Cancer Cell, Volume *25*

Supplemental Information

MLL3 Is a Haploinsufficient 7q Tumor Suppressor in Acute Myeloid Leukemia

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Supplemental Data

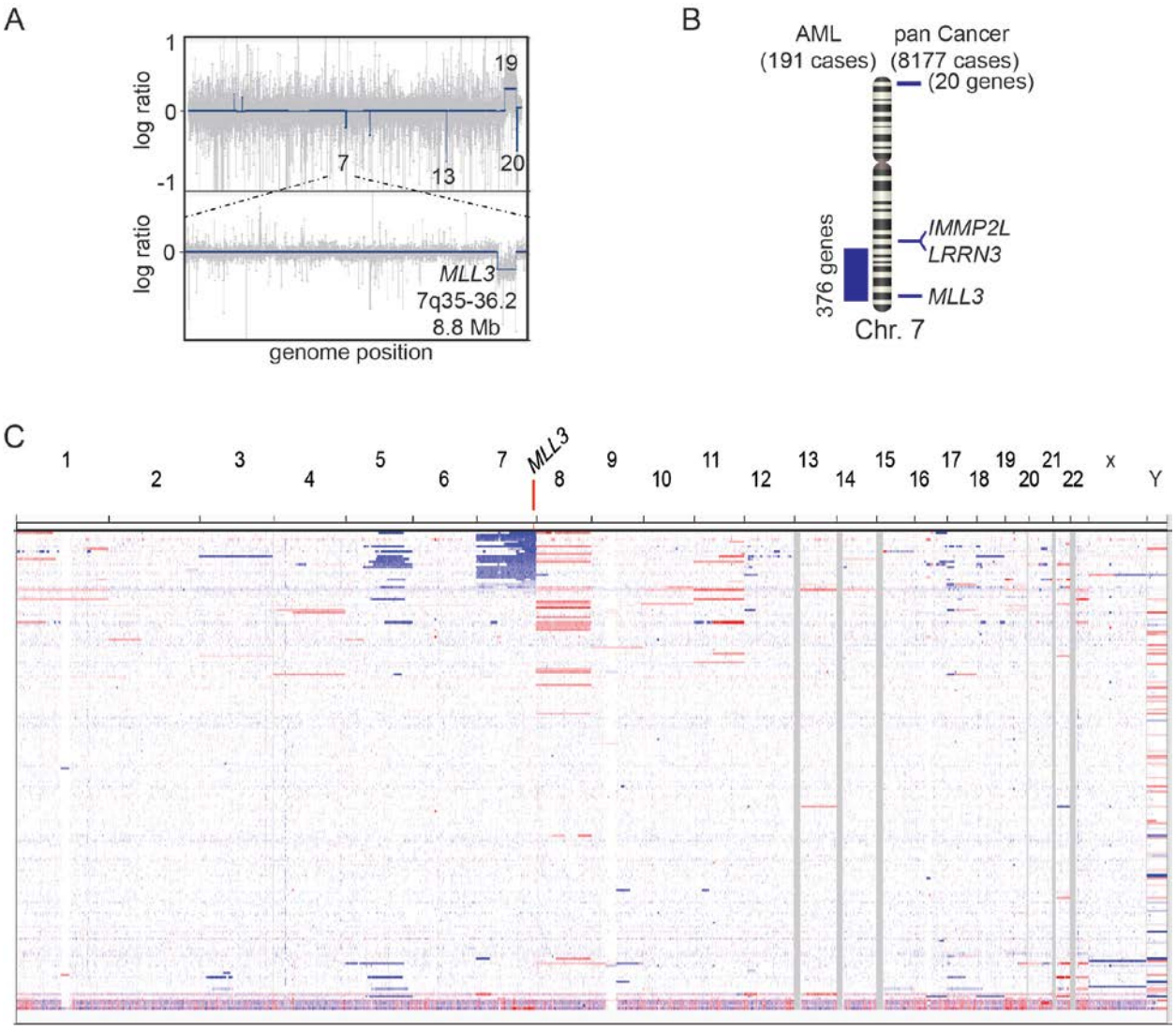

Figure S1, related to Figure 1.

A. ROMA plots of one AML case depicting copy number changes. Data plotted are the normalized fluorescence log ratio for each probe (85K).Top plot: whole genome view; left to right; chromosome 1-22, X, Y. Bottom plots: High resolution of chromosome 7, showing a submicroscopic deletion of *MLL3*. B. *MLL3* deletions in AML and pan Cancer (data from TCGA TumorScape). C. Overview of copy number events in TCGA AML cohort. Copy number events from 200 AML samples (TCGA) sorted on *MLL3* deletion status (24 samples).

