Supplementary Material and Methods

Real-time quantitative PCR (RQ-PCR) for human *EVI1* and *MDS-EVI1*

RNA isolation, cDNA synthesis were performed as described.[1-3] A diagnostic assay was developed to detect EVI1 expression in AML patients.[3] In summary, Real-time quantitative PCR (Q-PCR) was performed using human EVI1 forward and reverse primers plus probes located on exon 14 and 15. The $\Delta\Delta$Ct method was used to calculate relative expression using PBGD (Porphobilinogen deaminase) as a reference gene and the EVI1 overexpressing ovarian carcinoma cell line SKOV3 served as a calibrator; all cases with Ct values > 30.5 were discarded.[2,3] Optimal cut-off levels for EVI1 positivity were determined on a cohort of 458 patients. A cut-off of 0.1 was determined: cases with an expression $\geq$ 0.1 relative to the EVI1 expression of SKOV3 were labelled as $EVI1^{pos}$ and cases with a relative expression < 0.1 were determined $EVI1^{neg}$.
Primers and probe sequences are shown in Table S1.

RQ-PCR of murine genes

mRNA expression of *Evi1*, *Mds-Evi1*, *Meis*1 and *Prdm16* in mBM cells or colonies was determined by RQ-PCR as described previously.[4] mRNA expression was normalized against *HPRT* (hypoxanthine-guanine phosphoribosyl transferase) and mBM transduced with pMSCVpuro (EV) were used as a calibrator. All primer sequences are listed in Table S2.

Colony assays

Retrovirally infected mononucleated mBM ($1x10^5$ cells/dish) were plated in 1% methylcellulose medium (M3231; Stem Cell Technologies, Vancouver, Canada) supplemented with 10 ng/ml IL3, 10 ng/ml IL6, 10 ng/ml GM-CSF and 20 ng/ml SCF (Peprotech EC, London, UK) and selective antibiotics. Colonies were counted after 7 days of culture, and were subsequently plated at a concentration of 2x104 cells per dish, for 3 additional rounds of weekly replating (Figure S4A). During every replating, colonies were scored, and remaining cells were harvested for RNA isolation and cytospins. After 4 rounds of plating, cells were cultured in IMDM containing 10% FCS supplemented with 10 ng/ml IL3, 10 ng/ml IL6, 10 ng/ml GM-CSF and 20 ng/ml SCF to establish multiclonal cell lines. Clonal MLL-AF9 mBM cell lines were isolated from an additional round of low density Methocult culture (Figure S5A).

Cell culture of MLL-AF9 cell line 4166 and MLL-AF9 mBM clones

The 4166 cell line was established from spleen cells of a leukemic MLL-AF9 knock-in mouse model and its characterization is described previously.[5] 4166 cells were routinely maintained in IMDM containing 15% FBS and 2ng/ml murine IL3.Myeloid colony-forming assays were performed with 4166 cells and various clonal MLL-AF9 mBM cell lines in methylcellulose medium under myeloid conditions, and colonies were counted and scored after 7 days of culture as previously described.[5]

Western blot analysis

Cells were lysed in Carin buffer (20 mM Tris-HCL, 137 mM NaCl, 10 mM EDTA, 50 mM NaF, 1% Triton, 10% glycerol) containing protease inhibitors. EVI1 protein expression in colonies of MLL-AF9 transduced mBM cells was detected with polyclonal antibodies directed against the C-terminal or N-terminal part of murine EVI1 (kind gift of Prof. JN Ihle). EVI1 protein levels in AML samples were detected by a rabbit polyclonal EVI1

antibody (#2265, Cell Signaling, Danvers, USA). For the western blot analysis performed on 4166 cells the following antibodies were used: EVI1 (C-20, SC-8707-R, Santa Cruz Biotech., Santa Cruz, CA), Caspase-3, -8, -9 or -12, PARP (Cell Signaling) and HSP90 (BD Biosciences, San Jose, CA). Equal loading of protein was determined by reprobing the blots with an antibody against Actin (Santa Cruz, or Sigma-Aldrich, St. Louis, MO).

Retroviral transduction of murine Bone Marrow (mBM) cells

Retroviral constructs harboring the MLL fusions: pMSCVneo MLL-AF6, pMSCVpuro MLL-AF9, pMSCVneo MLL-AF9, pMSCVpuro MLL-ENL and pMSCVpuro E2A-PBX, were published previously.[6,7] Retroviral supernatants were produced in 293T cells co-transfected with the packaging vector pCL-Eco together with pMSCV constructs, using FuGENE6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. Retroviral supernatants were collected 2 days after transfection and filtered through a 0.45µm cellulose acetate filter. Lin^- cells[8] or Ficoll gradient purified mononuclear mBM cells were transduced twice on two consecutive days with the aid of RetroNectin (Lonza, Basel, Switzerland) mediated viral transduction.

ChIP-on-chip experiments

Chromatin immunoprecipitations on cross-linked material of MLL-AF9 mBM clones with either isotype-IgG control (#2729, Cell Signaling) or H3K79me2 (Abcam, Cambridge, UK; antibody ab3594) antibodies were performed according to the ChIP protocol available on the Millipore website http://www.millipore.com/userguides/tech1/mcproto407.
Immunoprecipitated material was de-crosslinked and 10 µl was amplified using a Whole Genome Amplification kit (WGA2/WGA3; Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequent fragmentation of amplified DNA, labeling and hybridization to Affymetrix Mouse promoter 1.0R arrays was done according to manufacturer's recommendations (Affymetrix, Santa Clara, CA, USA). Visualization of enriched regions was carried out using Affymetrix Integrated Genome Browser (IGB) software.

Knockdown of murine *Evi1 or Dot1L* using lentiviral shRNAs

Commercial nontarget control (SHC002), *Evi1* short hairpin RNA (SH1152; CCCAATCACCAAGTGAAGTTA), *Dot1L* short hairpin RNAs (SH6319: GTCCAGTTTGTACTGTCAATA; SH6320: GCTGACCTACAATGACCTGAT; SH6322: CGGCAGAATCGTATCCTCAAA) or PLKO1.EV lentiviral vectors were selected from the mission shRNA library (Mission© library; Sigma-Aldrich, Zwijndrecht, The Netherlands). After initial pilot studies testing of more than 10 different shRNAs against *Evi1*, SH1152 was selected as the only shRNA leading to more than 50% reduction of murine EVI1 protein expression (EB, MH and RD, data not shown).
ShRNA lentivirus was produced in 293T cells after co-transfection of SHC002, SH1152, or PLKO1.EV together with the packaging plasmids pSPAX2 and pMDG.2. Culture supernatants containing pseudovirus were harvested 48–72 h post-transfection. Lentivirus containing culture supernatants were filtered through a 0.45µM filter (Millipore Bedford, MA) and concentrated by ultracentrifugation at 25,000 rpm for 2 hours at 4°C, and viral pellets were resuspended in serum-free IMDM. Viral titers were determined by transducing NIH3T3 cells using diluted culture supernatants and counting the number of colonies after 13 days of culturing in the presence of puromycin. A multiplicity of infection (MOI) of 100 was used in subsequent experiments by spin transduction, or RetroNectin mediated transduction.

Retroviral transduction of defined hematopoietic cell populations

Bone marrow from 6-8 week old C57Bl/6 females was used to isolate HSC as Lin(CD3e CD4 CD8a CD19 CD45R CD127 Ter119 Gr1)- CD34- Sca1+ c-Kit+ and GMP as Lin- Sca1- c-Kit+ CD34+ CD16/32+ (eBiosciences) using a FACSAria cell sorter (BD Biosciences). Freshly isolated HSC and GMP were retrovirally transduced with MLL-AF9, after 16 hours the cells were plated in semisolid media (M3234) supplemented with 10ng/ml of each IL-3 and IL-6 and 20ng/ml SCF. The cells were replated every 7 days for 3 weeks, which allows for non MLL-AF9 expressing cells to die out. At the end of third plating the cells were collected, total RNA was extracted using Trizol (Invitrogen) and reverse-transcribed (BioLine). cDNA was used in quantitative SYBR green based PCR reaction (BioLine) with *Evi1* specific primers Evi1F ctcgaagccttcaggaacac and Evi1R agcttcaagcgggtcagtta.

Evi1 promoter luciferase assay

For promoter luciferase experiments, per well 0.5 x10^6 Jurkat cells were seeded in 24-well plates. Cells were transfected using X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN, USA) with 1μg expression plasmid, 1μg pGL3-EVI1 (harboring the human EVI1 promoter[9], kind gift of Kinuko Mitani) and 100ng pGL3 Renilla. After 48 hours of culture, cells were harvested and luciferase activity was measured using Dual-Luciferase reporter reagents (Promega, Madison, WI, USA).

Supplementary References

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6. Chueng N, Chan LC, Thompson A, et al. Protein arginine-methyltransferase-dependent oncogenesis. *Nat Cell Biol.* 2007; (10):1208-15.

7. Wong P, Iwasaki M, Somervaille TC et al. Meis1 is an essential and rate-limiting regulator of MLL leukemia stem cell potential. *Genes Dev.* 2007; 21(21):2762-74.

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SUPPLEMENTARY TABLES

Table S1. Primer sequences

Primer Sequence quantitative pcr	
hEVI1ex14-F	AGTGCCCTGGAGATGAGTTG
hEVI1ex15-R	TTTGAGGCTATCTGTGAAGTGC
Probe EVI1	CCCCAGTGAGGTATAAAGAGGA
hPBGD-F	GGCAATGCGGCTGCAG
hPBGD-R	GGGTACCCACGCGAATCAC
Probe PBGD	CATCTTTGGGCTGTTTTCTTCCGCC
hMDSEVI1-F	GAAAGACCCCAGTTATGGATGG
hMDSEVI1-R	GTACTTGGCCAGCTTCCAACA
probe MDSEVI1	TCTTAGACGAATTTTACAATGTGAAGTTCTGCATAGATG
mMeis1-F	AGCGGTGGCCATACTTCAC
mMeis1-R	CACTGTTGTCCAAGCCATCA
mHPRT-F	AGCCTAAGATGAGCGCAAGT
mHPRT-R	ATGGCCACAGGACTAGAACA
mEVI1ex11-F	CCAATCTTGACAGACACCTTGAA
mEVI1ex12-R	GGTTGCTGTTCCCGATGAAATT
mMDSEVI1-F	CCCTCCCTCCACATTCAAGA
mMDSEVI1-R	GGTCCTCTTCACTCTTCATGAAC
mPRDM16-F	CCACCAGCGAGGACTTCAC
mPRDM16-R	GGAGGACTCTCGTAGCTCGAA
mDOT1L-F	TGGAGAACTATGTCCTGATCGAC
mDOT1L-R	GTGCCGCAGAAGTCCATTG
mHoxA9-F	GAGAGCGGCGGAGACAAGCC
mHoxA9-R	TCGTACCTGCGGTCCCGTGT

Primer Sequences ChIP Q-PCR	
hu-ChIP-Evi1-k4 FW	CAAAGGGTCCGAATGTGACT
hu-ChIP-Evi1-k4 REV	ATCTGAATTCCTGGCACTGG
huChIPHoxA9_k4peakFw	TGTCCTCTTGTCCCCCTTCT
huChIPHoxA9_k4peakRev	CAGGTTGGGAAAGAAAGACGAA
huChIPbAct_k4peakFw	GTGGACATCTCTTGGGCACT
huChIPbAct_k4peakRev	AGGGCAGTTGCTCTGAAGTC
ChIPmEvi1prom1 FW	ACAAATCCTGCCTGGAGAAA
ChIPmEvi1prom1 Rev	ATCTCCAGAGTGGTTCGGAAG
ChIPmEvi1prom2 FW	ACATGTTTCTGGGGATGGTG
ChIPmEvi1prom2 Rev	CGAGGGAGAAAGGGAGCTAT
ChIPmEvi1Exon1#1 FW	CCCTACCTTCGCACACTTTC
ChIPmEvi1Exon1#1 Rev	GAGCCGGGTTTGGTGTATTT
ChIPmEvi1Exon1#2 FW	CGAAATGTTAGCGGGTGTTC
ChIPmEvi1Exon1#2 Rev	TTCGCTTACCTTCCGATTTC
ChIPmEvi1Exon9 FW	GCAATTGAGAACATGGCAGA
ChIPmEvi1Exon9 Rev	CTGCAGGTGTAGCGCTCTTT
ChIPmEvi1Exon13 FW	TAAAAAGGCTTTGGCAACCA
ChIPmEvi1Exon13 Rev	GGCACCAGCTTCTTCGTAGT
ChIPmHoxA9 FW	ATGTTGACTGGCGATTTTCC

ChIPmHoxA9 Rev	AGAGAACTGCCAACCCTCAA
ChIPmHoxC8 FW	CAGGCTCAGCCCTGACTTAC
ChIPmHoxC8 Rev	CCAGCCCTAGCTAATGGACA
ChIPm_b_actin FW	GATGCTGACCCTCATCCACT
ChIPm_b_actin Rev	ATGAAGAGTTTTGGCGATGG

Primer sequences for detection of Common MLL-Fusions

Forward MLL	CGTCGAGGAAAAGAGTGA
ENL Rev	TACCCCGACTCCTCTACTT
ELL Rev	CCCATGACTGGAGACATACT
AF6 Rev	TCCCGATCATCTTTGTTC
AF9 Rev	ATGTTTCCAGGTAACTCTGTAGT
AF10 Rev	CTGGAAATTTGCATTTGTAA

Table S2.
Patient characteristics of 102 *MLL* rearranged AML patients**.** Per patient, age, sex, FAB type, karyotype, *MLL* rearranged fusion gene, relative expression (RE) of *EVI1* scoring of *EVI1* (over)expression (+) or no *EVI1* expression (-) are shown. The karyotyping was performed according to the International System for Human Cytogenetic Nomenclature (1995) for each patient (N.D. Not Determined).

