MLL Fusion Protein-Driven AML is Selectively Inhibited by Targeted Disruption of the MLL-PAFc Interaction

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Supplemental Information

Supplemental methods:

Proliferation assays and colony assays

MA-*Cdc73fl*-CreER and EH-*Cdc73*-CreER and control cell lines (MA-CreER, MA- *Cdc73fl,* EH-CreER and EH-*Cdc73fl*) cell lines were seeded in growth media at 25,000 cells/ml. Excision of the *Cdc73* allele was achieved through addition of 5nM 4-hydroxytamoxifen (4-OHT) (Sigma). Control cells received vehicle ethanol. A Trypan blue (Gibco) exclusion assay was performed for daily counts of living cells. Primary bone marrow colony assays were performed by isolating $lin^{-}c\text{-}kit^{+}$ cells from 8 week old C57Bl/6 mice using the EasySep progenitor isolation kit (Stem Cell Technologies). Following two rounds of spinoculation, the indicated number of cells were plated in semi-solid methylcellulose (Stem Cell Technologies) with the addition of 10 ng/ml IL6, GM-CSF, 100 ng/ml SCF (R&D Technologies), 1 mg/ml G418 and 2 □g/ml puromycin. After 7 days colonies were scored and replated for secondary and tertiary plating.

Chromatin Immunoprecipitation

ChIP was performed as described previously {Muntean, 2010 #26}. Briefly, 30 x 10^6 cells were fixed in 1% paraformaldehyde following 48 hour EtOH or 100nM 4-OHT treatment and snap frozen. Cells were lysed in SDS buffer (Millipore) and diluted 1:10 in dilution buffer (Millipore). 1% of diluted lysis is retained for input controls. Immunoprecipitations were performed using primary antibodies specific for MLL^{C} (gift from Dr. Yali Dou), Flag (Sigma F7425), PAF1 (Bethyl A300-172A), Parafibromin (Bethyl A300-170A), histone H3 (Abcam ab1791), H3K4me3 (Abcam ab8580), H2Bub (Millipore clone 56), H3K79me2 (Abcam 3594) and RNA pol II (Covance 8WG16). Precipitated DNA was purified using a Qiagen PCR purification kit and subjected to q-RT-PCR with TaqMan fluorescent labeling with primers and q-RT-PCR probes. Binding was quantitated as follows: $\Delta C_T = C_T(\text{input}) - C_T(\text{Chromatin IP})$, $\%\text{total} = 2^{\Delta C_T}$. Primer/Probe (FAM-TAMRA) sequences can be found in SI.

ChIP primer probe sets

Primer/Probe (FAM-TAMRA) sequences are as follows: Meis1-1 probe – AATCTCCATTTCTTTCCACTCTGCAGACCC, Meis1-1F – GCCTCTTTAGGGCAATCTATAGCTT, Meis1-1R – CACTTTGCCTTCCTCCAAACC; Meis1-2 probe – CCGGCGCTGACTCGTGCAGAC, Meis1-2F – CTGCCGCTGGTGTGAACTAG, Meis1-2R – CGGAGCCTCCTAAGACAGCTT; Meis1-3 probe – TTCCTTTCCTCCGGCCCTACGTCC. Meis1-3F – TCAAAGTGACAAAATGCAAGCA, Meis1-3R – CCCCCCGCTGTCAGAAG; Meis1-4 probe – CATTCACCACGTTGACAACCTCGCC, Meis1-4F – GAAGAAGACAGAACGGACGATCA, Meis1-4R – GCCACTCCAGCTGTCAATCA; Meis1-5 probe – CAAACCCCTAGAGCCCATTCTTATCCGC, Meis1-5F – TTCTGCAGCCAGCTCTACCA, Meis1-5R – GGTGTTCCCCCACATGTCA; Meis1-6 probe – CTCTTCCAAGTGTGCCTGTATGTGTTCCG, Meis1-6F – GCATAAATCCGAGAGGCCTTT, Meis1-6R – GACACTACCCTCGCTTTGAACAG; Meis1-7 probe – TAGGACGTTCTCCTTGCCGCTCGG, Meis1-7F – GGAGGGCTTTTGTCTGTTTTTAAA, Meis1-7R – AAGAATTTGCCCCACCTTACC; Meis1-8 probe – CTGCTGCCCGAGTTCCCACTGTG, Meis1-8F – ACAAGGAACGCGTAGTAAATGAGA, Meis1-8R – ACTAGGGAGCCAAGTGCAAAAC; Meis1-9 probe – TCTGGCATGAAGAAGCCACACCCA, Meis1-9F – CCCCTCTGGCAACCTACAGTT, Meis1-9R – CCACTAGAGAAAGCAGCAGCAA; Meis1-10 probe – CTCAGGTCTCTCTGTTCCTGCCCCTG, Meis1-10F – GGATTGTGCGAGCCTTGCT. Meis1-10R – GAGGAAAAGCTAAAACAGAATTAAACG.