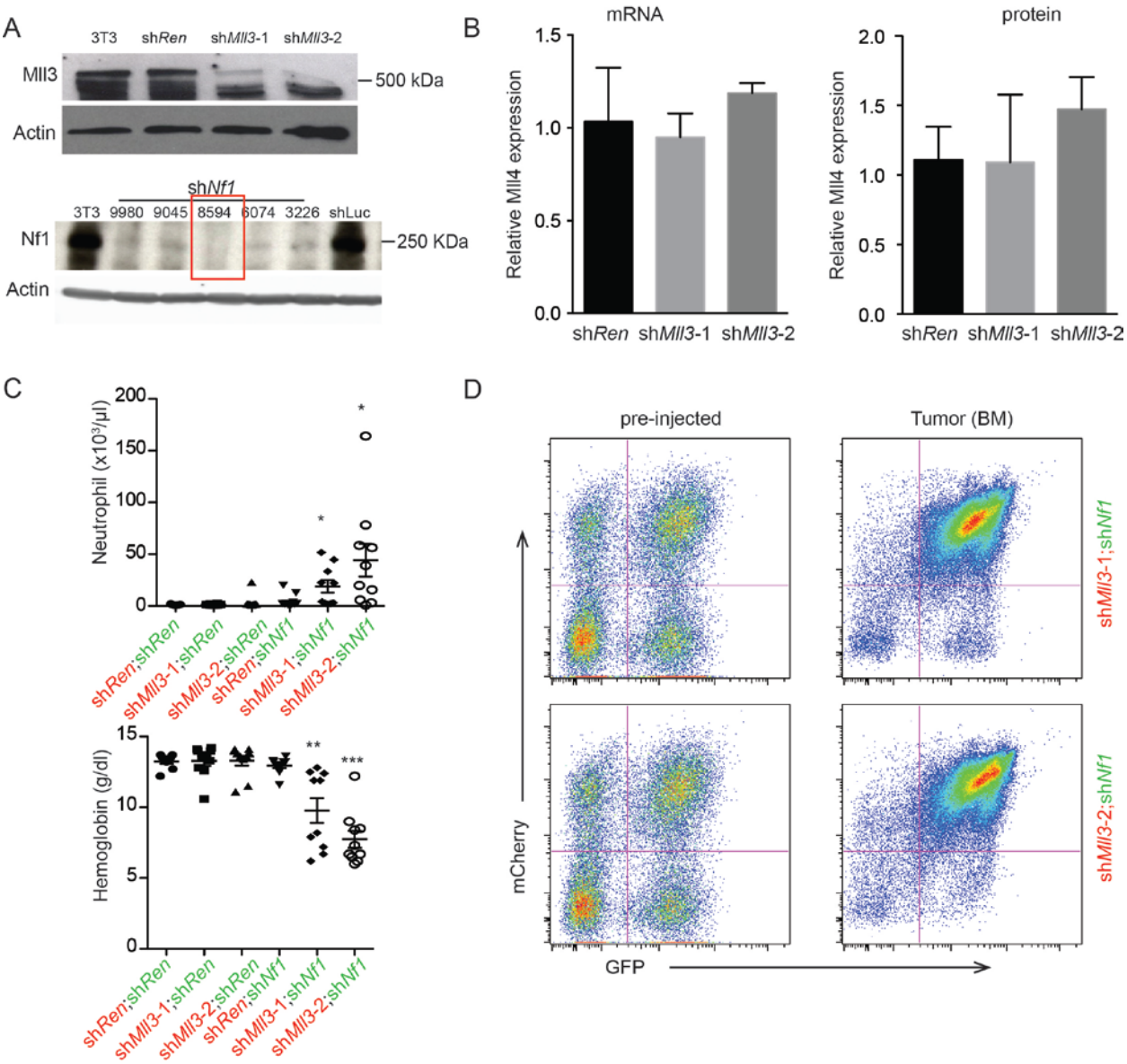

Figure S2, related to Figure 2.

A. Up, immunoblotting showing knockdown efficiency of Mll3 protein in tumor cells by two independent *Mll3* shRNAs in NIH-3T3 cells, relative to untransduced or cells transduced with a neutral shRNA (*Ren*); bottom, immunoblotting showing knockdown efficiency of Nf1 protein in NIH-3T3 cells, relative to untransduced or cells transduced with sh*Ren*. The red gate indicates the shRNA (8594) used in this study. B. The mRNA and protein levels of Mll4 in sh*Mll3*;sh*Nf1*;*p53*$^{-/-}$ AML. Left, Mll4 mRNA levels of HSPC with sh*Ren* or sh*Mll3* by qPCR,