Sample	Age	Sex	FAB	G-banding	*MLL*-fusion	RE *EVI1*	*EVI1*+
642	48	M	M1	46,XY,t(10;11)(q22;q23)	*MLL-AF10*	0.44	+
114	30	M	M4	46,XY,t(10;11)(q11;q23)[8]/45,XY,add(1)(p36), t(10;11)(q11;q23),der(12)t(12;18)(p11;q11),-18[11]	*MLL-AF10*	0.45	+
2535	38	F	M5	47, XX, i(8)(q10), +19 [1]/48, idem, +i(8) [2]/49, idem, +i(8), +22 [4]/50, idem, +i(8), +i(8), +22 [2]/46, XX [1].	*MLL-AF10*	0.0	-
2281	20	M	M4	46,XY[58]; Cryptic rearrangement	*MLL-AF10*	0.0	-
292	50	F	N.D.	47,XX,add(7)(p22),inv(8)(p23p13),+8,del(10)(p11), der(11)t(11;10;7)(pter->11q23::?->?::q23->pter),del(19)(q13)	*MLL-AF10*	0.0	-
336	37	M	M4	46,XY,ins(10;11)(p13;q23q13)[12]/48,XY,+8, ins(10;11)(p13;q23q13),+19[7]/46,XY[1]	*MLL-AF10*	0.05	-
582	31	M	M5	46,XY,ins(10;11)(p13;q13q23)	*MLL-AF10*	0.01	-
3317				47,XX,+add(1)(p1?2)[24]/46,XX[3]	*MLL-AF10* *MLL-ELL*	0.0	-
2255	45	M	M1	46,XY,t(4;11)(q21;q23)[18]	*MLL-AF4*	0.0	-
524	38	F	N.D.	46,XX,t(4;11)(q21;q23),del(20)(q13)[7]/46,XX,t(4;11)(q21;q23), del(5)(q31q33),del(12)(p11),del(20)(q13)[19]	*MLL-AF4*	0.0	-
15017	34	M	M4	46,XY,t(4;11)(q21;q23)[21]	*MLL-AF4*	0.0	-
818	60	F	M0	48,XX,t(6;11)(q27;q23),+21,+21[9]	*MLL-AF6*	2.22	+
590	59	F	M1	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	0.67	+
675	58	F	M4	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	3.75	+
18	41	F	M1	46,XX,t(6;11)(q26;q23)	*MLL-AF6*	1.92	+
964	34	F	M4	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	0.5	+
2207	31	M	M1	46,XY,t(6;11)(q25;q23)	*MLL-AF6*	0.7	+
6238	30	F	M4	46,XX,t(6;11)(q27;q23)[28]	*MLL-AF6*	1.1	+
15018	30	M	M4	46,XY,t(6;11)(q27;q23)	*MLL-AF6*	6.5	+
549	28	F	M0	46,XX,t(6;11)(q27;q23)	*MLL-AF6*	0.42	+
14294	19	M	M5	46,XY,t(6;11)(q26;q22)	*MLL-AF6*	0.6	+
5351	67	F	M4	46,XX,t(6;11)(q27;q23)[36]	*MLL-AF6*	0.39	+
2196	39	M	M5	NULL; Cryptic rearrangement	*MLL-AF6*	2.2	+
2272	35	F	M5	47,XX,+8,add(9)(34)[19]/47,XX,+8,add(17)(q25)[1]/48,XX,+8,+8, add(17)(q25)[1]/46,XX[36] ; Cryptic rearrangement	*MLL-AF6*	0.0	-
15015	40	M	M5	46,XY[35],46,XY,t(6;11)(q26orq27;q23)[6]	*MLL-AF6*	0.0	-
889	22	F	M5	45,XX,t(6;11)(q27;q23),-8,+2x i(8)(q10)	*MLL-AF6*	0.0	-
14454	72	F	N.D.	45,XX,add(1)(p?),der(2)t(2;6)(p16;q13)t(2;6)(q32;p12), add(5)(q21),der(5)t(5;18)(q31;?),?inv(7)(q31q35), der(9)t(9;11)(q22~31;q13),del(12)(q13q23),-17,-18,+mar	*MLL-AF9*	0.1	+
14290	62	F	M1	46,XX,t(9;11)(p22;q23)[16]	*MLL-AF9*	0.7	+
14456	61	F	Sec	46,XX,t(9;11)(p22;q23)[21]	*MLL-AF9*	2.6	+
7072	61	M	M4	46,XY,der(11)(q2?)[3]/46,XY[18] .ish t(9;11)(p22;q23)	*MLL-AF9*	0.1	+
464	60	F	N.D.	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	4.38	+
2682	57	F	M4	46,XX,t(2;9;11)(p13;p22;q23)	*MLL-AF9*	1.0	+
14457	51	M	N.D.	46,XY,t(9;11)(p22;q23)[18]	*MLL-AF9*	0.7	+
709	51	M	M4	46,XY,t(4;11;9)(q27;q23;p22)	*MLL-AF9*	4.6	+
1055	46	M	M4	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	8.15	+
485	45	F	N.D.	46,XX,t(9;11)(p22;q23)[13]/47,XX,t(9;11)(p22;q23),+19[2]	*MLL-AF9*	6.52	+
716	43	F	M0	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	4.46	+

Sample	Age	Sex	FAB	G-banding	*MLL*-fusion	RE *EVI1*	*EVI1*+
587	34	M	M4	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	4.32	+
589	32	F	M5	46,XX,t(9;11)(p22;q23)[3]/47,XX,t(9;11)(p22;q23),+13[8]	*MLL-AF9*	2.13	+
2288	31	M	M4	45,XY,-7,t(9;11)(p21;q23)[33]	*MLL-AF9*	1.7	+
375	23	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	1.42	+
261	23	F	M5	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	6.95	+
15014	17	F	N.D.	47,XX,t(9;11)(p22;q23),+der(9)t(9;11)(p22;q23)	*MLL-AF9*	2.0	+
6720	38	F	M1	NN; Cryptic rearrangement	*MLL-AF9*	2.95	+
6945	33	M	M4	46,XY/47,XY,+?8 (9%)/48,XY,+mar1,+mar2 (90%); Cryptic rearrangement	*MLL-AF9*	11.77	+
7416	55	M	M4	48, XY, +9, +21 [9]; Cryptic rearrangement	*MLL-AF9*	2.97	+
2749	38	M	M5	46,XY[32]; Cryptic rearrangement	*MLL-AF9*	0.0	-
6882	37	M	M5	NN; Cryptic rearrangement	*MLL-AF9* *MLL-ELL*	0.0	-
6955	31	F	M2	47,XX,+8[29]/46,XX[1] Cryptic rearrangement	*MLL-AF9* *MLL-ELL*	0.0	-
6976	31	F	M0	46,XX[32] ; Cryptic rearrangement	*MLL-AF9*	0.0	-
7178	28	M	M5	46,XY,del(9)(q?),add(10)(q?24),add(19)(p13 or q13)[cp]; Cryptic rearrangement	*MLL-AF9*	0.0	-
14293	66	F	M5	51-52,XX,+4[2],+8,t(9;11)(p22;q23)[3],+13,+16[2],+21[2], +22[2][cp4]/46,XX[3]	*MLL-AF9*	0.0	-
14289	59	M	M5	46,X,-Y,+8,t(9;11)(p21;q23),t(18;20)(q21;p12)[13]	*MLL-AF9*	0.0	-
2541	57	F	M5	45,XX,-7[3]/45,idem,t(9;11)(p21;q23)[20]/46,XX[3]	*MLL-AF9*	0.0	-
799	56	M	M5	47,XY,+8,t(9;11)(p22;q23),inv(16)(p13q22),del(17)(p11)	*MLL-AF9*	0.0	-
210	53	F	M5	47,XX,+8,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
649	52	F	M5	46,XX,t(9;11)(p22;q23)[10]/47,XX,t(9;11)(p22;q23),+8[4]	*MLL-AF9*	0.01	-
944	50	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
6364	49	M	M5	46,XY,t(9;11)(p22;q23)[19]	*MLL-AF9*	0.0	-
65	48	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
274	48	F	M5	46,XX,t(9;11)(p21;q23)	*MLL-AF9*	0.0	-
2694	46	M	M5	46,XY,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
7166	44	F	M5	46,XX,t(9;11)(q22;q23)[11]/46,XX[4]	*MLL-AF9*	0.0	-
3316	44	F	M5	46,XX,t(9;11)(p22;q23),t(10;12)(q21;q24)[22]	*MLL-AF9*	0.0	-
5358	40	F	M5	46,XX,add(6)(q2?5),t(9;11)(p22;q23)[20]/	*MLL-AF9*	0.0	-
350	37	F	M5	46,XX,t(9;11)(p22;q23)	*MLL-AF9*	0.0	-
413	36	F	N.D.	47,XX,t(9;11)(p21;q23),+21	*MLL-AF9*	0.01	-
14288	35	M	M5	46,XY,inv(2)(q1?1q35),t(9;11)(p22;q23)[38]	*MLL-AF9*	0.0	-
1083	35	F	N.D.	46,XX,t(9;11)(p21;q23)	*MLL-AF9*	0.01	-
348	34	F	M5	47,XX,+8,t(9;11)(p22;q23)[9]/46,XX[2]	*MLL-AF9*	0.0	-
14295	30	M	M5	47,XY,+8,t(9;11)(p22;q23)[22]	*MLL-AF9*	0.0	-
15013	30	M	M3	47,XY,+8,der(9)t(9;11)(q;q),t(15;17)(q22;q21)	*MLL-AF9*	0.0	-
15019	29	M	M5	50,XY,+5,+6,t(9;11)(p21;q23),+19,+22[4]	*MLL-AF9*	0.0	-
691	28	M	M5	46,XY,t(9;11)(p22;q23)[10]/47,XY,+8,t(9;11)(p22;q23)[5]	*MLL-AF9*	0.02	-
14453	28	M	N.D.	46,XY,der(9)ins(9;?)(q12;?)del(9)(q12;q2?2),t(9;11)(p22;q23)[16]	*MLL-AF9*	0.0	-
2285	18	F	M5	46,XX,t(9;11)(p21~22;q23)[57]	*MLL-AF9*	0.0	-
15016	64	M	NA	46,XY,t(11;19)(q23;p13)	*MLL-ENL*	4.3	+
14460	57	F	NA	46,XX,t(11;19)(q23;p13)[29]	*MLL-ENL*	0.3	+
360	55	F	N.D.	46,XX,t(11;19)(q23;p13)	*MLL-ENL*	0.11	+
60	52	F	N.D.	46,XX,t(11;19)(q23;p13)	*MLL-ENL*	0.31	+
126	48	F	M4	46,XX,t(11;19)(q23;p13)[9]/46,XX[1]	*MLL-ENL*	3.02	+
14459	75	F	M4	46,XX,t(11;19)(q23;p13)[5]	*MLL-ENL*	0.0	-
465	45	M	M5	46,XY,t(11;19)(q23;p13)	*MLL-ENL*	0.0	-
572	39	M	M5	45,X,-Y,t(11;19)(q23;p13)	*MLL-ENL*	0.0	-
143	39	M	M1	46,XY,t(2;8)(p12;q24),add(7)(q32),?t(10;11;19)(p13;q23;p13)	*MLL-ENL*	0.0	-