q-RT-PCR

Expression profiling was performed by extracting RNA from cells using TRIzol reagent (Invitrogen). cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Relative quantitation of q-RT-PCR product was performed using the comparative ΔΔCt method (described in ABI Prism 7700 Sequence Detection System User Bulletin No. 2) and Taqman fluorescent labeling and the ABI 7500 PCR Detection System. Taqman primer probe sets were purchased from Applied Biosystems for mouse *Hoxa9, Hoxa7*, *Meis1* and *Bcl2.* MLLDN expression was detected using the SYBR green detection and two primer sets. Data in figure 4 was obtained using the primer set: MLL-F – 5' GCTCAGAAGATGCTGAACCTC and MLL-R – 5' TCGATCGACGTCCTTTCTTT. Data is Figure S6 was obtained using the primer set: MycDN-F – 5' GCATCAATGCAGAAGCTGAT and MycDN-R – 5' CATGGCCCCTACCTTTCTCT.

In Vivo leukemogenesis

Lin- bone marrow cells isolated from 8-10 week old C57Bl/6 mice injected with 5-FU at 150 mg/kg. Harvested lin- cells were retrovirally transduced with combinations of MSCV, MSCV-MLL-AF9, MigR1 and

MigR1-MLLDN by two rounds of spinoculation. 500,000 cells were injected intravenously through the tail vein to cohorts of lethally irradiated (900 rads) C57Bl/6 recipients (MSCV-MigR1 n=5, MSCV-MigR1-MLLDN n=5, MA-MigR1 n=10, MA-MLLDN n=10). Mice were irradiated in the Experimental Irradiation Core of the University of Michigan Comprehensive Cancer Center. They received 9 Gy delivered as 250kV X-rays by a Phillips RT250 orthovoltage unit (Kimtron Medical, Oxford CT) at a dose rate of approximately 2 Gy/min. Recipient mice were maintained on antibiotics for 2 weeks after transplantation.

In Vivo Bone Marrow Reconstitution

$Lin^{-}c\text{-}kit^{+}$ bone marrow cells were harvested from 5-FU (150 mg/kg) treated C57Bl/6 mice using EasySep progenitor isolation kit (Stem Cell Technologies). Cells were infected by spinoculation with MigR1 or MigR1-MLLDN viral supernatant generated from transiently transfected Plat-E cells in the presence of Polybrene (4□/ml) for 90 minutes at 3200 RPM. After two rounds of spinoculation 1.25x10^5 cells were injected into lethally irradiated (900 rads) C57Bl/6 mice by I.V. injection into the tail vein. Mice were irradiated in the Experimental Irradiation Core of the University of Michigan Comprehensive Cancer Center. They received 9 Gy delivered as 250kV X-rays by a Phillips RT250 orthovoltage unit (Kimtron Medical, Oxford CT) at a dose rate of approximately 2 Gy/min. Mice were bled every four weeks by tail vein nick and collection of peripheral blood into EDTA treated capillary tubes. Red cells were lysed with ammonium chloride solution (Stem Cell Technologies) followed by staining with APC conjugated anti-mouse Cd11b (BD Pharmingen). Cells were incubated for 15 minutes in the dark and washed followed by analysis on an LSRII using FACSDIVA software (BD Biosciences). LSK and hematopoietic lineage analysis was performed by isolating the bone marrow cells and thymus from injected mice at 16 weeks post cell injection and preparing single cell suspensions. Cells were treated with red cell lysis buffer as described above followed by staining with the following anti-bodies: Sca-1-PercpCy5.5, cKit-APC, LIN PE, CD11b PE-CY7, GR-1 APC, B220 FITC and TCRB-APC. Lineage markers were identified with anti-CD11b, Gr1, CD11c, B220, CD19, CD3, TCRb, TCRgd, CD8, NK1.1 and Ter119. Cells were analyzed on a FACSCanto using FACSDIVA software. Data analysis was performed using FlowJo (Treestar). Secondary transplants were performed by injecting 1 x 10^6 primary BM cells isolated from mouse 702 that received MigR1 transduced cells and mouse 713 that received MLLDN transduced cells. Cells were injected into lethally irradiated recipients as described above. PB Cd11b+ cells were monitored for GFP expression by flow cytometry for 16 weeks post injection.