normalized to actin; right, Mll4 protein levels of *p53*$^{-/-}$ tumor cells with shRen or shMll3 by western blotting normalized to actin. C. The neutrophil counts and hemoglobin levels in the peripheral blood of mice transplanted with sh*Mll3*;sh*Nf1*;*p53*$^{-/-}$ HSPCs. As shown in Fig 2A, *p53*$^{-/-}$ HSPCs were co-infected with GFP-linked and mCherry-linked shRNA and then transplanted into sublethally irradiated recipient mice. The neutrophil counts (up) and hemoglobin (bottom) in peripheral blood of transplanted mice were measured using Hemavet at 8 weeks after transplant or upon death of leukemia-bearing sh*Mll3*;sh*Nf1*;*p53*$^{-/-}$ recipients if they died before 8 weeks. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. D. Representative flow cytometry plots of pre-injected HSPCs and tumor cells (from BM of sick mice) transduced with GFP-sh*Nf1* and mCherry-sh*Mll3*. B and C show mean±SD.

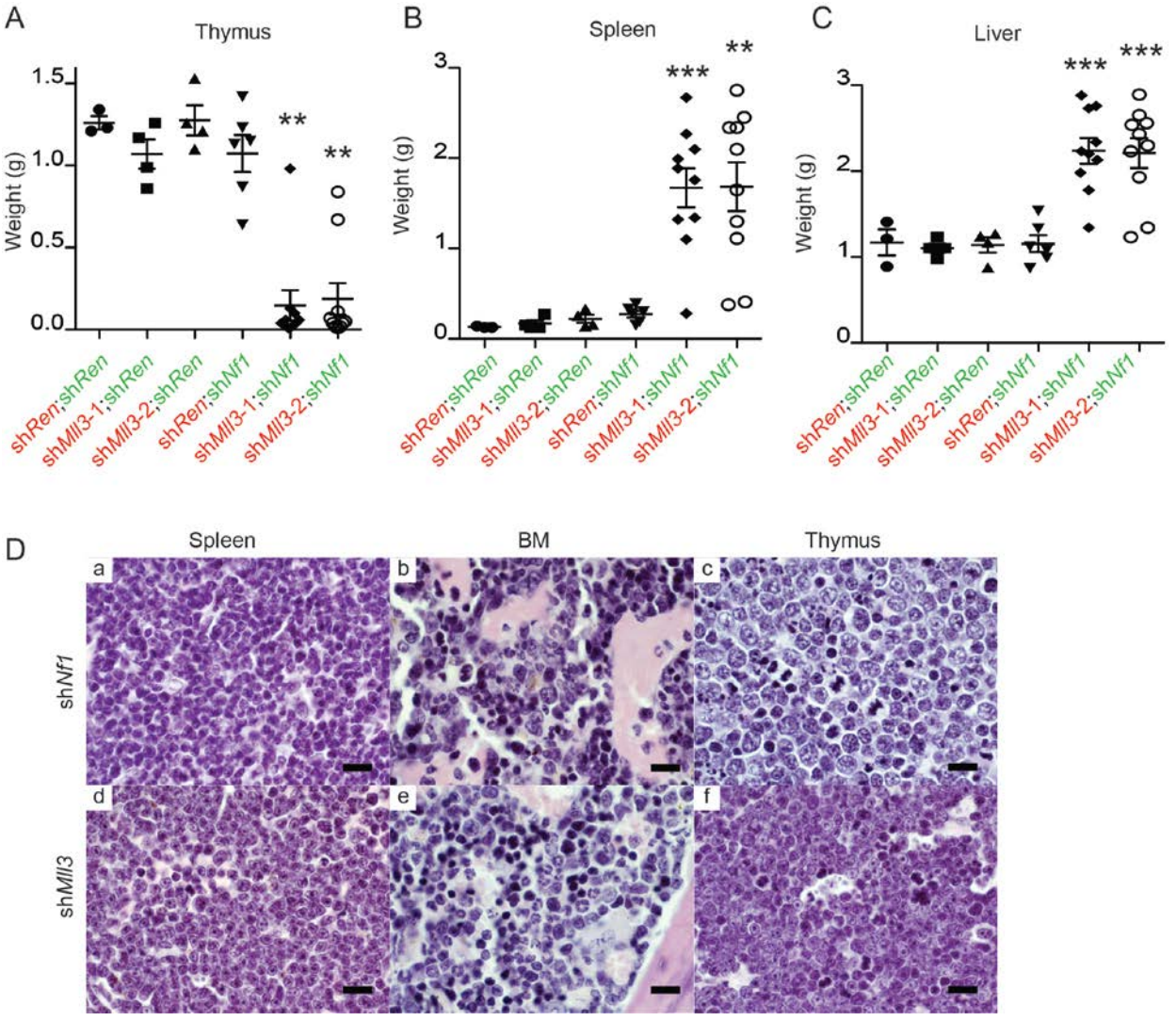

Figure S3, related Figure 3.