Sample	Age	Sex	FAB	G-banding	*MLL*-fusion	RE *EVI1*	*EVI1*+
14291	38	F	N.D.	47,XX,+X,t(11;19)(q23;p13)[23]	*MLL-ENL*	0.0	-
3322	35	F	M5	47,XX,+8,t(11;19)(q23;p13)[25]	*MLL-ENL*	0.0	-
14462	34	F	M5	47,XX,+8,t(11;19)(q23;p13)[25]	*MLL-ENL*	0.0	-
6981	27	M	M5	46,XY	*MLL-ELL*	3.2	+
7306	51	M	M5	46,XY,t(11;19)(q23;p13.1)[15]/46,XY[5]	*MLL-ELL*	0.3	+
3328	41	F	M5	46,XX,t(11;19)(q23;p13)[21]	*MLL-ELL*	0.8	+
7053	29	F	M1	46,XX,t(1;3)(p13:q29),del(5)(q13q33),del(11)(p13)[16]/92,XXXX, del(5)(q13q33)x2,del(12)(p11.2)x2[4]/46,XX[4]	*MLL-ELL*	0,0	-
3289	48	M	M2	46,XY,t(11;19)(q23;p13)[20]	*MLL-ELL*	0.01	-
374	49	M	M4	46,XY,t(11;17)(q23;q21)	*Other*	0.12	+
247	47	F	M2	46,XX,t(7;11)(p11;q23)	*Other*	0.29	+
2229	43	M	M4	47, XY, +11 [14]/ 46, XY [13]	*Other*	0.0	-
363	56	F	M4	46,XX,t(11;22)(q23;q13)[18]/46,XX[3]	*Other*	0.0	-
7171	22	M	N.D.	48,XY,der(11)del(11)(p13)del(11)(q13q23),+21,+21[17]/46,XY[3]	*Other*	0.0	-
2302	43	F	N.D.	N.D.	*Other*	0.0	-
1102	55	M	N.D.	46,XY,t(11;21)(q23;q11)	*Other*	0.0	-
613	47	F	M4	46,XX,t(1;11)(q21;q23),del(20)(q11)[4]/46,XX[2]	*Other*	0.05	-
7307	40	F	M1	46,X,ins(X;11)(q13;q23q22)[1]/48,idem,+6,+19[33]/46,XX[7]	*Other*	0.0	-

Table S3
Distribution of CD14- , CD34+CD38+, CD34+CD38- in sorted fractions of M5 human leukemias, corresponding to Figure S2.
Percentages are given of the CD14- population and the percentages of the CD34+CD38+ and CD34+CD38- populations within the CD14- population.

	Sample	CD14- (%)	CD34+CD38+ (%)	CD34+CD38- (%)
3q	2747	14.7	79.3	10.97
MLL, non MLL-AF9	2275	22.1	9.09	2.97
MLL, non MLL-AF9	2220	26.5	0.43	0.16
MLL, non MLL-AF9	3221	10.5	0.13	0.47
MLL, non MLL-AF9	2261	6.5	0.52	0.48
MLL-AF9	14288	73.5	5.11	0.85
MLL-AF9	2285	50.1	0.20	0.01
MLL-AF9	5358	21.8	0.02	0.00
MLL-AF9	14293	28.5	23.33	56.88

Legends to Supplementary Figures S1 to S13:

Figure S1

Acute Myeloid Leukemia patients with an 11q23 translocation either expressing *EVI1* (*EVI1*^{pos}) or not (*EVI1*^{neg}) show a distinct gene expression signature.

Pairwise Pearson's correlation clustering of 35 11q23 AMLs, using 1455 probe sets (with standard deviation > 4, based on 506 AMLs). Color of boxes represents Pearson's correlation coefficient: with deeper red corresponding to higher positive correlation and deeper blue corresponding to higher negative correlation.

Figure S2

Hematopoietic stem and progenitor cells in FAB M5 human leukemias do not express *EVI1* mRNA.

A) Example of the gating strategy for sorting of CD14-/CD34+/CD38- (immature stem cells) and CD14-/CD34+/CD38+ (progenitor cells) from leukemic cells of FAB M5 AML samples. Table S3 depicts the relative percentages of the CD34+/CD38- and CD34+/CD38+ fractions within the CD14- population. From these two fractions mRNA was isolated, and B) *EVI1* expression of these FAB M5 leukemia samples was determined by RQ-PCR. A 3q26 AML sample was used as positive control. The Relative *EVI1* expression was measured in duplicate and normalized against *EVI1* expression of the cell line SKOV3.

Figure S3

Aberrant expression of EVI1 mRNA in sorted CD34/CD38 fractions of MLL-AF9 rearranged AMLs.

Bone marrow cells from two MLL-AF9 rearranged AMLs were FACS sorted for the distinct CD34/CD38 populations, with subsequent isolation of mRNA. Relative expression was calculated using PBGD as a reference gene and normalized against the ovarian carcinoma cell line SKOV3.[3] Each measurement was carried out in triplicate and standard deviation is shown per measurement. Note that in the upper panel the relative EVI1 expression in the CD34+/CD38+ population was not determined, since no cells were present in that fraction of this AML sample.

Figure S4

High *Evi1* expression in mBM cells transformed by MLL-Fusion proteins.

A) Schematic representation of the experimental setup to study *in vitro* the transforming effect of various MLL-fusions transduced into normal mBM cells and its correlation with *Evi1* expression. B) Transformation of normal mBM cells with MLL-Fusions leads to colony formation in methocult with sustained replating capability as compared to an empty vector (EV) control. C) Harvested colonies from MLL-fusion transformed mBM cells showed high *Meis1* and *Evi1* mRNA expression as compared to EV colonies.

Figure S5

Generation and characterization of MLL-AF9 transformed, clonal mBM cell lines.

A) Schematic illustration of the experimental strategy to generate clonal cell lines of MLL-AF9 transduced mononucleated normal mBM cells. B) These clones could be separated in an *Evi1*^{pos} and *Evi1*^{neg} group based on their *Evi1* mRNA expression, and they retained their *Evi1* expression status after repeated colony cultures (D). Relative *Evi1* mRNA expression of 1 represents equal *Evi1* levels compared to a reference

sample of mBM transduced with empty vector. There was no difference in the growth capacity of *Evi1*^{pos} and *Evi1*^{neg} clones as judged by colony (C) or cell suspension (E) cultures.

Figure S6
H3K79me2 enrichment on the *Evi1* and *Mds1* promoter region of *Evi1*^{pos} MLL-AF9 cells.
ChIP-on-chip experiments for H3K79me2 vs. IgG control on *Evi1*^{pos} and *Evi1*^{neg} MLL-AF9 transformed mBM clones respectively clone #1 and clone #3. The *Evi1*^{pos} clone #3 displayed H3K79me2 enrichment on the *Evi1* and *Mds1* promoter, whereas the *Evi1*^{neg} clone #1 did not. Note that both clones exhibited clear H3K79me2 enrichment on MLL-AF9 target genes, like the *HoxA* Cluster genes (B) and *Meis1* (C), whereas a non-target gene, *Myt1*, did not show any enrichment (D).

Figure S7
Enrichment of H3K79me2 mark on the *Evi1* promoter of 4166 cells.
ChIP using H3K79me2 antibody on 4166 cells showing enrichment of H3K79me2 around the TSS of the murine *Evi1* promoter as compared to normal mBM. As controls enrichment on the *HoxA9* promoter and lack of enrichment of *HoxC8* promoter are shown.

Figure S8
***Dot1L* knockdown in MLL-AF9 cell line 4166 leads to reduced Evi1 mRNA expression.**
Murine cell line 4166 was transduced with shRNAs against *Dot1L*, selected on puromycin, and cultured until 72 hours after virus transduction. Subsequent RQ-PCR shows downregulation of *Evi1, HoxA9* and *Meis1* after *Dot1L* knockdown (n=3, error bars represent SD).

Figure S9
Expression of MLL-AF9 maintains expression of *Evi1* in HSC but does not reactivate *Evi1* expression in GMP.
Quantitative reverse transcription PCR (qRT-PCR) of relative *Evi1* mRNA levels in HSC and GMP expressing MLL-AF9, two independent experiments (#1; #2). Results were normalized to *Gapdh* as a reference gene. Error bars represent standard deviation of triplicates. N/D = not detectable.

Figure S10
Decreased colony formation of MLL-AF9 clones after *Evi1* knockdown.
ShRNA against *Evi1* and a control (SHC) were transduced in *Evi1*^{pos} and *Evi1*^{neg} MLL-AF9 clones, resulting in reduced *Evi1* mRNA expression (A). *Evi1* knockdown resulted in reduced colony growth of *Evi*1^{pos} clones, whereas *Evi1*^{neg} clones were unaffected, showing that the reduced growth was not due to off-target effects of the used shRNA (B). Experiments (n=3) with standard deviation (SD) are shown.

Figure S11
4166 cells with reduced levels of Evi1 are more sensitive to cytostatic drugs *in vitro*.

MLL-AF9 cell line 4166 was transduced with *Evi1* shRNA or SHC and selected for 3 days on puromycin. Subsequently dose-response curves were generated as described in material and methods, to measure sensitivity to either idarubicin (A) or cytarabine(B) alone, or in combination with a predetermined, fixed concentration of 0.047 µM cytarabine (C).

Figure S12

Co-expression of *Evi1* and *MdsEvi1* in MLL-AF9 transformed mBM clones.

Parallel upregulation of *Evi1* and *MdsEvi1* in $Evi1^{pos}$ MLL-AF9 transduced mBM (A). $Evi1^{neg}$ MLL-AF9 transformed mBM displayed no clear differential higher transcript as compared to the rest MLL-AF9 mBM clones (B).

Figure S13

MLL-fusion proteins transactivate the promoter of EVI1.

Relative luciferase activity from reporter gene assays using an EVI1-luc reporter co-transfected with expression constructs harboring empty vector (EV), MLL-fusions, or E2A-PBX. Luciferase activity normalised against Renilla activity and calibrated to empty vector control with mean value ± SD from 3 independent experiments are shown.