Supplemental Figures

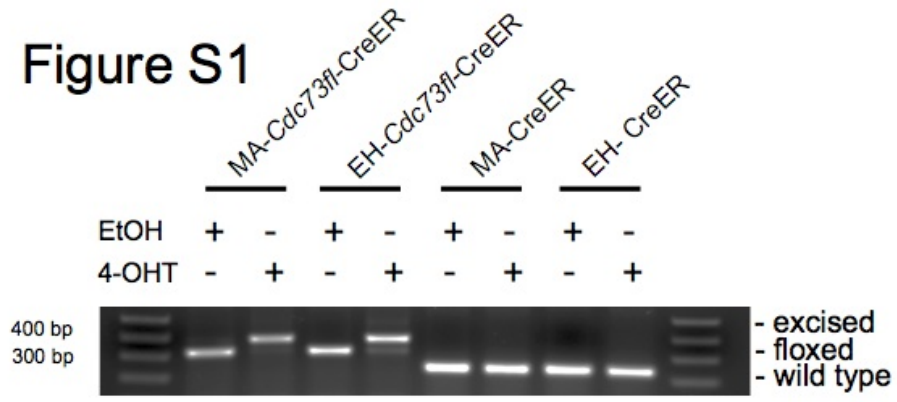

Figure S1 – Genotyping of *Cdc73* floxed and wild type cell lines

Genotyping for the floxed, excised and wild type *Cdc73* allele was performed on genomic DNA isolated from the MA-*Cdc73fl*-CreER, EH-*Cdc73fl*-CreER and control MA-CreER and EH-CreER cell lines following 48 hours of ethanol (EtOH) or 5nM 4-OHT treatment. PCR product sizes are: floxed *Cdc73* allele = 304 bp; excised *Cdc73* allele = 369 bp.

Figure S2

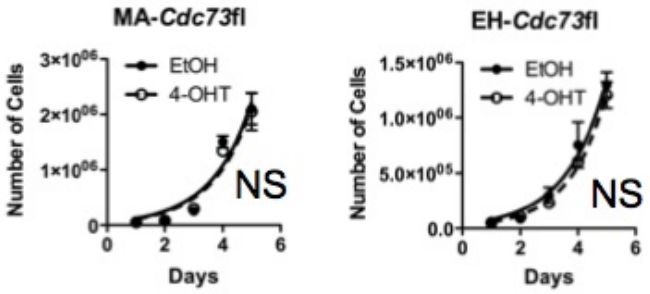

Figure S2 – Proliferation assays performed with control cell lines

MA-*Cdc73fl* and EH-*Cdc73fl* parental cell lines lacking CreER were grown for 5 days in liquid media in the presence of either EtOH or 100nM 4-OHT. Trypan blue exclusion assays were performed to monitor cell proliferation and the total number of living cells are plotted for each day. Statistical analysis performed using two way Anova. NS=Not Significant ($p>0.05$).

Figure S3

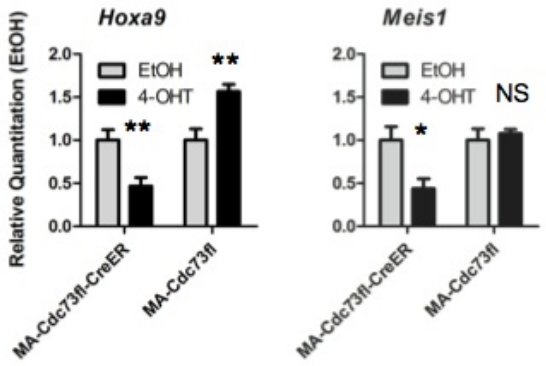

Figure S3 – 4-OHT treatment does not alter MLL fusion protein target gene expression.

q-RT-PCR was performed on the MA-*Cdc73fl*-CreER, EH-*Cdc73fl*-CreER and parental MA-*Cdc73fl* and EH-*Cdc73fl* cell lines 48 hours after EtOH or 100nM 4-OHT treatment. Expression levels for *Hoxa9* and *Meis1* are shown relative to EtOH treatment. Results show 4-OHT treatment alone does not reduce target gene expression. Statistical analysis performed using unpaired two tailed *t* test. **p<0.005, *p<0.05, NS=Not Significant (p>0.05).