A-C. The weights of spleen and liver of mice transplanted with sh*Nf1* and sh*Mll3* infected HSPCs. As shown in Fig 2A, *p53*$^{-/-}$ HSPCs were co-infected with sh*Ren* or sh*Nf1* and sh*Ren* or sh*Mll3* and then transplanted into sublethally irradiated recipient mice. The weight of thymus (A), spleen (B) and liver (C) were measured at sacrifice, showing mean±SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. D. Histological analysis of spleen (a and d), BM (b and e) and thymus (c and f) of control recipient mice (upper: sh*Nf1;*sh*Ren;p53*$^{-/-}$; lower: sh*Mll3*;sh*Ren*; *p53*$^{-/-}$) upon death. Scale Bar: 12 μm.

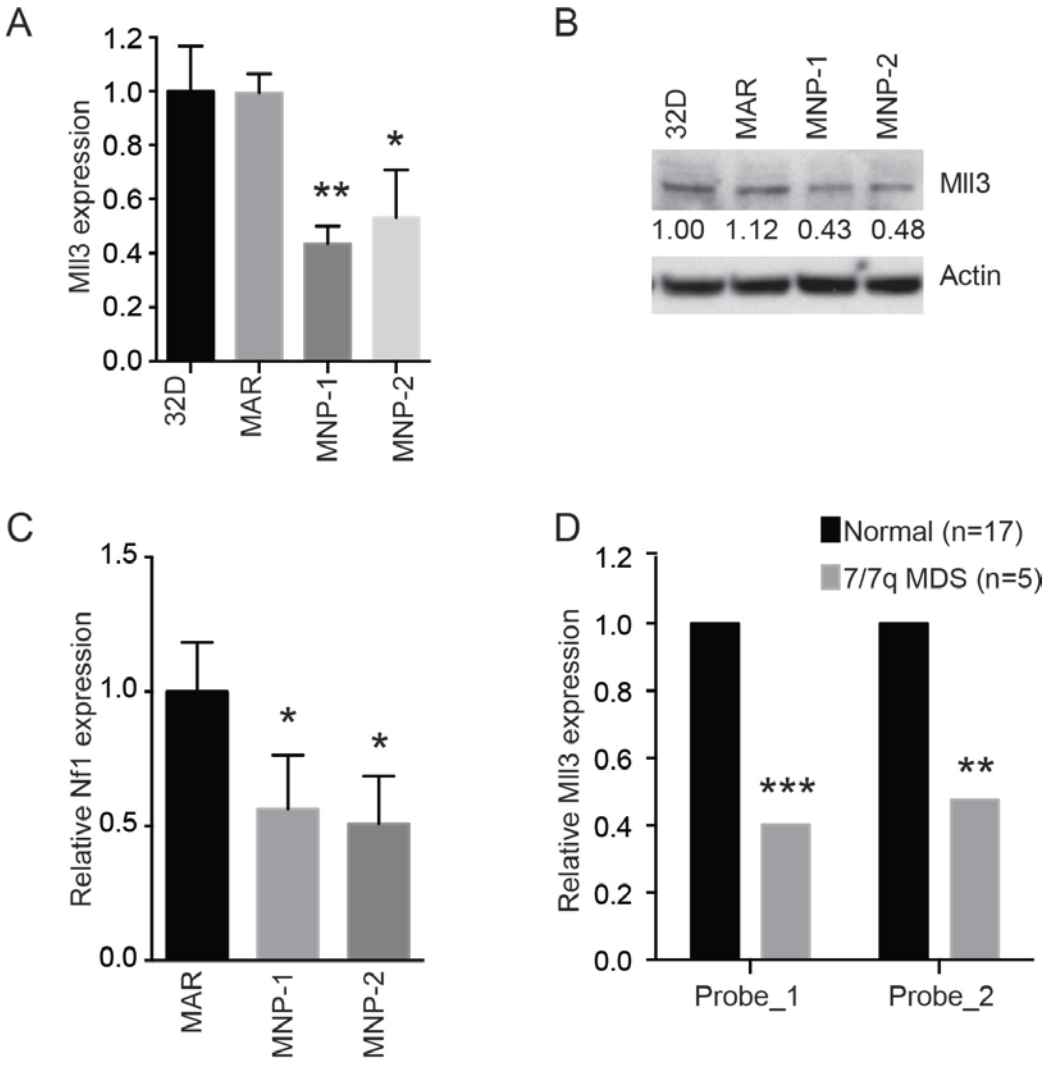

Figure S4. Related to Figure 4.

