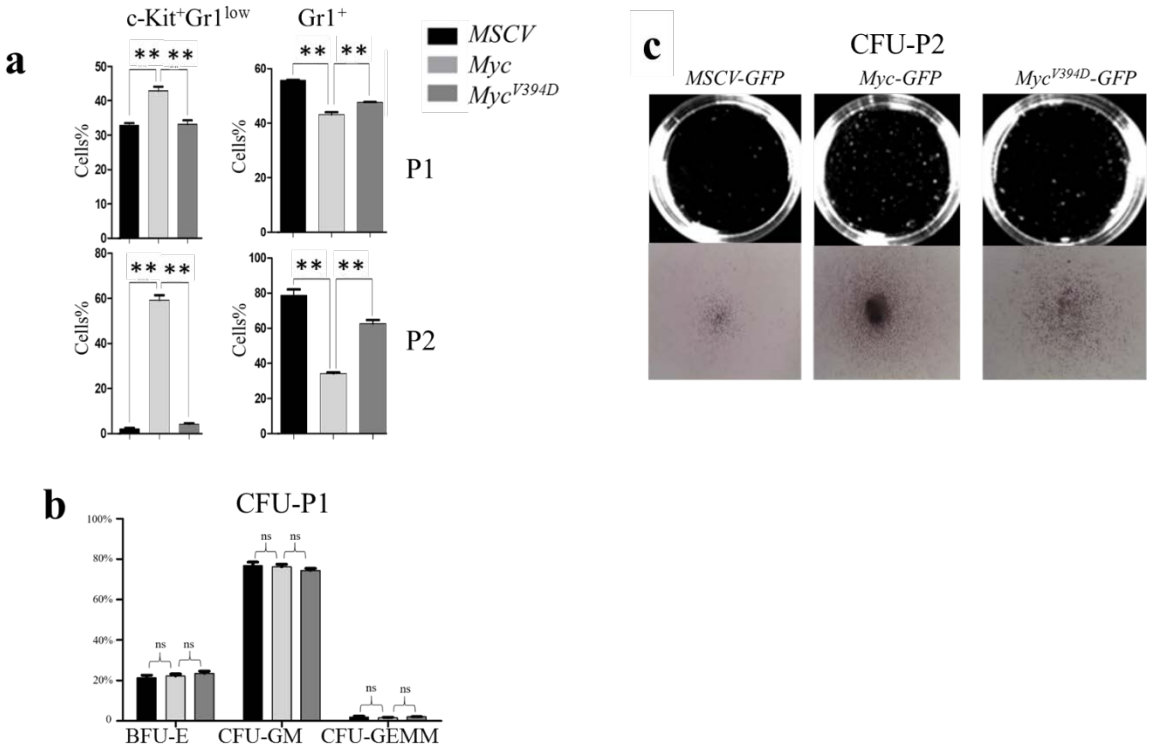


Figure S5 (associated with Figure 2). Representative phenotypic analysis of the PB of transplanted recipient mice by flow cytometry. All mice that received *Myc*-HSPCs developed MDP or AML with a phenotype the same as #2 MPD mouse or #3 AML mouse, whereas most mice which received *Myc*^{V394D}-HSPCs had normal hematopoiesis the same as #4 mouse and are comparable to mice transplanted with *MSCV*-HSPC #1. Only 3/20 mice that received *Myc*^{V394D}-HSPCs developed MPD with a phenotype the same as #5 mouse. The MPD which developed in mice that received *Myc*^{V394D}-HSPCs was not as severe as that which developed in mice that received *Myc*-HSPCs.