Figure S4

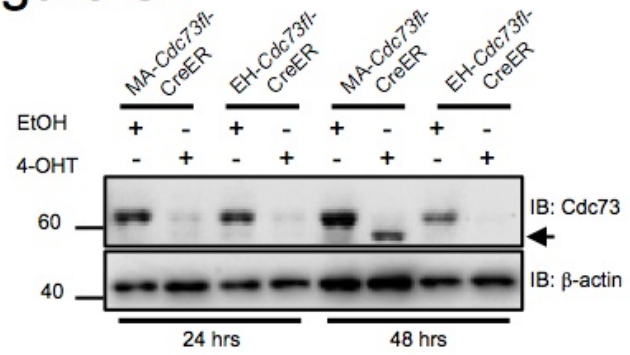

Figure S4 – Undetectable Cdc73 protein levels with increased 4-OHT treatment

Western blotting was performed on whole cell lysate for the MA-*Cdc73fl*-CreER and EH-*Cdc73fl*-CreER cell lines treated with EtOH or 100nM 4-OHT for 24 or 48 hours. The Cdc73 protein is undetectable following 48 hours of 4-OHT treatment. β-actin serves as a loading control. Arrow indicates an unidentified band.

Figure S5

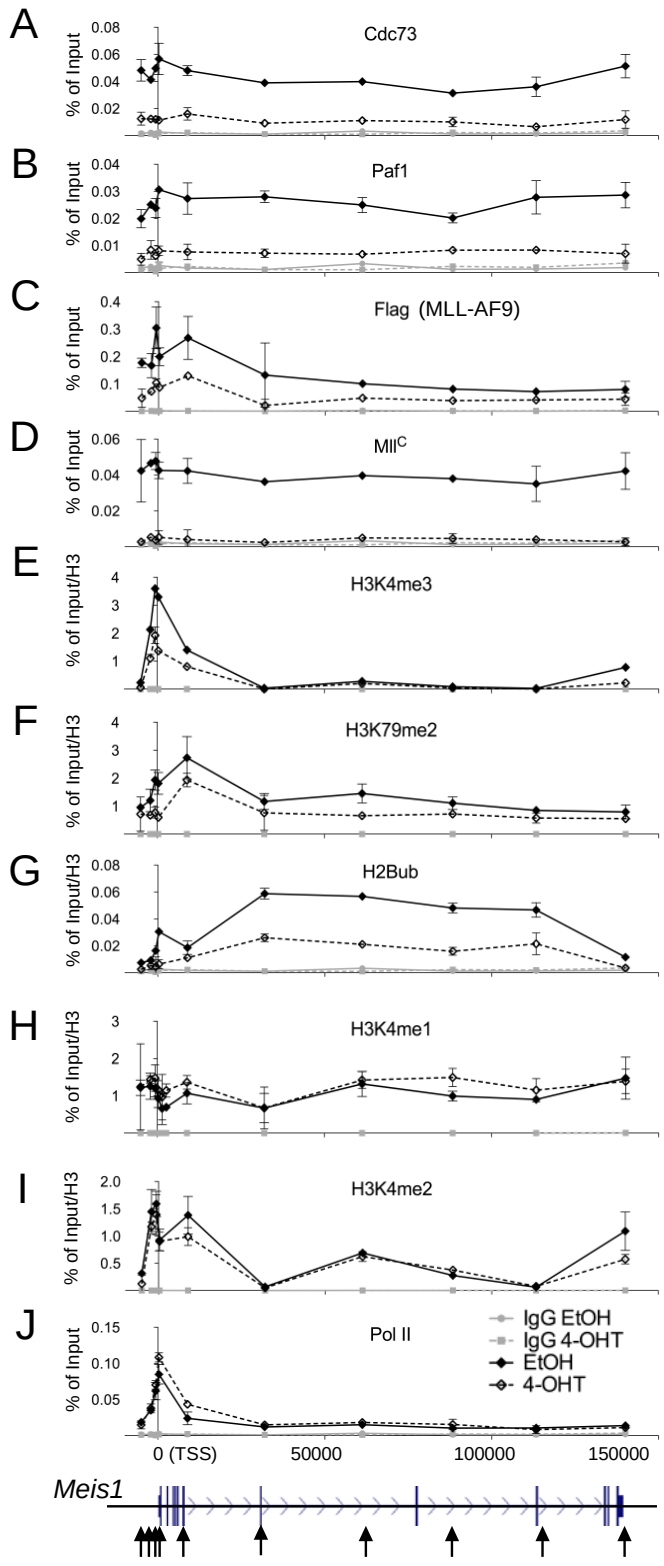
A
Cdc73
% of Input
0.08
0.06
0.04
0.02
B
Paf1
% of Input
0.04
0.03
0.02
0.01
C
Flag (MLL-AF9)
% of Input
0.4
0.3
0.2
0.1
D
MllC
% of Input
0.06
0.04
0.02
E
H3K4me3
% of Input/H3
4
3
2
1
F
H3K79me2
% of Input/H3
4
3
2
1
G
H2Bub
% of Input/H3
0.08
0.06
0.04
0.02
H
H3K4me1
% of Input/H3
3
2
1
I
H3K4me2
% of Input/H3
2.0
1.5
1.0
0.5
J
Pol II
% of Input
0.15
0.10
0.05
IgG EtOH
IgG 4-OHT
EtOH
4-OHT
0 (TSS)
50000
100000
150000
Meis1