A-B. Mll3 mRNA (A) and protein (B) levels were quantitated in sh*Mll3*;sh*Nf1*;*p53*$^{-/-}$ AML (MNP) compared to mouse myeloid cell line 32D and *MLL/AF9;Nras*^{G12D} AML (MAR). Mll3 mRNA and protein levels were measured by RT-qPCR and western blotting, respectively. C. Nf1 protein levels of MAR and MNP AML cells by western blotting normalized to actin. D. Expression levels of MLL3 in human MDS HSC with -7/del(7q) compared to normal HSC. Data from NCBI GSE19429 (Originally by Fellagatti et al., *Leukemia* 2010). A and C show mean±SD. .

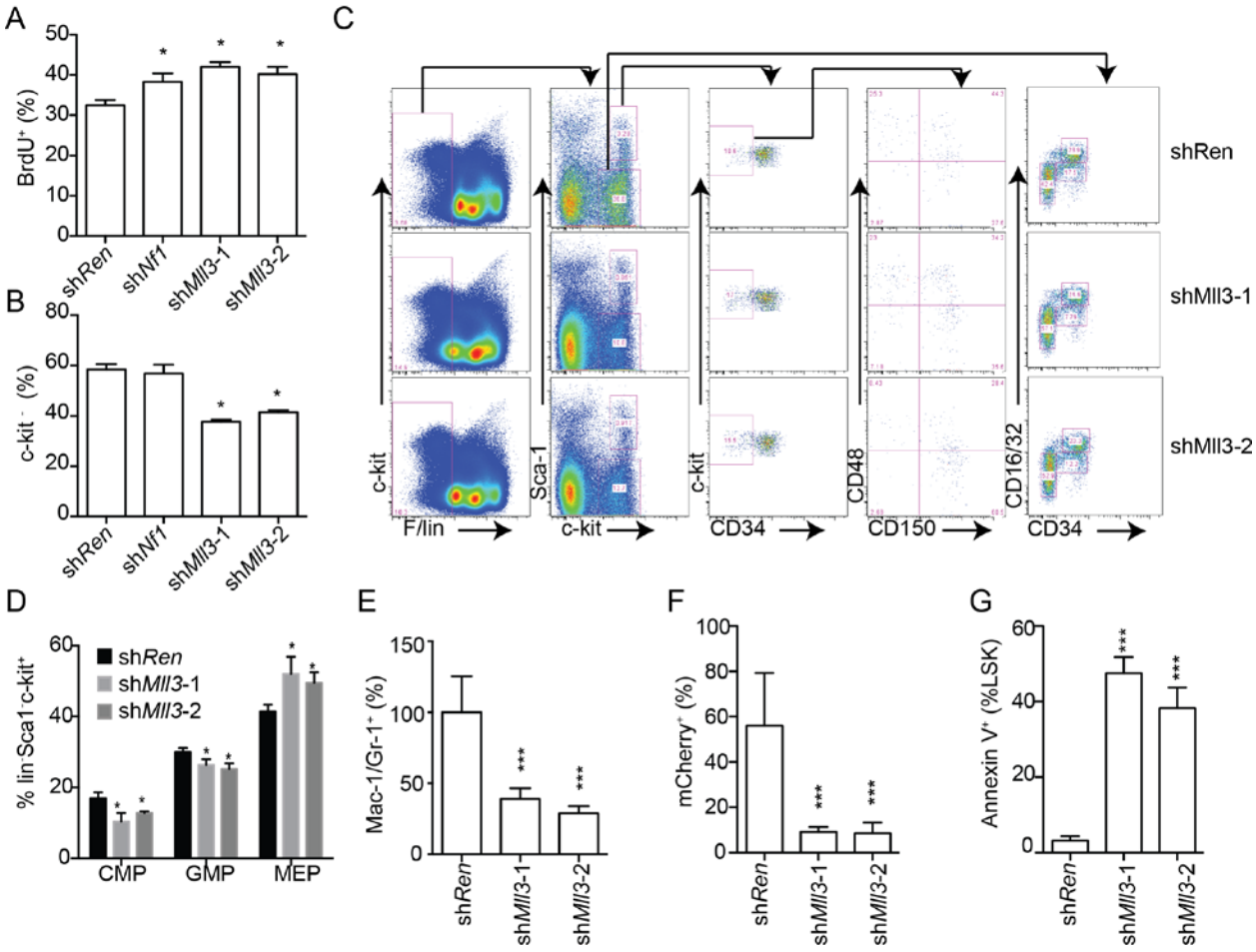

Figure S5, related to Figure 5.