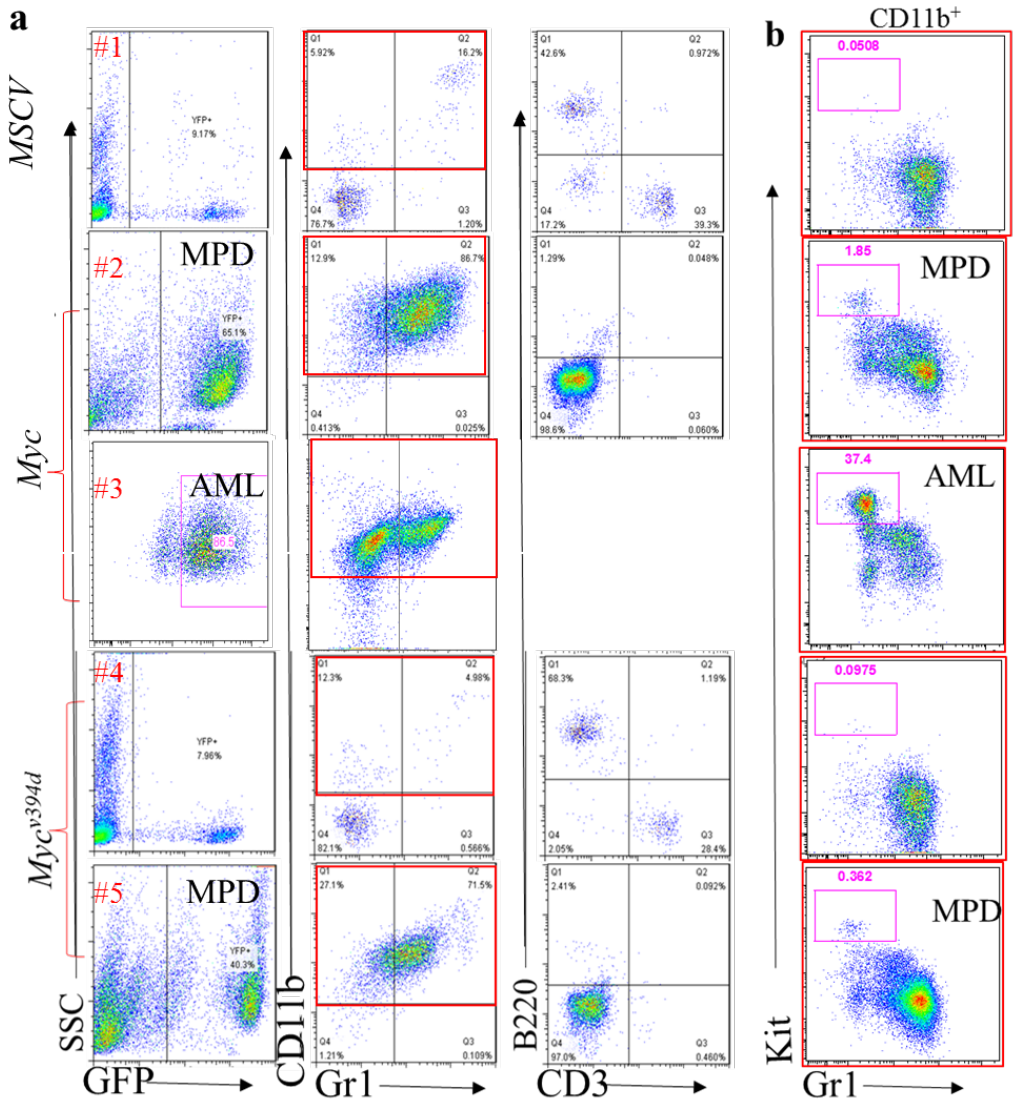


Figure S6 (Associated with fig. 3-7). Schematic diagram of the experimental procedures for the studies in Figures 3-7. **a.** c-Kit^{+} HSPCs were collected from *Cre^{ERT}Myc$^{fx/fx}$* mice and transduced with the *MLL-AF9* leukemia fusion gene. The transduced HSPCs were injected into recipient mice for AML development. The AML cells were collected from BM of the leukemic mice and then transduced with *MSCV*-GFP, *Myc-GF*P or *Myc^{V394D}-GFP*, respectively. Such transduced AML cells were used in the experiments shown in **Figs. 3-7** with 4-OHT (for *in vitro* studies) or TAM (for *in vivo* studies) treatment to induce endogenous *Myc* deletion. **b-e**. Qualification control studies of the transduced AML cells. The proliferation of transduced AML cells was examined by BrdU (**b**). CFC of the transduced AML cells was evaluated by methylcellulose culturing (**c**). The