Figure S5 – ChIP assays in MA-*Cdc73fl*-CreER cells

Chromatin immunoprecipitation (ChIP) assays were performed on the MA-*Cdc73fl*-CreER cell line treated with either EtOH (solid black lines) or 100nM 4-OHT (dotted black lines) for 48 hours. The *Meis1* locus (transcribed from left to right) is shown schematically with arrows indicating the site of primer probe sets recognizing the promoter and coding region of *Meis1*. Solid and dotted gray lines indicate background signals detected with normal IgG in EtOH and 4-OHT treated cells respectively. ChIP experiments were performed for A) Cdc73, B) Paf1, C) Flag (MLL-AF9 fusion protein), D) MLL^{C}, E) H3K4me3, F) H3K79me2 G) H2Bub and H) H3K4me1 I) HeK4me2 and J) RNA pol II. All binding is shown as a percentage of 1% input chromatin except E, F, G, H and I that are then divided by total histone H3 (mean ± SD; n = 3 experimental triplicate).

Figure S6

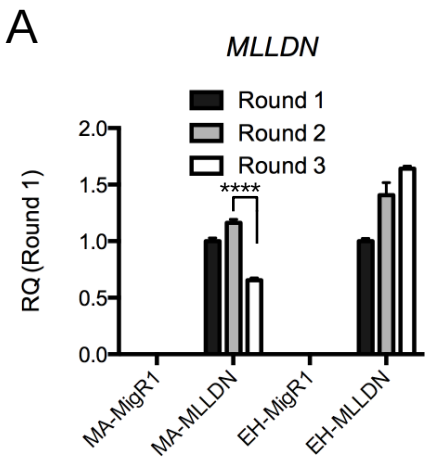

Figure S6 – MLLDN expression is silenced in MLL-AF9 leukemic cells

A) RNA was isolated from cells collected after each round of colony assays performed with cells transduced with MLL-AF9, E2A-HLF and either MigR1 or MigR1-MLLDN. cDNA was generated and used in q-RT-PCR experiments to determine the expression level of MLLDN using transcript specific primers. Data shows a specific down regulation of MLLDN expression in leukemic cells bearing the MLL-AF9 fusion protein in contrast to those transformed by E2A-HLF. Statistical analysis performed using unpaired two tailed *t* test. ****$p<0.0005$.

Figure S7

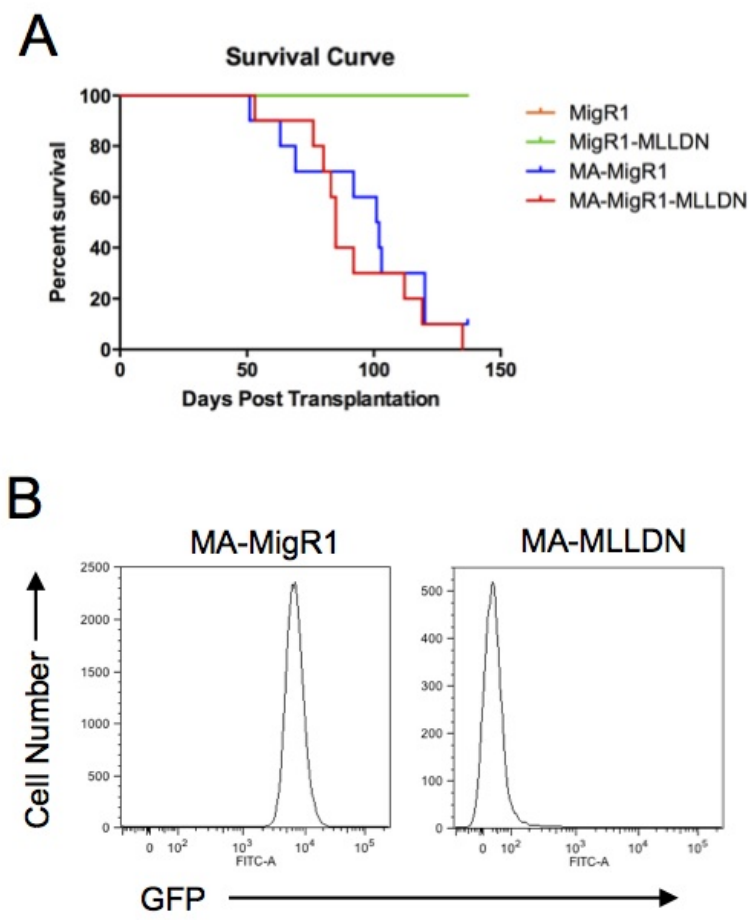

Figure S7 – Expression of MLLDN does not change disease latency in an *in vivo* MLL-AF9 leukemia mouse