A-B. *p53*$^{-/-}$ HSPCs were transduced with indicated shRNAs and cultured in liquid medium with IL3, IL6 and SCF for 5 days and then analyzed for BrdU incorporation ratio in the c-kit^{+} population (A) and the percentage of c-kit^{-} populations in the total population (B). C-E. Flow cytometry analysis of BM cells from sh*Ren*; *p53*$^{-/-}$ and sh*Mll3*; *p53*$^{-/-}$ recipient mice at 6 weeks after transplant. C. Representative flow plots showing the gates of Flt3^{-}lin^{-}, FLSK (Flt3^{-}lin^{-}Sca-1^{+}c-kit^{+}), LT-HSC (Flt3^{-}lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{-}CD34^{-}), ST-HSC (Flt3^{-}lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{+}CD34^{-}), MPP (Flt3^{-}lin^{-}Sca-1^{+}c-kit^{+}CD150^{-}CD48^{+}CD34^{-}), CMP (Flt3^{-}lin^{-}Sca-1^{-}c-kit^{+}CD34^{+}CD16/32^{-}), GMP (Flt3^{-}lin^{-}Sca-1^{-}c-kit^{+}CD34^{+}CD16/32^{+}) and MEP (Flt3^{-}lin^{-}Sca-1^{-}c-kit^{+}CD34^{-}CD16/32^{-}) in mCherry+ BM cells. D. The percentage of CMP, GMP and

MEP in mCherry^{+}Flt3^{-}lin^{-}Sca1^{-}c-kit^{+} MP cells at 6 weeks after transplant. n=3. E. Relative counts of myeloid lineage cells (Gr1^{+} and/or Mac-1^{+}) in sh*Mll3* recipient mice compared to that in sh*Ren* control mice. n=5. F. Reconstitution by mCherry+ donor cells in BM at 10 weeks after transplantation. G. Annexin V staining of mCherry+ LSK cells (C). n=3. A-B and D-G show mean±SD. *: $p<0.05$; ***: $p<0.001$.

Table S1, related to Figure 6. Provided as an excel file.

Raw data: the raw data of Illumina microarray gne expression analysis in Fig 6;

Downregulated genes: the list of downregulated genes in Fig 6A;

Upregulated genes: the list of upregulated genes in Fig 6A;

Hematopoietic early progenitor: the gene set "hematopoietic early progenitor" used for GSEA in Fig 6E;

Hematopoietic mature cell: he gene set "hematopoietic mature cell" used for GSEA in Fig 6E;

Leukemia stem cell UP: the gene set "leukemia stem cell up" used for GSEA in Fig 6F;

Leukemia stem cell DN: the gene set "leukemia stem cell down" used for GSEA in Fig 6F;

Human MDS vs. normal HSC DN: the gene set "human MDS vs. normal HSC down" used for GSEA in Fig 6G;

Human 7q vs. NK MDS DN: the gene set "human -7/del(7q) vs. normal karyotype MDS down" used for GSEA in Fig 6G.