success of endogenous *Myc* deletion of was examined by PCR (**d**) and Western blotting (**e**). Increased Myc protein levels in both *Myc* and *Myc^{V394D}*-AML cells before and after 4-OHT induction compared to *MSCV* transduction was demonstrated by Western blotting. * indicates $p<0.05$.

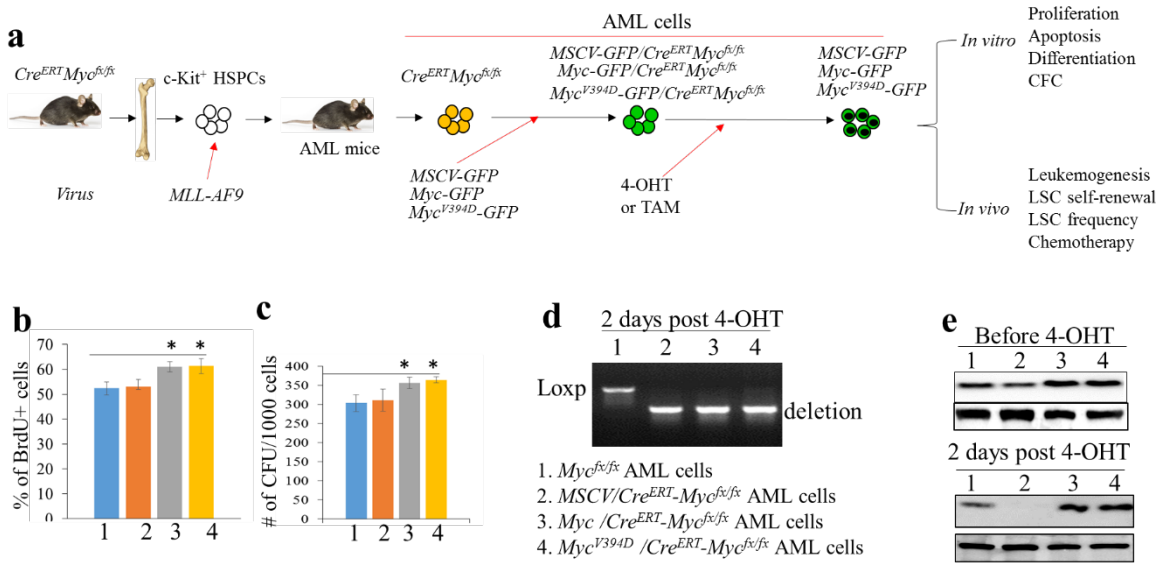


Figure S7 (Associated with fig. 3). Comparing the proliferation of Kit^{+}CD11b^{low}, Kit^{+}CD11b^{high} and Kit^{-}CD11b^{high} populations among* MSCV-, Myc- *and* Myc^{V394D}*-AML cells. *MSCV-, Myc-* and *Myc^{V394D}*- AML cells were treated with BrdU 48 hours after deletion of endogenous *Myc*. Kit^{+}CD11b^{low}, Kit^{+}CD11b^{high} and Kit^{-}CD11b^{high} populations were sorted by FACS 2 hours after BrdU treatment and fixed for BrdU labeling. The percentages of BrdU^{+} cells were analyzed by flow cytometry. Data are a summary of 3 biological triplicated experiments.