Figure S1

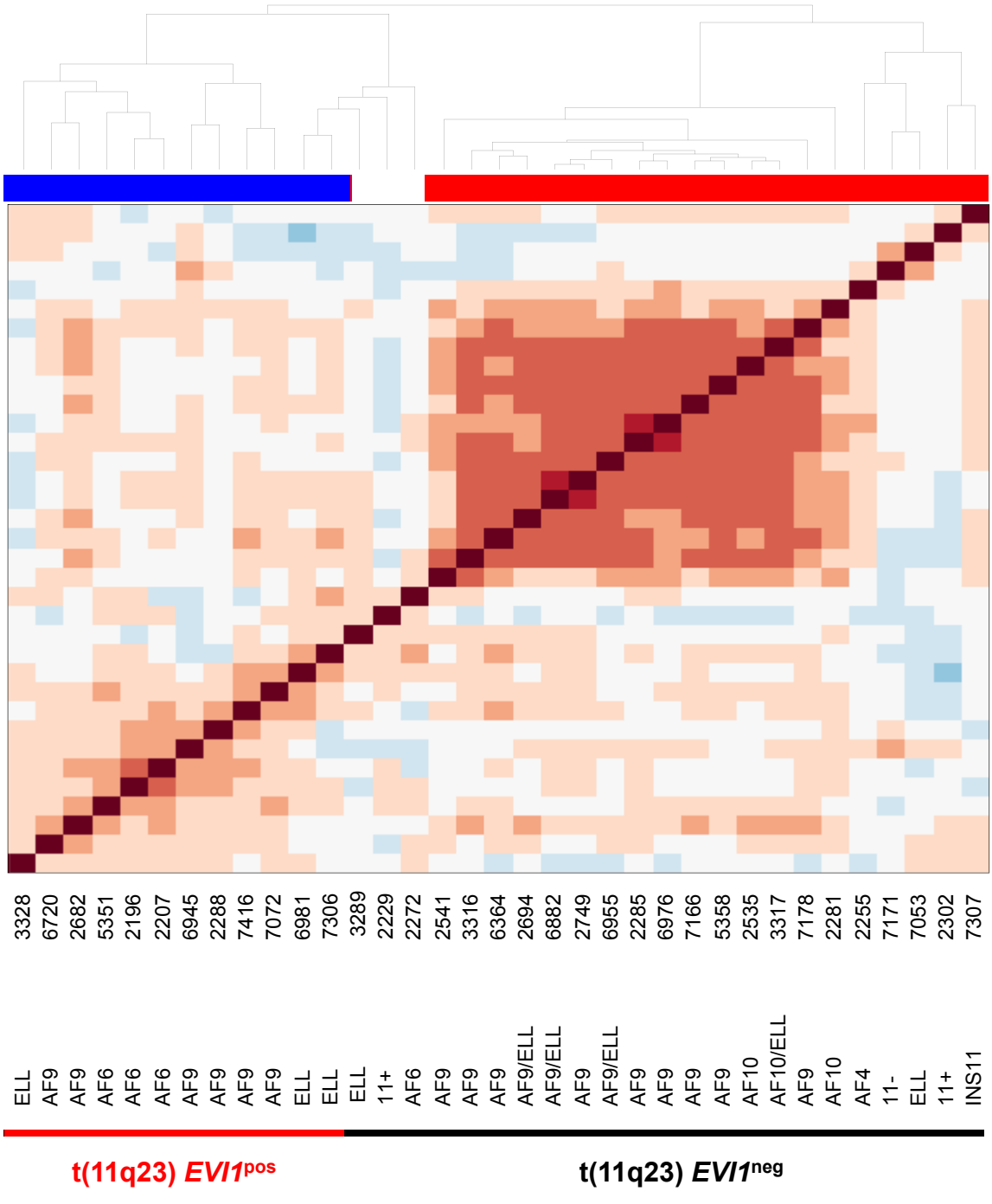
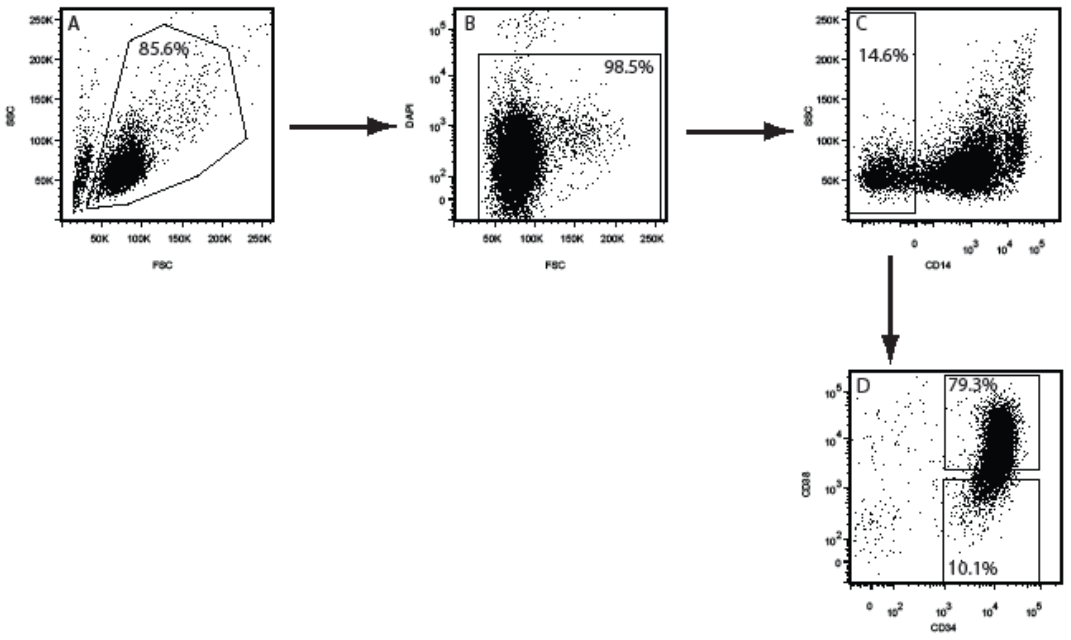

3328 6720 2682 5351 2196 2207 6945 2288 7416 7072 6981 7306 3289 2229 2272 2541 3316 6364 2694 6882 2749 6955 2285 6976 7166 5358 2535 3317 7178 2281 2255 7171 7053 2302 7307
ELL AF9 AF9 AF6 AF6 AF6 AF9 AF9 AF9 AF9 ELL ELL ELL 11+ AF6 AF9 AF9 AF9 AF9/ELL AF9/ELL AF9 AF9/ELL AF9 AF9 AF9 AF9 AF10 AF10/ELL AF9 AF10 AF4 11- ELL 11+ INS11
t(11q23) EVI1pos
t(11q23) EVI1neg

Figure S2

A


A
85.6%
SSC
FSC
B
98.5%
DAPI
FSC
C
14.6%
SSC
CD14
D
79.3%
10.1%
CD38
CD34

B

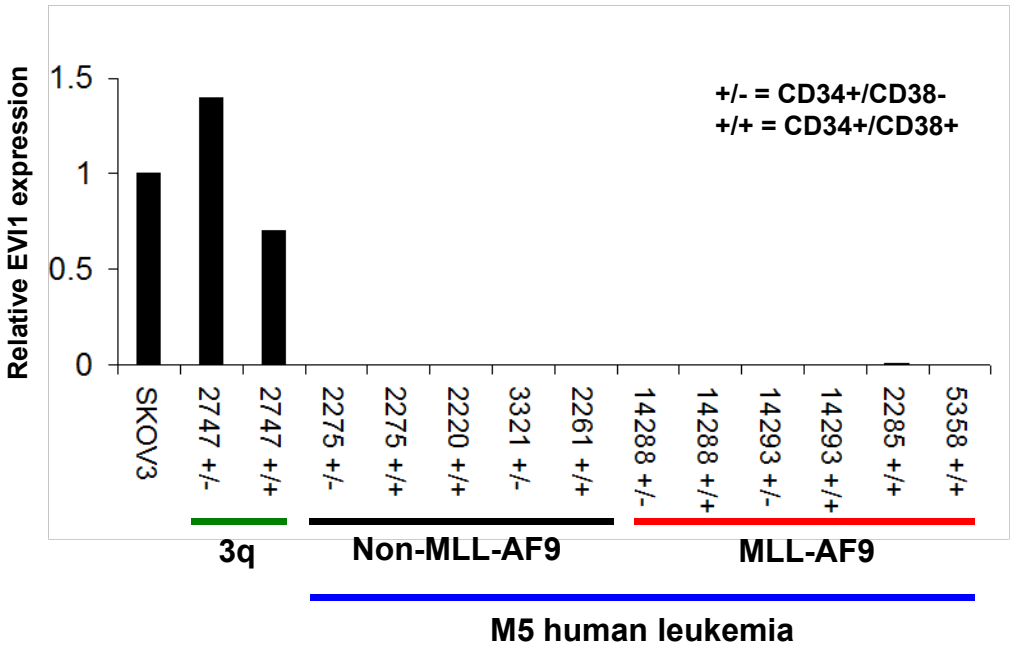
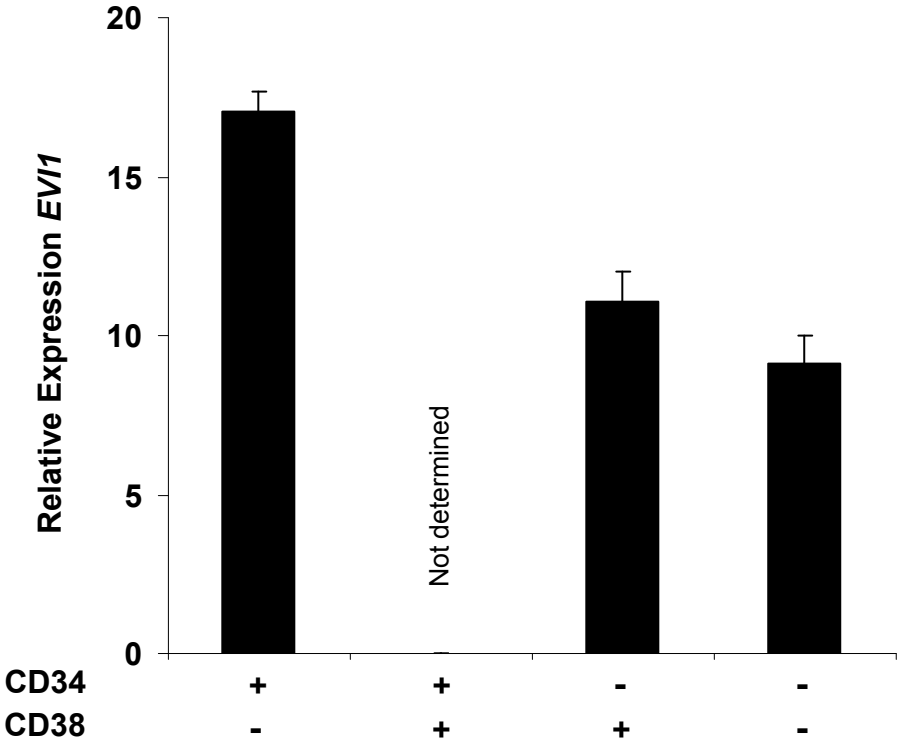

Relative EVI1 expression
1.5
1
0.5
0
+/- = CD34+/CD38-
+/+ = CD34+/CD38+
SKOV3
2747 +/-
2747 +/+
2275 +/-
2275 +/+
2220 +/+
3321 +/-
2261 +/+
14288 +/-
14288 +/+
14293 +/-
14293 +/+
2285 +/+
5358 +/+
3q
Non-MLL-AF9
MLL-AF9
M5 human leukemia