A) C57Bl/6 mice were injected with lin- c-kit+ mouse bone marrow cells co-transduced with MigR1, MigR1-MLLDN, MLL-AF9/MigR1 or MLL-AF9/MLLDN. Survival rates of recipient mice are shown in a Kaplan-Meier curve. B) Flow cytometry was performed on bone marrow cells isolated from leukemic mice injected with either MLL-AF9/MigR1 or MLL-AF9/MLLDN. Data shows a strong selective pressure against MLLDN expression in MLL-AF9 leukemic cells compared to empty MigR1.

Figure S8

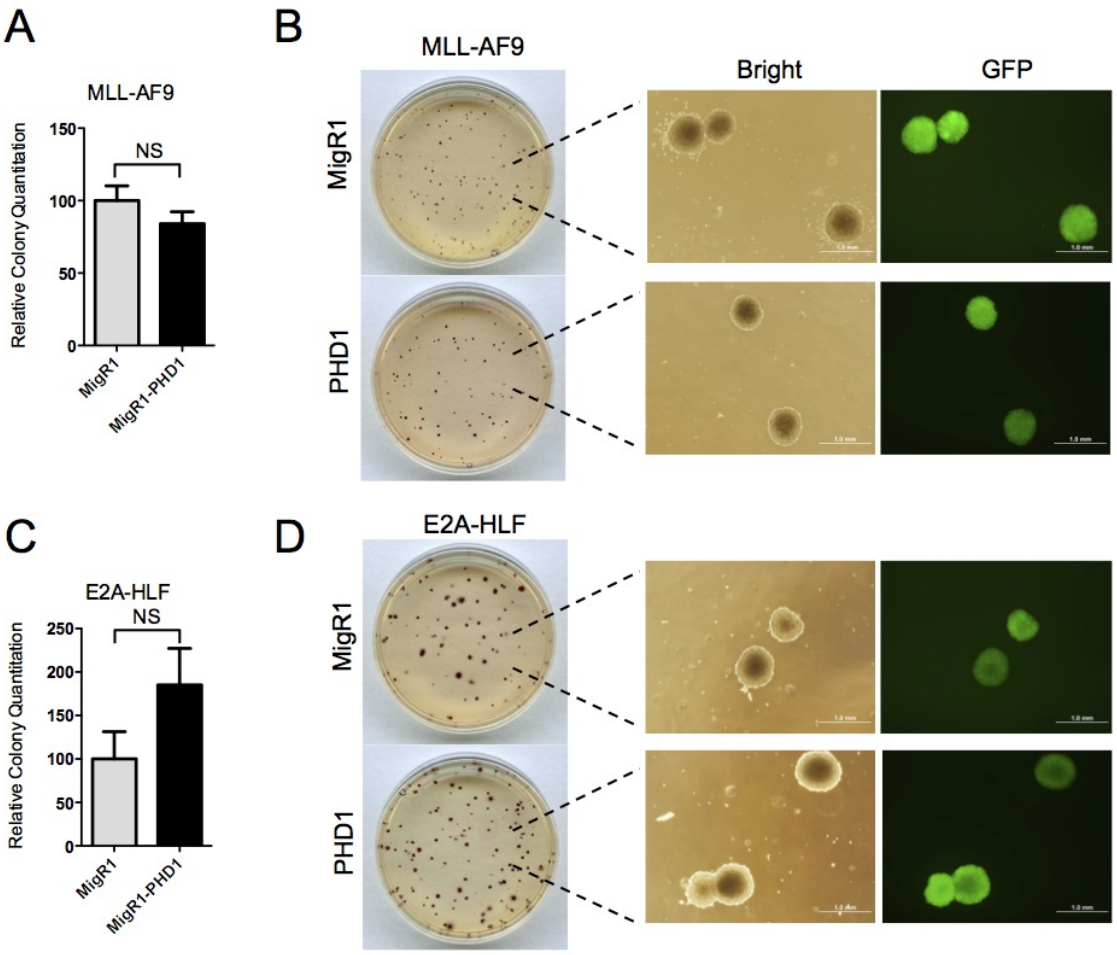

Figure S8 – Expression of PHD1 does not affect the growth of MA or EH cells

Colony assays were performed with the MA and EH cell lines transduced with MigR1 or the MigR1-PHD1 retrovirus. Sorted GFP positive cells were grown in semi-solid media. A and C) The number of colonies is shown relative to each cell line transduced with empty MigR1. 5 x 10^5 cells were plated for MLL-AF9 colony assays and 1 x 10^5 cells were plated for E2A-HLF colony assays. Statistical analysis performed using unpaired two tailed *t* test of duplicate experiments. NS = Not Significant ($p>0.05$). B and D) INT stained plates of experiments described in A and C shows no change in colony formation in both MA or EH cells following transduction with PHD1. Similar GFP positivity in resultant PHD1 transduced colonies compared to MigR1 transduced colonies suggests no selective pressure against the expression of PHD1 and confirming the specificity of MLLDN on inhibition of MA cell proliferation. Scale bar = 1mm.