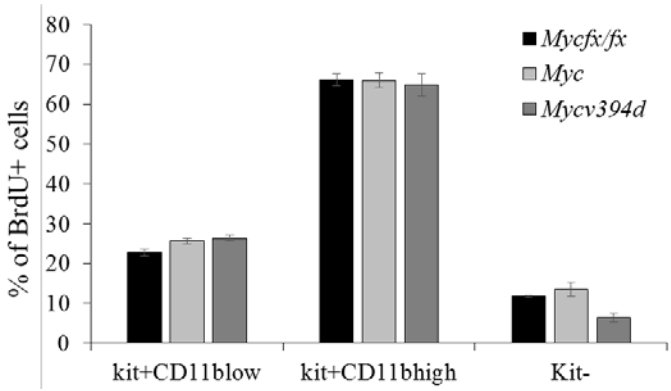


Figure S8 (Associated with fig. 3). Increased phagocytosis of Myc^{V394D}-AML cells compared to Myc-AML cells. *Myc^{V394D}*-AML cells and *Myc*-AML cells were collected on day 7 after the deletion of endogenous *Myc*. **a.** Phagocytosis of the cells were examined by using EZCell™ Phagocytosis Assay Kit (Red E. coli) following the instruction provided by the vender (Biovision); or **b.** Cells were incubated with APC-conjugated beads for 2 hours. Phagocytosis of the AML cells were analyzed by flow cytometry to detect APC^{+} cells. Data are a summary of 3 biological triplicated experiments. ** indicates p<0.01.

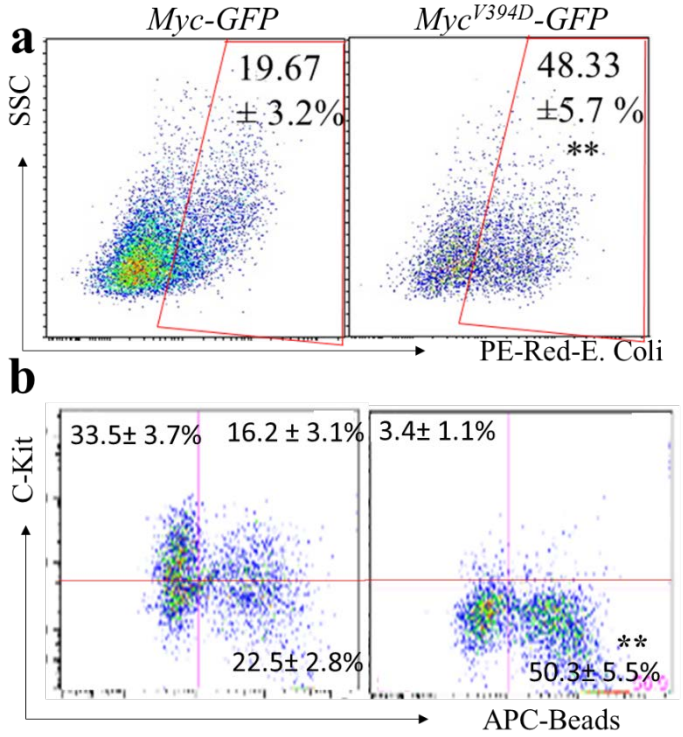


Figure S9 (Associated with fig. 3g). Comparing the role of* Myc *and* Myc^{V394D} *in the replating CFC of* PML-RARα*-transduced HSPCs. c-Kit^{+} HSPCs were collected from *Cre^{ERT}Myc$^{fx/fx}$* mice and co-transduced with *PML-RARa*-GFP plus *MSCV*-mCherry, *Myc*-mCherry, or *Myc^{V394D}*-mCherry, respectively. The transduced HSPCs were purified by FACS and seeded into methylcellulose with or without 4-OHT induction. CFUs were counted 7 days after plating. Cells from the CFUs were collected for 2nd and 3rd replatings. ** indicates p<0.01.