Figure S3


20
15
10
5
0
Relative Expression EVI1
Not determined
CD34
CD38
+
-
+
+
-
+
-
-

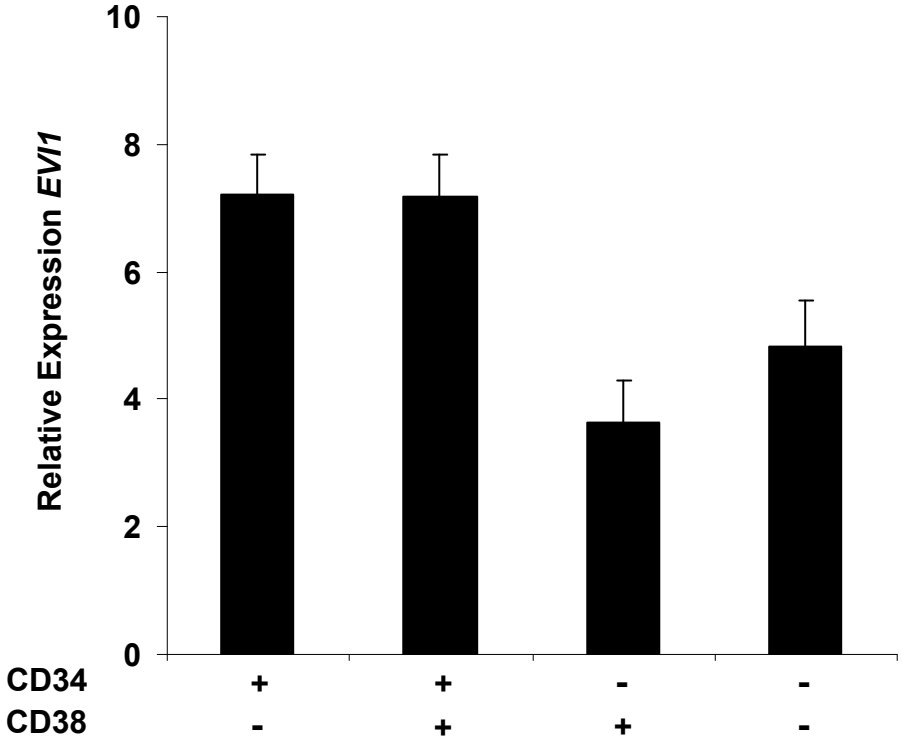
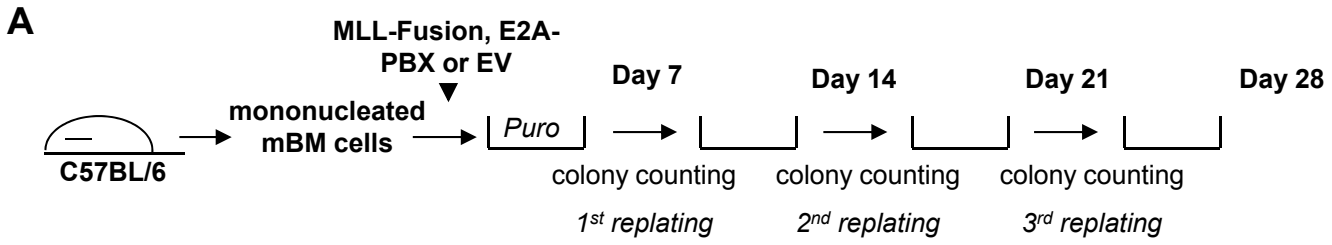
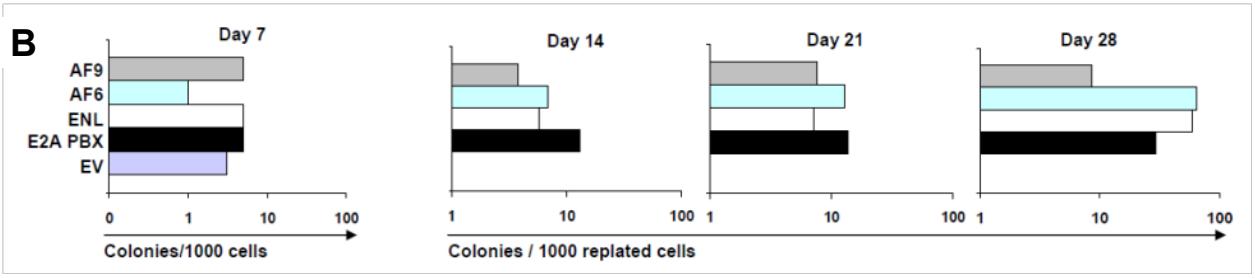

10
8
6
4
2
0
Relative Expression EVI1
CD34
CD38
+
-
+
+
-
+
-
-

Figure S4


A
MLL-Fusion, E2A-PBX or EV
Day 7
Day 14
Day 21
Day 28
mononucleated mBM cells
Puro
C57BL/6
colony counting
1st replating
colony counting
2nd replating
colony counting
3rd replating


B
Day 7
Day 14
Day 21
Day 28
AF9
AF6
ENL
E2A PBX
EV
0
1
10
100
Colonies/1000 cells
Colonies / 1000 replated cells

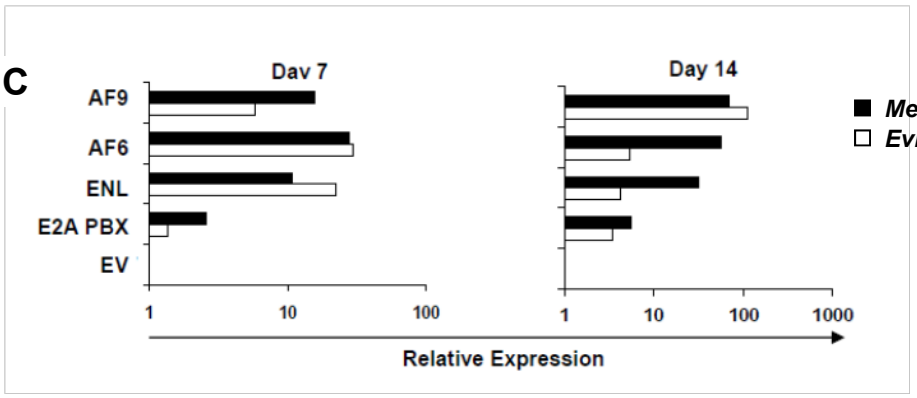
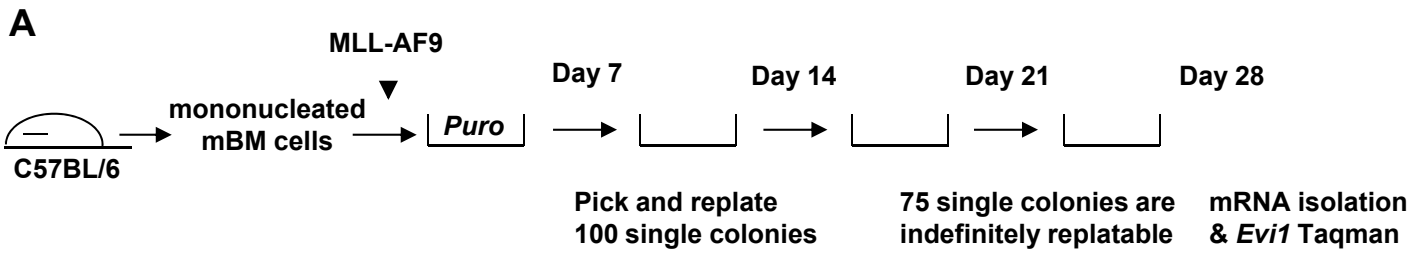
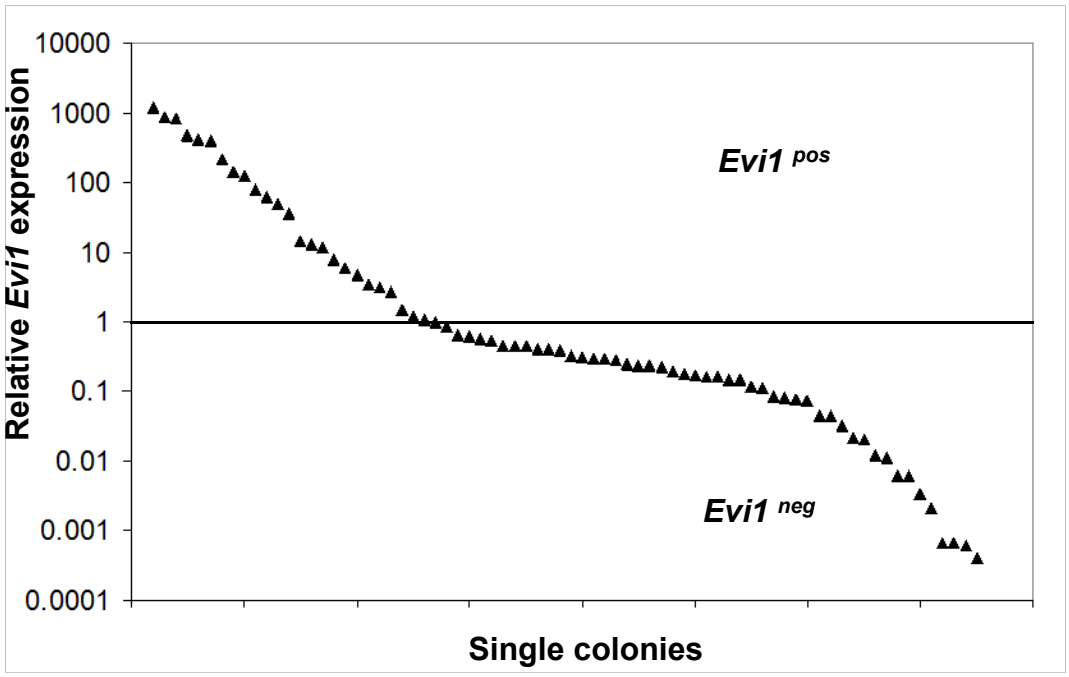

C
Dav 7
Day 14
AF9
AF6
ENL
E2A PBX
EV
Meis1
Evi1
1
10
100
1000
Relative Expression

Figure S5


A
MLL-AF9
Day 7
Day 14
Day 21
Day 28
mononucleated
mBM cells
Puro
C57BL/6
Pick and replate
100 single colonies
75 single colonies are
indefinitely replatable
mRNA isolation
& Evi1 Taqman


B
Relative Evi1 expression
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
Evi1 pos
Evi1 neg
Single colonies

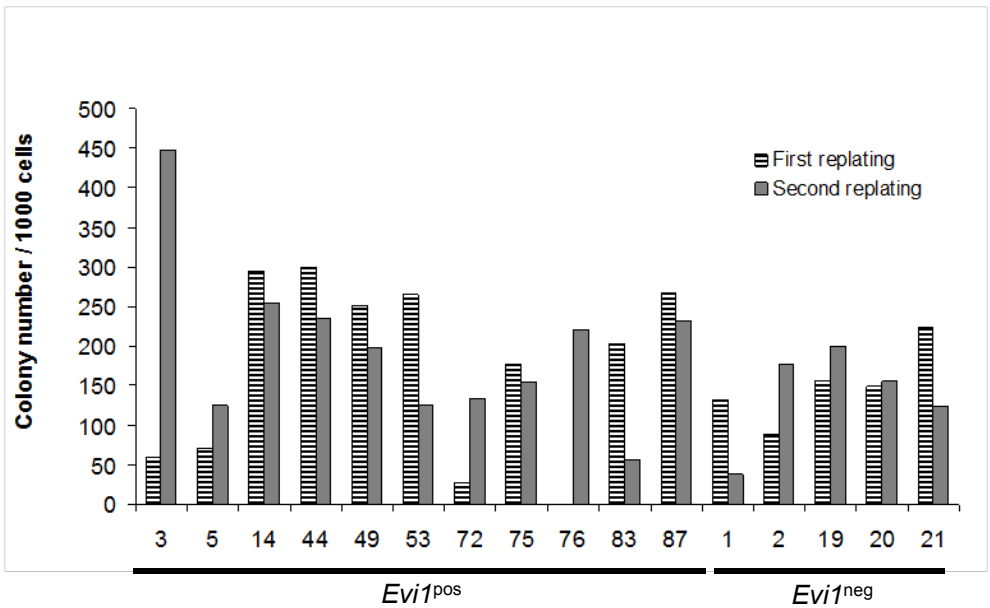
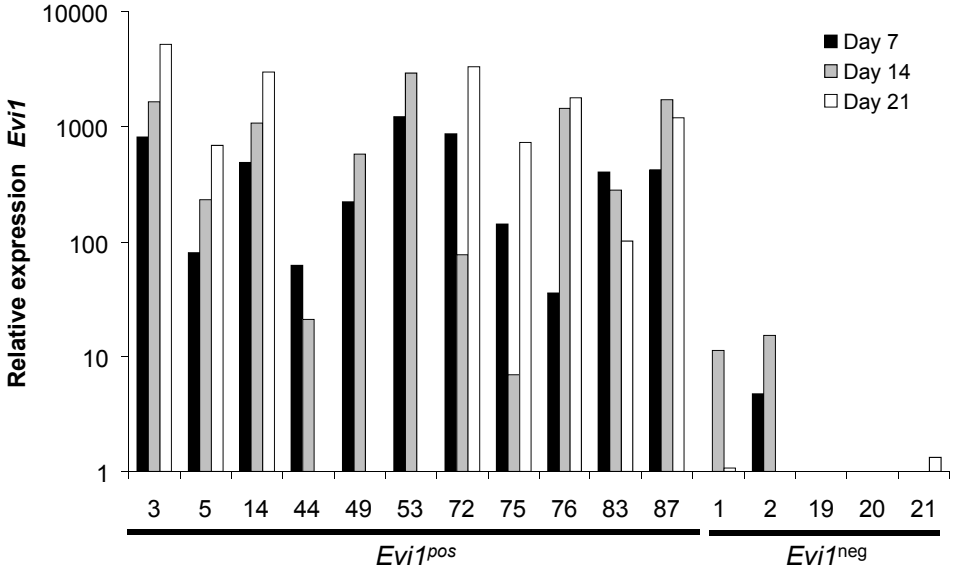