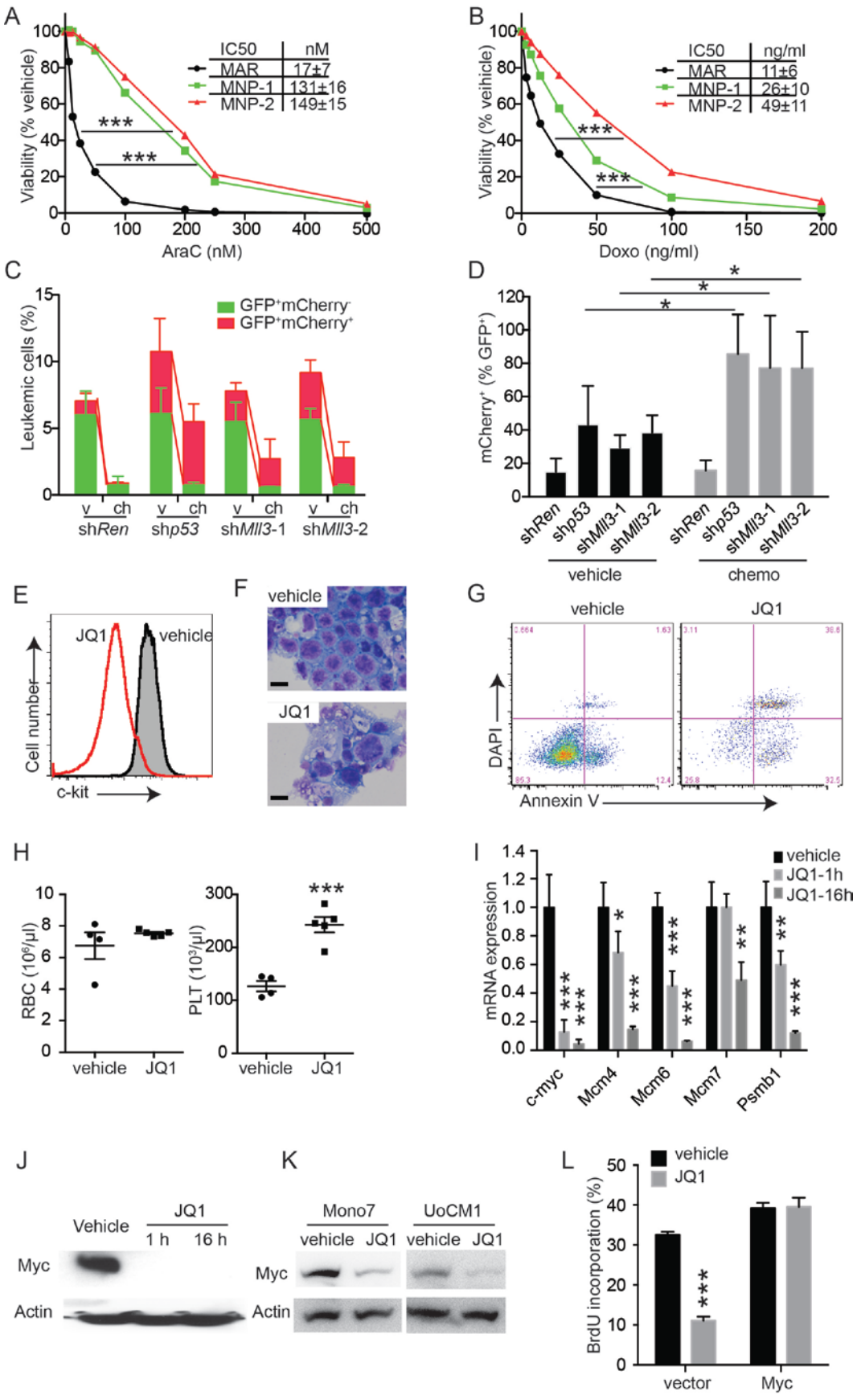

Figure S6, related to Figure 7.

A-B. sh*Mll3*;sh*Nf1*;*p53*$^{-/-}$ (MNP-1 for sh*Mll3*-1 and MNP for sh*Mll3*-2) and *MLL/AF9*;*Nras*^{G12D} (MAR) AML cells were cultured in vitro and treated with indicated concentrations of AraC (A) or Doxo (B) for 3 days. The numbers of cells were counted and normalized to vehicle-treated cells. Shown is the average of 3 independent experiments and the inset panels give the IC_{50}. ***: $p<0.001$, two-way ANOVA analysis. C-D. *In vivo* drug response of *AML/ETO*;*Nras*^{G12D};*GFP* AML with or without Mll3 knockdown. *AML/ETO*;*Nras*^{G12D};*GFP* AML cells were transduced with sh*Ren*, sh*p53* or sh*Mll3* (MLS-mCherry) and then transplanted into sublethally irradiated syngeneic recipients. 4 weeks after transplant, the recipient mice were treated with vehicle (V, n=3) or AraC (100mg/kg, 5 days) and Doxo (3mg/kg, 3 days) (Ch, n=4). The recipients were sacrificed and their BM cells were analyzed 4 days after treatment. C. Shown percentages of GFP$^+$mCherry$^-$ and GFP$^+$mCherry$^+$ AML populations in the BM. D. The relative fold changes of mCherry$^+$ vs. GFP$^+$ AML cells in vehicle or chemo-treated mice. E. The expression level of c-kit on MNP AML treated with vehicle or 100 nM JQ1 for 2 days in vitro measured by flow cytometry. F. Cytospin showing differentiation following JQ1 treatment. G. Annexin V/DAPI staining of MNP AML treated with vehicle or 100 nM JQ1 for 2 days in vitro. Shown flow plots representative of 3 independent experiments. Scale bar: 12 μm. H. Related to Figure 7F-G, MNP recipient mice were treated with vehicle or 50mg/kg/day JQ1 by gavage for one week starting at 5 days after transplant. RBC and platelet counts were measured for MNP mice treated with vehicle or JQ1, 12 days after transplantation. n=5. I. The transcript levels of Myc, Mcm4, Mcm6, Mcm7 and Psmb1 in MNP AML cells following 1 or 16 hours 250 nM JQ1 treatment. n=3. J. Western blotting showing Myc levels in MNP AML cells with 1 hour or 16 hours 250 nM JQ1 treatment. K. Western blotting showing Myc levels in human AML cells Mono7 and UoCM1 with 250 nM JQ1 treatment for 4 hours. L. BrdU incorporation ratios of MNP AML cells after 4 days of 50 nM JQ1 treatment. n=3. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Supplemental Experimental Procedures

HSPC culture, infection and electrophoresis. Fetal liver cells (FLC/HSPC) were isolated from E13.5-14.5 *p53*$^{-/-}$ embryos. FLC from multiple embryos were mixed and cultured in RPMI1640 medium with 10% FBS and 5% Penicillin-Streptomycin and supplemented with 5ng/ml IL-3, 50ng/ml IL-6 and 50ng/ml SCF (Schmitt et al., 2002). Retroviruses were produced by transfection of ecotropic Phoenix packaging cell line. HSPC were infected by spinoculation. Lonza 4D nucleofector system was used to transduce CRSIPR/Cas9 into HSPC with Nucleofector kit following manufactory's manual.