Figure S9

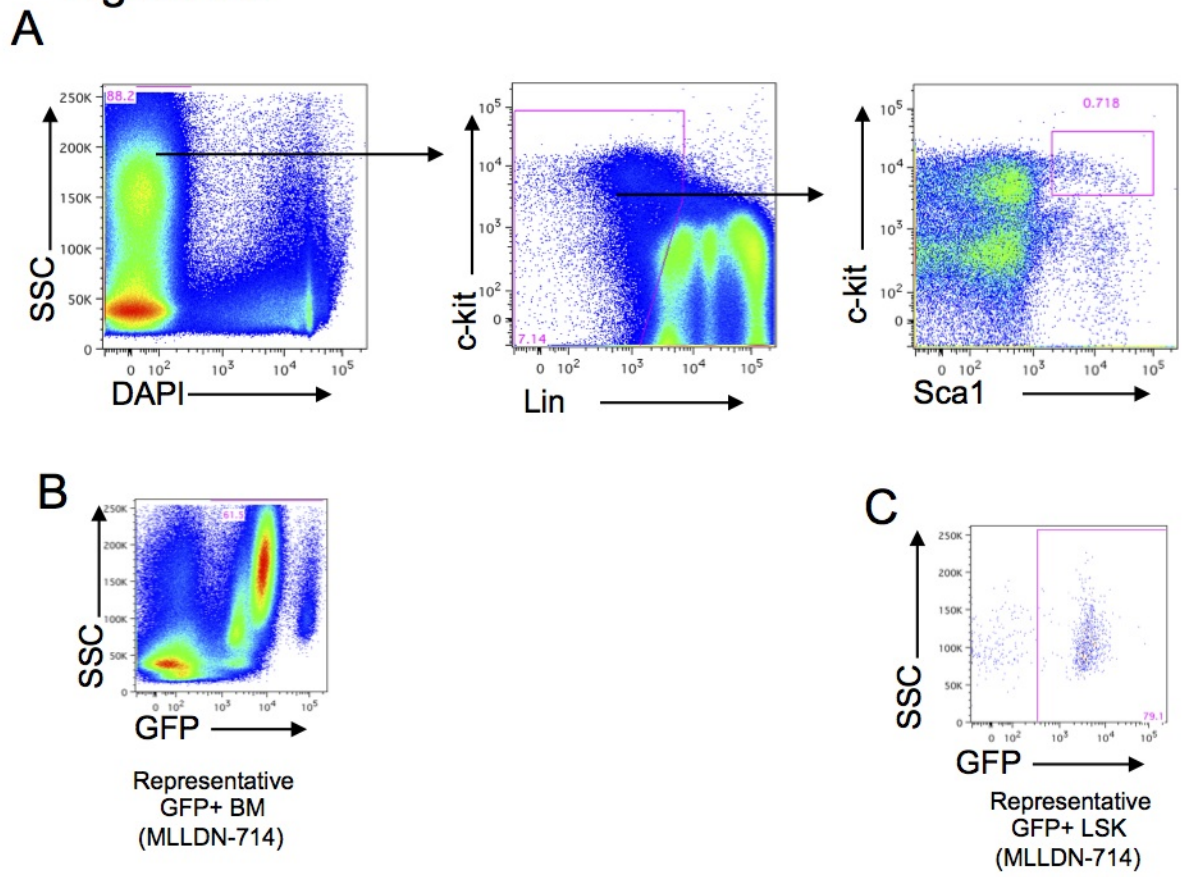

Figure S9 – Expression of MLLDN does not alter normal hematopoiesis

A) Viable (DAPI negative) bone marrow cells were analyzed for c-kit and expression of lineage markers. $Lin^{-}c\text{-}kit^{+}$ bone marrow cells were analyzed for Sca-1 expression to identify $Lin^{-}c\text{-}kit^{+}sca\text{-}1^{+}$ (LSK) cells. B) A representative mouse with GFP^{+} cells is plotted from total bone marrow from the MLLDN cohort. C) Representative GFP^{+} reconstituted mouse from the MLLDN cohort showing GFP expression in the LSK population of cells.