C
Colony number / 1000 cells
500
450
400
350
300
250
200
150
100
50
0
First replating
Second replating
3
5
14
44
49
53
72
75
76
83
87
1
2
19
20
21
Evi1pos
Evi1neg

Figure S5

D


Relative expression Evi1
10000
1000
100
10
1
Day 7
Day 14
Day 21
3 5 14 44 49 53 72 75 76 83 87 1 2 19 20 21
Evi1pos
Evi1neg

E

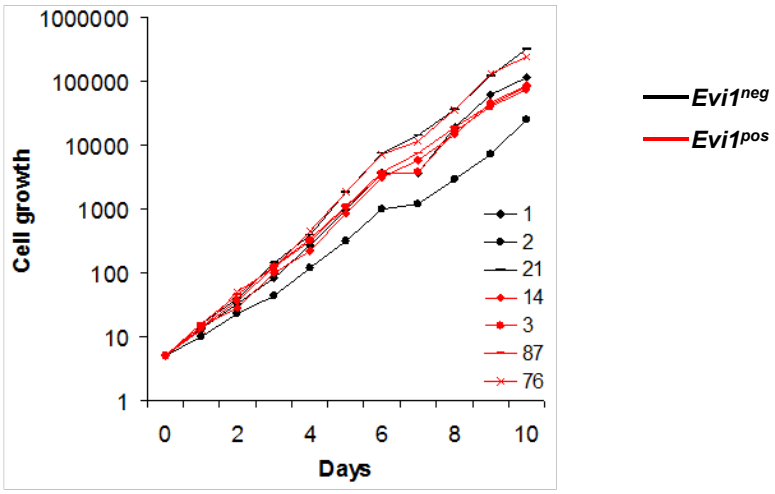
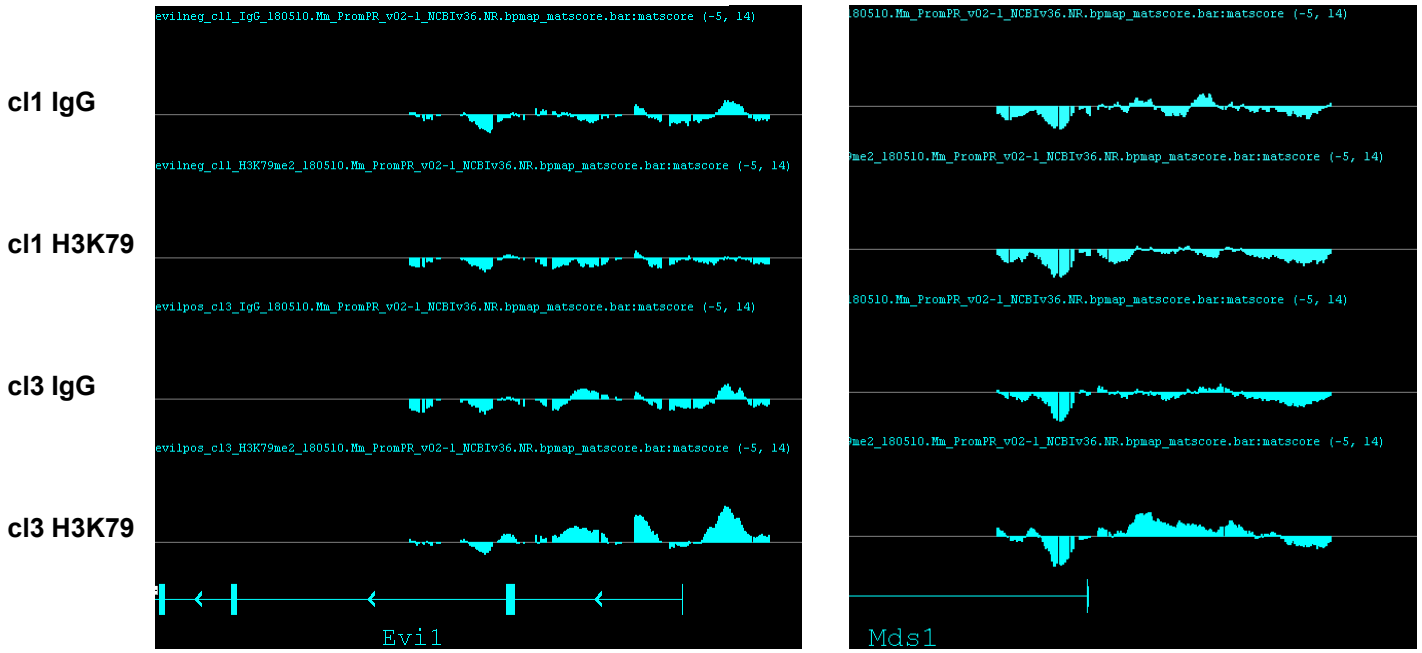

Cell growth
1000000
100000
10000
1000
100
10
1
0 2 4 6 8 10
Days
1
2
21
14
3
87
76
Evi1neg
Evi1pos

Figure S6

A


cl1 IgG
cl1 H3K79
cl3 IgG
cl3 H3K79
Evi1
Mds1

B

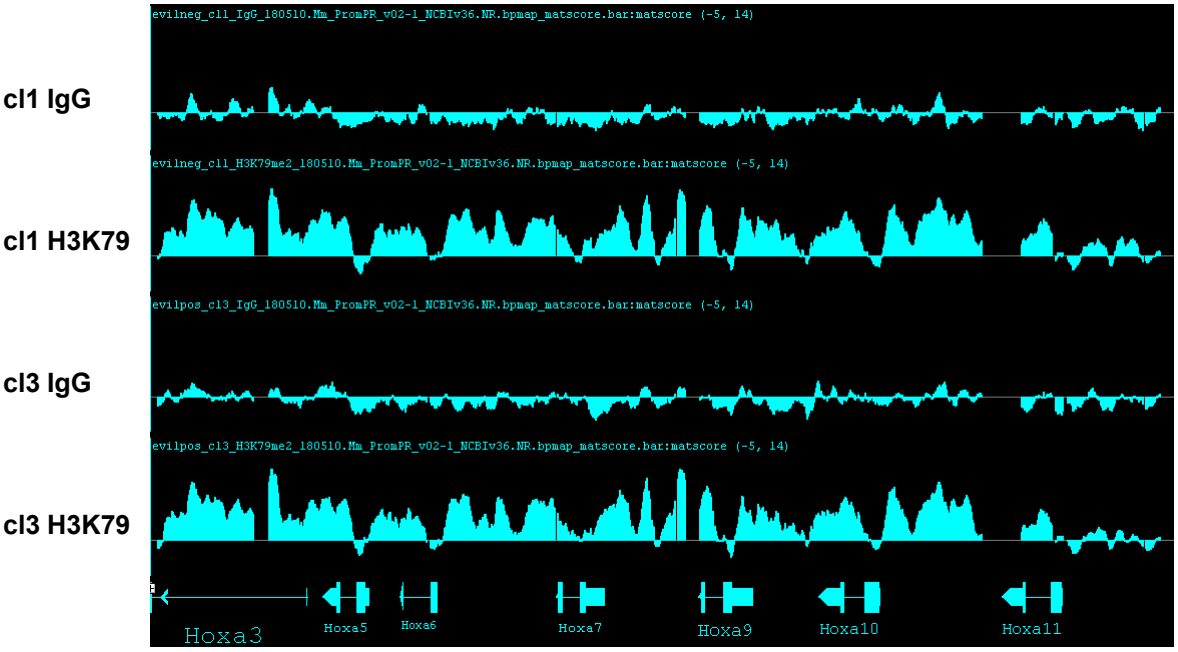
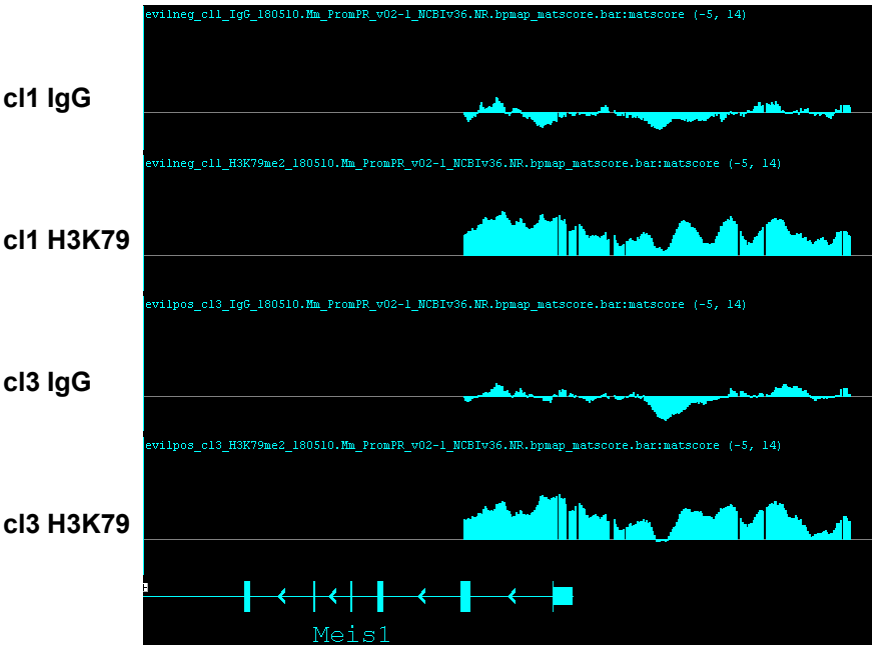

cl1 IgG
cl1 H3K79
cl3 IgG
cl3 H3K79
Hoxa3
Hoxa5
Hoxa6
Hoxa7
Hoxa9
Hoxa10
Hoxa11

Figure S6

C


evilneg_c11_IgG_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl1 IgG
evilneg_c11_H3K79me2_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl1 H3K79
evilpos_c13_IgG_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl3 IgG
evilpos_c13_H3K79me2_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl3 H3K79
Meis1

D

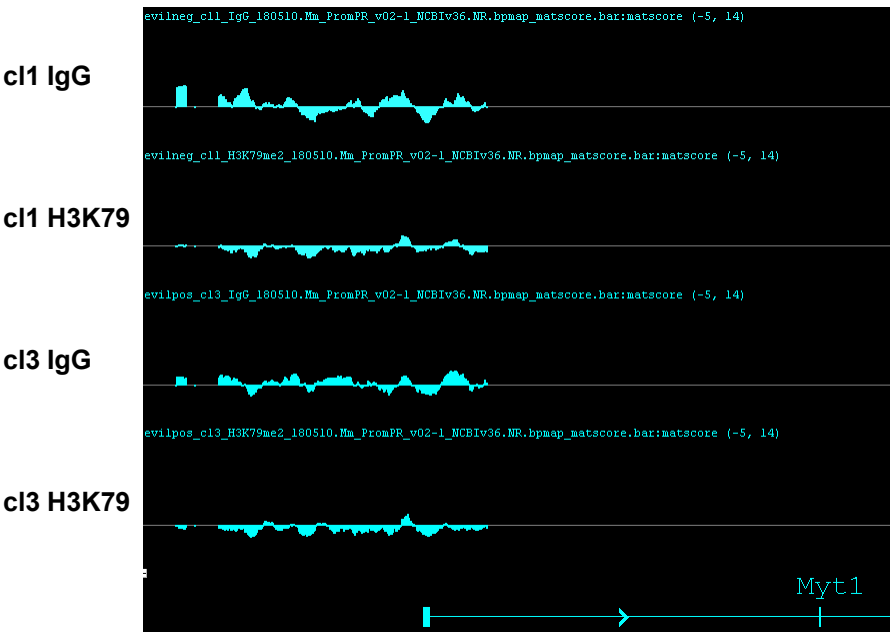

evilneg_c11_IgG_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl1 IgG
evilneg_c11_H3K79me2_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl1 H3K79
evilpos_c13_IgG_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl3 IgG
evilpos_c13_H3K79me2_180510.Mm_PromPR_v02-1_NCBIv36.NR.bpmap_matscore.bar:matscore (-5, 14)
cl3 H3K79
Myt1