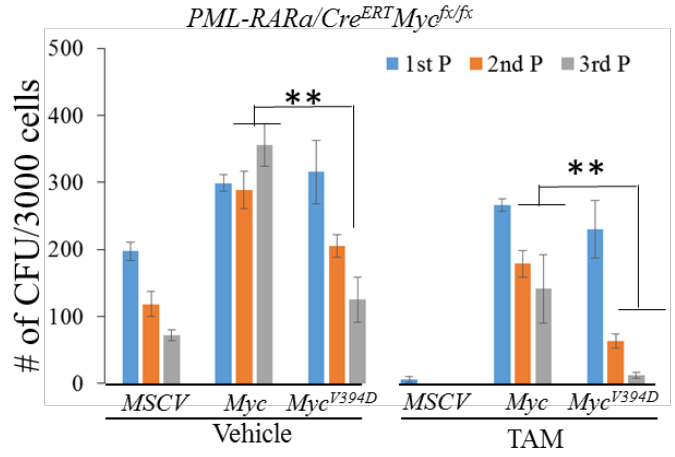
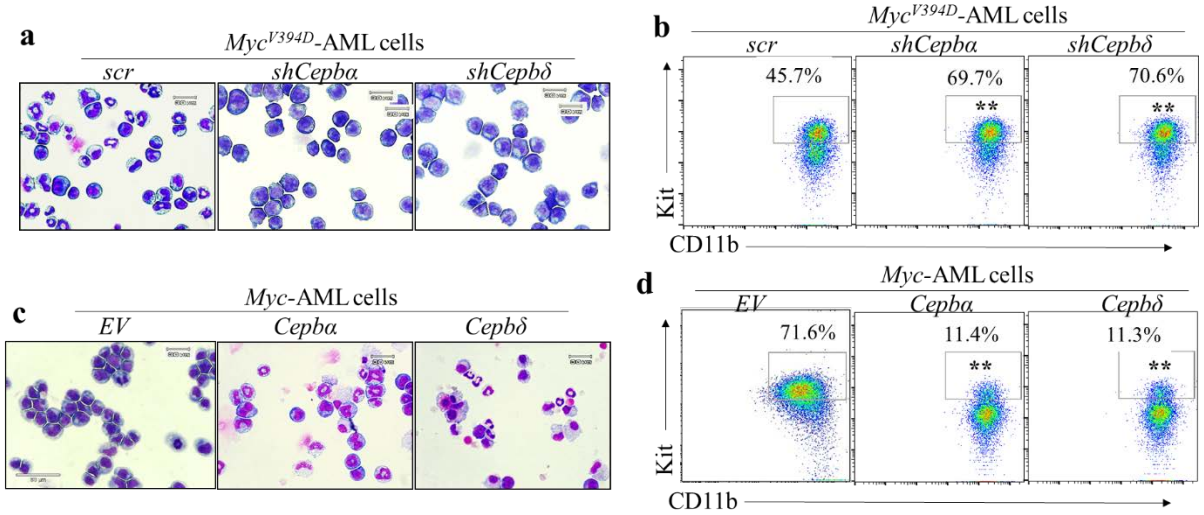


Figure S10 (Associated with fig. 7). Up-regulation of Cebpα and Cebpδ in* Myc^{V394D}*-AML cells contributes to the partial differentiation phenotype. As described in **Figure 7**, **a-b**, *Cebpα* or *Cebpδ* was knocked down in *Myc^{V394D}*-AML cells using shRNA specific for *Cebpα* (shCα) or *Cebpδ* (shCδ). The morphology (**a**) and c-Kit expression (**b**) of the gene knock-down cells were compared to scrambled shRNA (scr)-transduced *Myc^{V394D}*-AML cells. **c-d**. *Cebpα* or *Cebpδ* were over-expressed in *Myc*-AML cells by viral transduction. The morphology (**c**) and c-Kit expression (**d**) of the cells in which the specific gene was over-expression were compared to empty vector (EV)-transduced *Myc*-AML cells. ** indicates p<0.01.



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