Figure S10

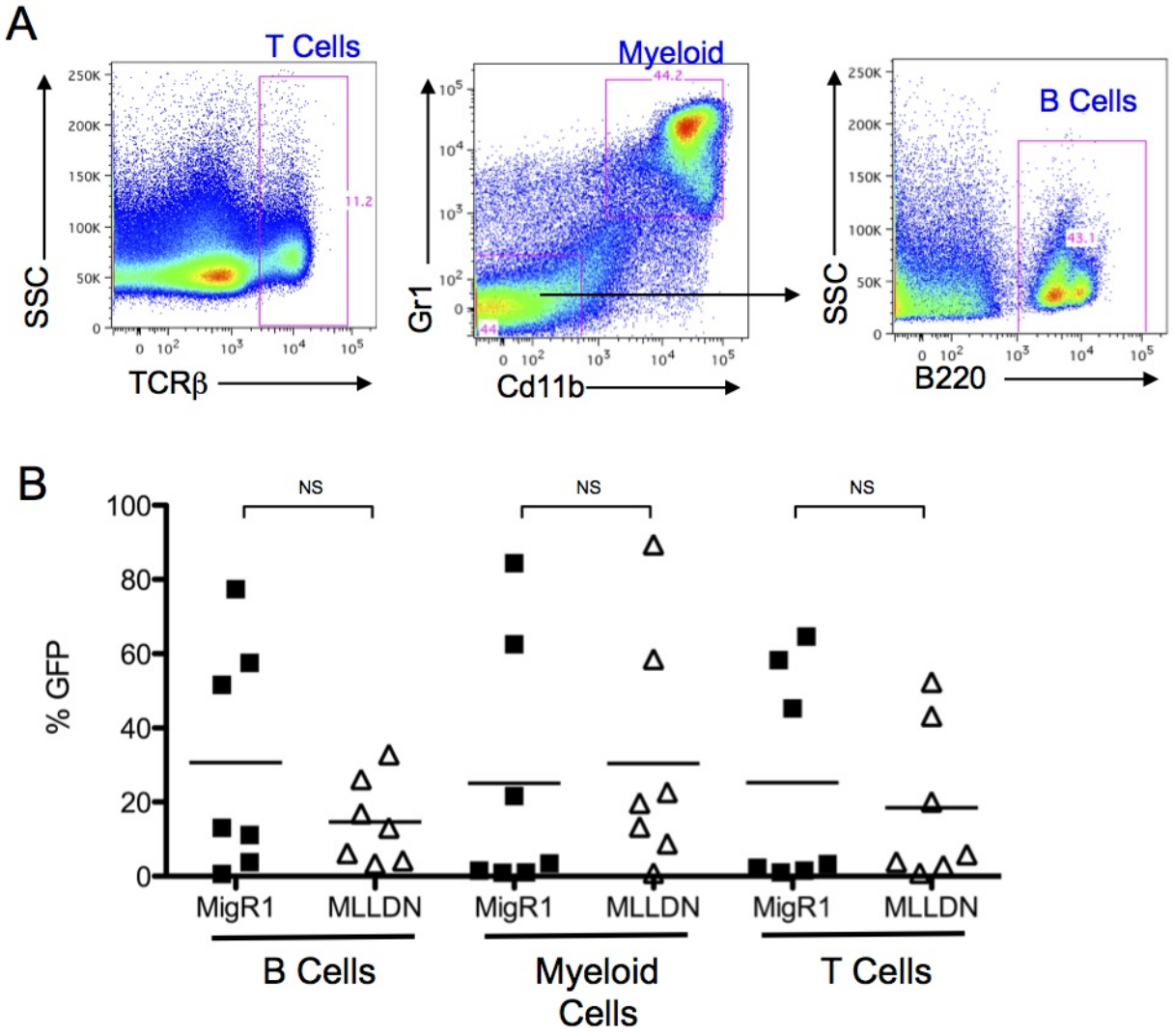

Figure S10 – Lineage cells of the hematopoietic system are not affected by MLLDN

A) Representative flow cytometry plots are shown for GFP$^+$ cells with the TCRβ$^+$ T cell population, Cd11b$^+$, Gr-1$^+$ myeloid population and the B220$^+$ B cell population. B) The percentage of GFP$^+$ B cells, Myeloid cells and T cells are plotted for each mouse in the MigR1 and MigR1-MLLDN cohort. Mean expression is indicated by the horizontal line. Although not statistically significant, decreased GFP expression is observed specifically in the B cell compartment. Statistical analysis performed using unpaired two tailed *t* test. NS = Not Significant ($p>0.05$).