Figure S7

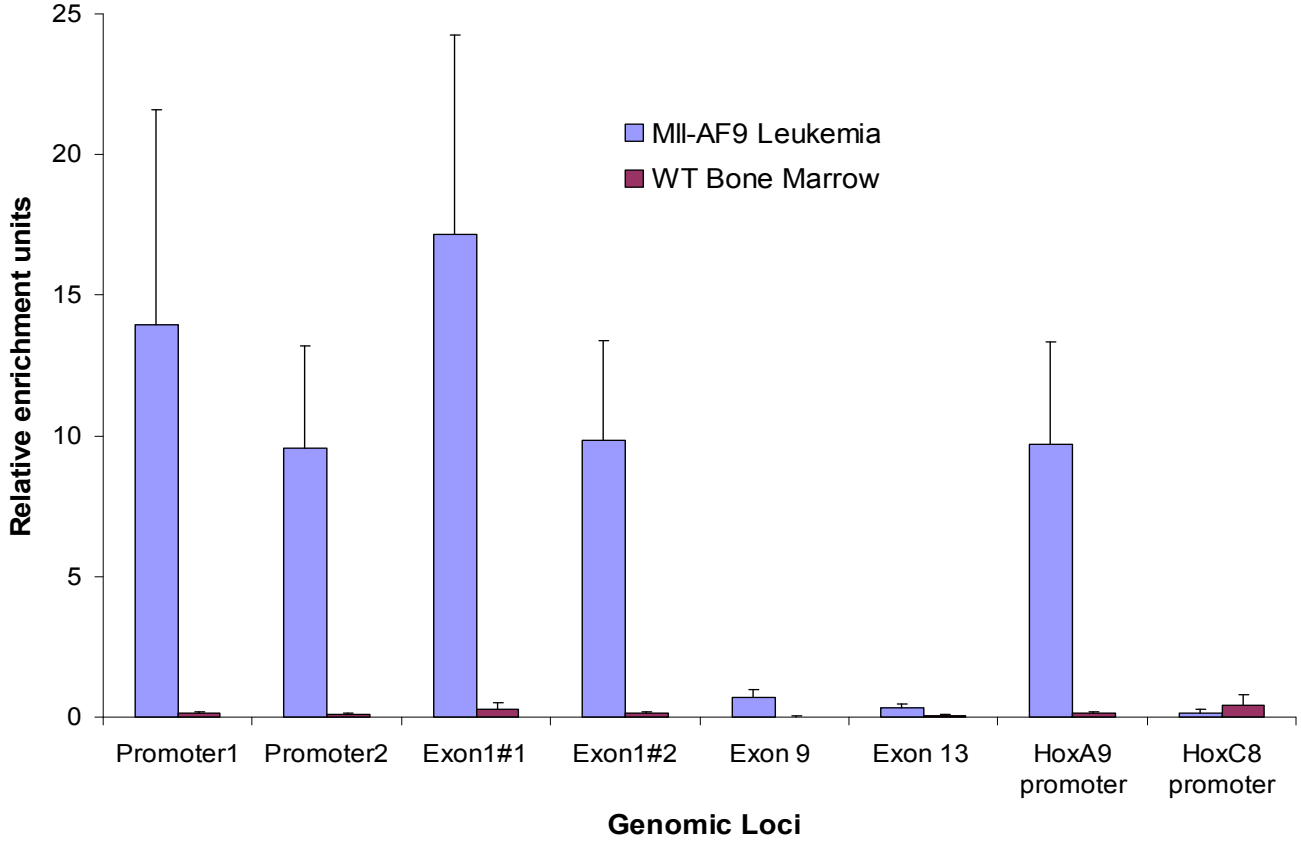

25
20
15
10
5
0
Relative enrichment units
Mll-AF9 Leukemia
WT Bone Marrow
Promoter1
Promoter2
Exon1#1
Exon1#2
Exon 9
Exon 13
HoxA9 promoter
HoxC8 promoter
Genomic Loci

Figure S8

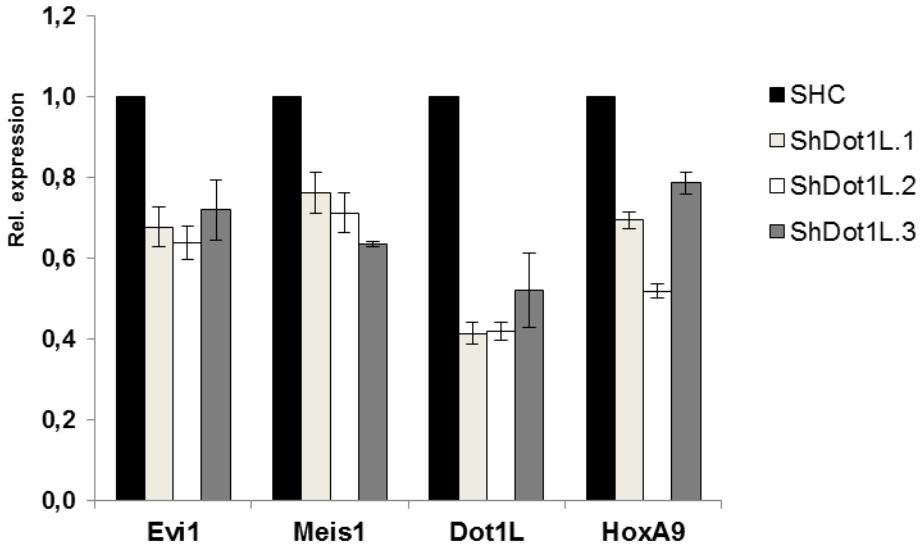

1,2
1,0
0,8
0,6
0,4
0,2
0,0
Rel. expression
Evi1
Meis1
Dot1L
HoxA9
SHC
ShDot1L.1
ShDot1L.2
ShDot1L.3

Figure S9

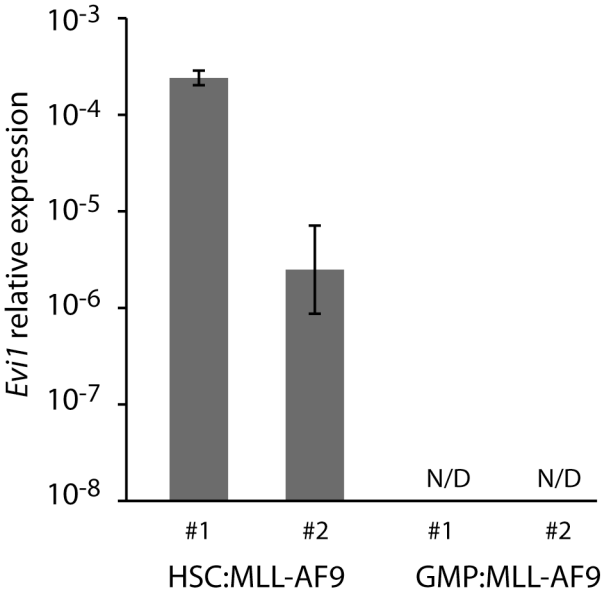
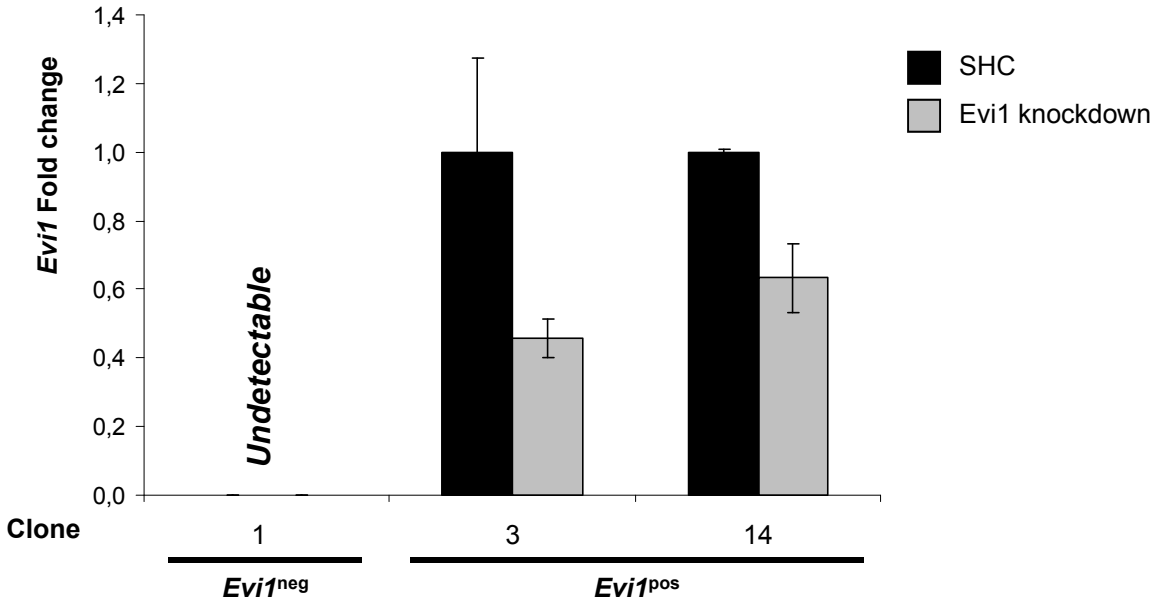

10-3
10-4
10-5
10-6
10-7
10-8
Evi1 relative expression
N/D
N/D
#1
#2
#1
#2
HSC:MLL-AF9
GMP:MLL-AF9

Figure S10

A



B

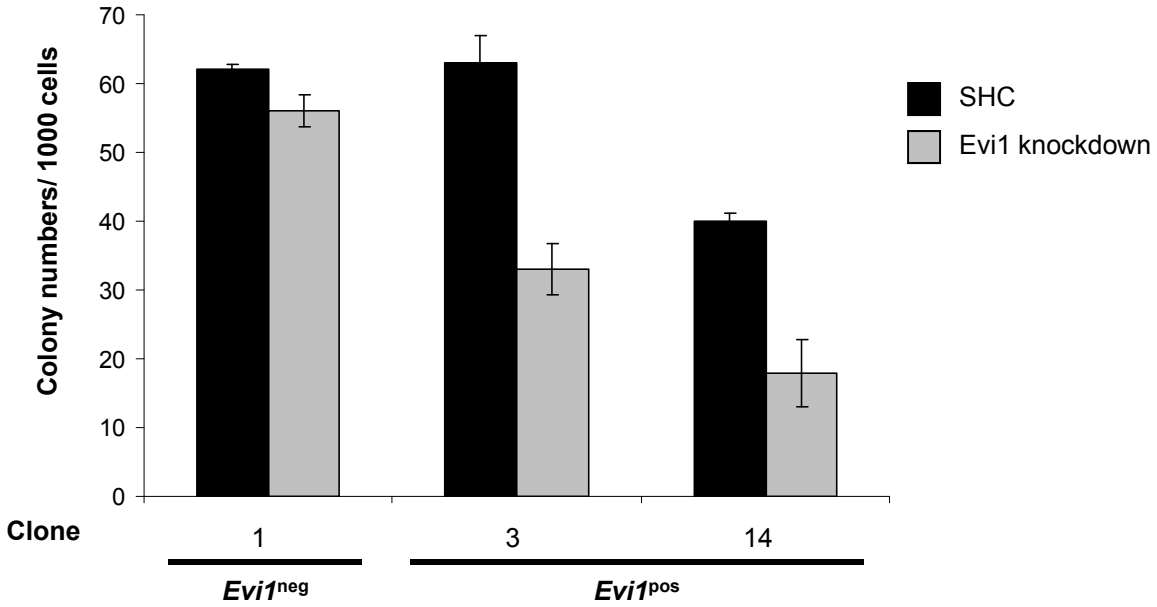
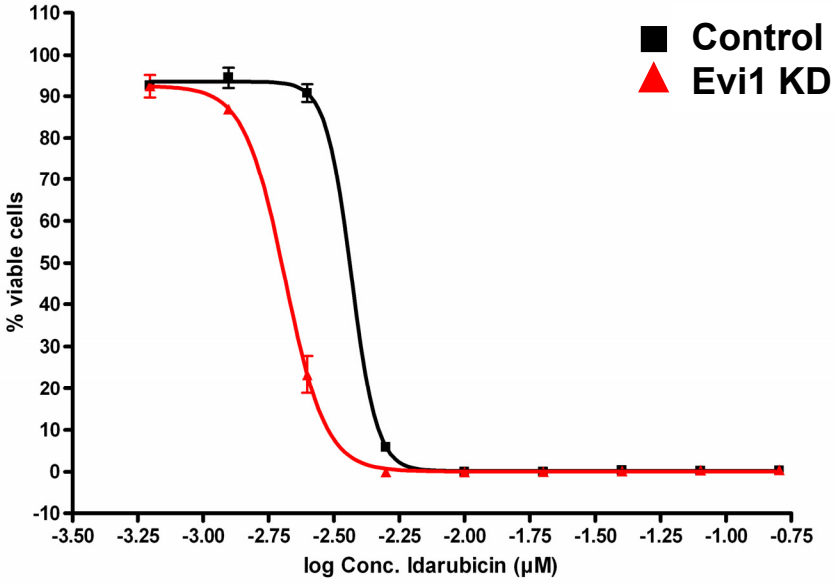