Pathology. Bone, spleen, thymus and liver were fixed in 10% formalin and sections were stained with hematoxylin and eosin. For peripheral blood, blood smear was prepared and May–Gru¨nwald (Sigma) and Giemsa (Sigma) stainings were performed according to manufacturer's protocols.

Flow cytometry. BM cells were harvested from the long bones (tibias and femurs) with HBSS without calcium or magnesium (Invitrogen), supplemented with 2% heat-inactivated fetal bovine serum. Peripheral blood was obtained from retro orbital puncture, and RBCs were lysed by ammonium chloride/ potassium bicarbonate buffer. Antibody staining, except Annexin V and BrdU staining, was performed at 4°C for 15 minutes with HBSS without calcium or magnesium (Invitrogen), supplemented with 2% heat-inactivated fetal bovine serum(Chen et al., 2008). Annexin V staining was performed in Annexin V staining buffer (eBioscience). BrdU labeling lasted 90 minutes and staining was performed according to the manufacturer's manual (BD Bioscience). All other antibodies were purchased from BD eBioscience. Flow cytometry analysis was performed on an LSR II (BD Biosciences), and FACS was performed on a FACSAria II (BD Biosciences).

Western blotting. Whole cell lysates were extracted with RIPA buffer (25 mM Tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and resolved by 4-20% precast SDS-PAGE gradient gel (Bio-Rad) electrophoresis. Mll3 and Nf1 antibodies were from Abcam. Histone methylation and acetylation western blotting were done with acid extracts from BM cells and antibodies against H3K4me, H3K4me2 and H3K4me3 were from Active Motif and H3 and H3K27ac antibodies were from Millipore.

Leukemia cell culture and in vitro drug Treatment. sh*Mll3*;sh*Nf1*;*p53*$^{-/-}$ AML cells were cultured in RPMI1640 medium with 10% FBS and 5% Penicillin-Streptomycin and supplemented with 5ng/ml IL-3, 50ng/ml IL-6 and 50ng/ml SCF and *MLL/AF9;Nras*^{G12D} AML cells with RPMI1640 medium with 10% FBS and 5% Penicillin-Streptomycin. Cells were treated with araC, Doxo or JQ1 at given concentrations for 3 days and then the viable cells were counted by Guava (Millipore).

Gene expression profiling and gene set enrichment analysis (GSEA). c-kit^{+};GFP^{+} BM cells were sorted from mice 6 weeks after transplantation of sh*Ren*;*p53*$^{-/-}$ or sh*Mll3*;*p53*$^{-/-}$ HSPC (sh*Ren* and sh*Mll3* were linked with GFP). Total RNA was extracted using Trizol reagent and microarray analysis was performed on Illumina mouseref-8 expression chip. Gene ontology analysis was performed with DAVID tools (http://david.abcc.ncifcrf.gov/tools.jsp). GSEA was performed with Broad's GSEA algorithm.

Chromatin immunoprecipitation (ChIP)-PCR. c-kit^{+};GFP^{+} BM cells were sorted from mice 6 weeks after transplantation of sh*Ren*;*p53*$^{-/-}$ or sh*Mll3*;*p53*$^{-/-}$ HSPC (sh*Ren* and sh*Mll3* were linked with GFP). Chromatin immunoprecipitation was performed as previously described (Bernt et al., 2011). Briefly, crosslinking was performed with 1% formalin, and DNA was fragmented by sonication. ChIP for H3K4me3 and H3K27me3 was performed

using antibodies specific to the respective modifications. Eluted DNA fragments were analyzed by quantitative PCR. The sequences of PCR primers for H3K4me3 were: Gadd45g-F: GCTTGTTCTTTCACAGGATGC; Gadd45g-R: CTTTGGCGGACTCGTAGAC; Il1r2-F: CACGTGATCGCTCCATTCT; Il1r2-R: CTCGTGTGCTGCAGGTT; Cpa3-F: CAGAAGCAGACTCCTAACCAG; Cpa3-R: CCTCCTTGGAGCACTTAACA, and for H3K27me3: Gadd45g-F: GCTTGTTCTTTCACAGGATGC; Gadd45g-R: CTTTGGCGGACTCGTAGAC; Il1r2-F: CTTCTGCCGCTTCTGCT; Il1r2-R: GTGACCACGTCCGACTTT; Cpa3-F: TCACGTTGGTCTTGGTGTTAAG; Cpa3-R: GCACAGGGACGATGGAAAG.

Supplemental References

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