Figure S11

A

	4166 SH	4166KD
EC50	0.003688	0.002055



B

	4166 SH	4166KD
EC50	0.2086	0.1332

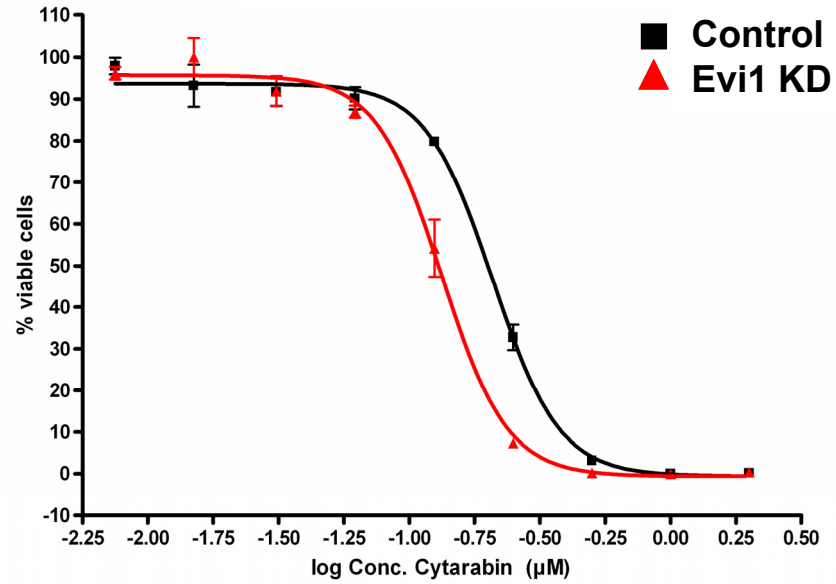


Figure S11

C

	4166 SH	4166KD
EC50	0.003859	0.002579

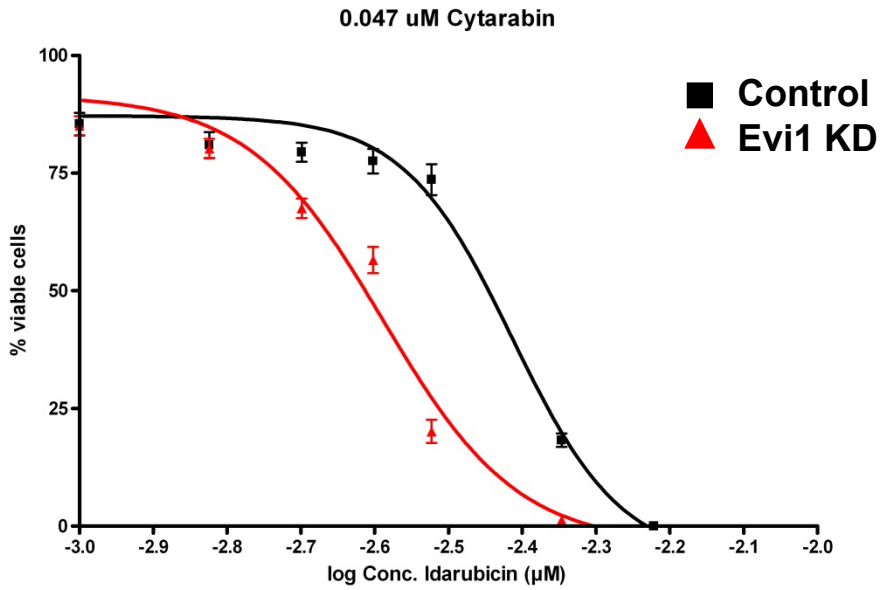
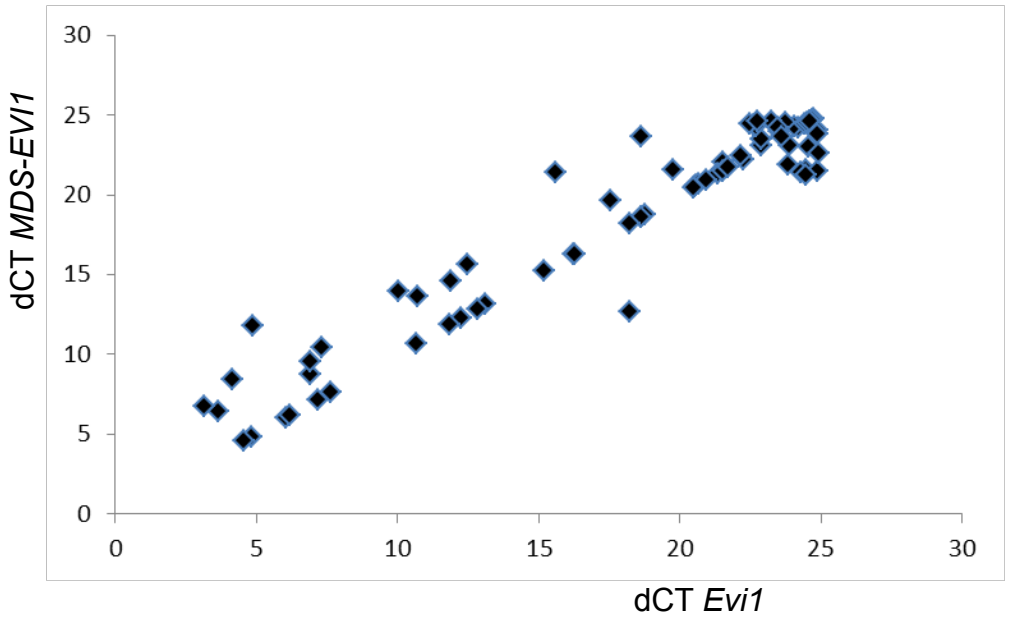


Figure S12

A



B

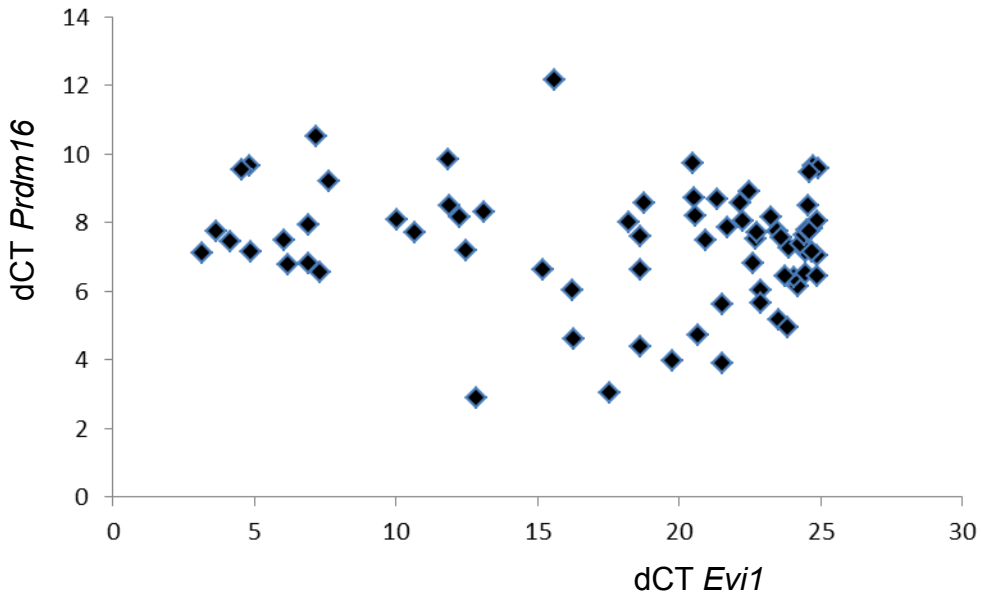
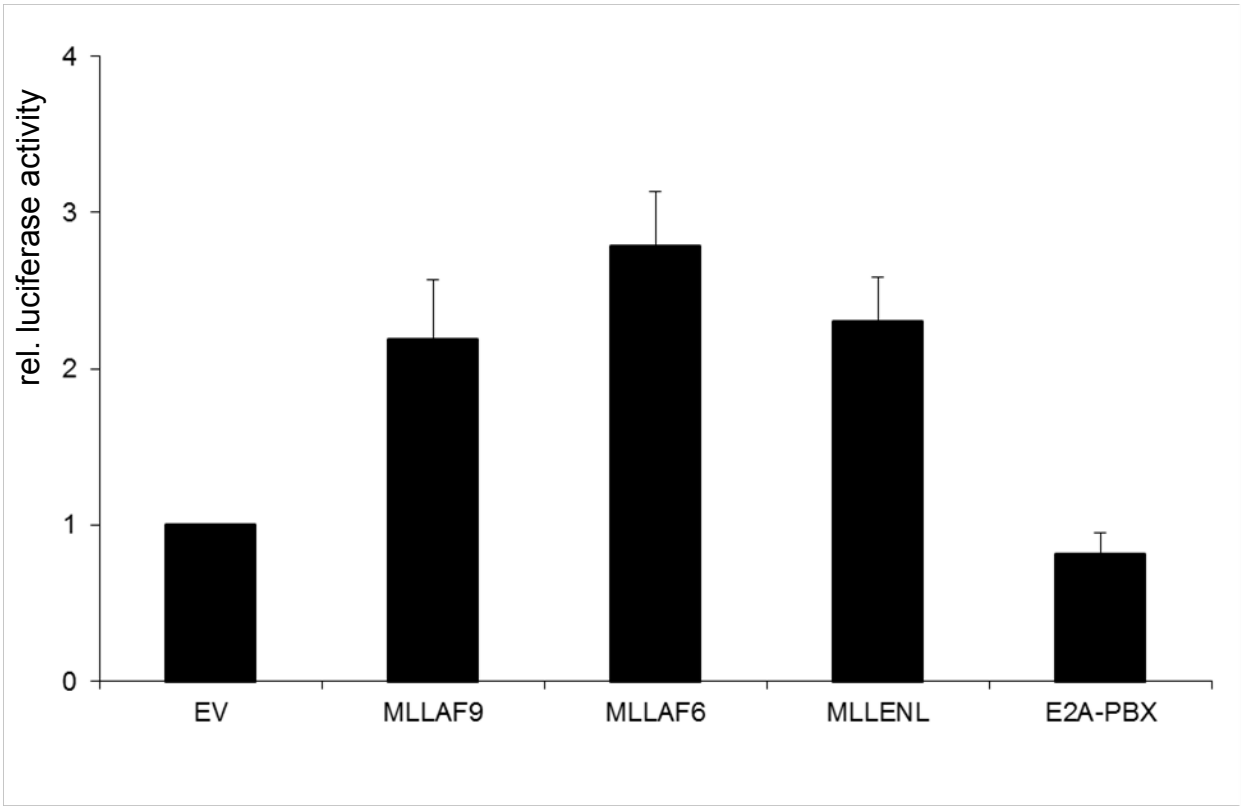


Figure S13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2
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EV
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MLLENL
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