Color legend is given below. A table indicating where individual clones have been presented is given on the right (Supplementary Figure abbreviated as S.Figure).

Supplementary Methods and Data

For evaluation of sgRNA-Cas9 efficacy we developed a modular reporter system based on disruption of the reading frame of a fluorescent protein (RFP657) (Figure 1A). Reporter fluorescence is lost upon out-of-frame deletion/insertion after NHEJ thus not detecting all cleavage events, however, for studies based on NHEJ mediated gene disruption these out of frame deletions/insertions are relevant and the reporter therefore provides a precise measurement of gene disruption events.
Off note, similar approaches using disruption of a fluorescent protein coding sequence have been used before for evaluation of genome editing or RNA interference tools and allow more rapid and quantitative testing of cleavage efficiency than standard T7-endonuclease assays.[15] In contrast, others have used homologous recombination to evaluate the cleavage efficacy of CRISPR-Cas9 or sgRNA-Cas9 systems, which is also limited in detection of cleavage efficacy, in particular when transfection rates are not provided.[4]
In our approach to model myeloid malignancy, we used this reporter assay to establish high efficacy sgRNAs for 8 genes that are recurrently mutated in myeloid malignancies (*Tet2, Runx1, Dnmt3a, Ezh2, Nf1, Smc3, p53* and *Asxl1*). The chosen genes have been described as leukemia promoting loss-of-function mutations and have been found co-mutated in various combinations.
Off note, Wang and colleagues used an efficient Tet2 target site to generate sgRNA:Cas9 based knock-out mice, which was located in the same exon as the one used in our study.[8] As its efficacy was proven, future studies may use sites described here or by Wang and colleagues.
We tested an average of 3 spacers per gene and found that about 60% of the spacers could reduce the Mean Fluorescence Intensity (MFI) of the reporter by more than 20%. About 30% of the sgRNAs reduced the MFI by more than 50%, thereby exceeding formerly published cleavage efficacies of TALENs (Figure 1C).
Comparison of our results for Cas9 cleavage efficacy is hampered by differences in applied methods. Former studies mostly employed transient transfection with limited information about transfection efficacies. The use of lentiviral delivery may increase

the efficacy of sgRNA:Cas9 genome editing due to prolonged expression. At the transduction rates achieved in our study (<30%) one can assume to be in a 1-2 vector copy number per cell. The employed EFS promoter delivers relatively low expression of the Cas9-eGFP cassette. This is in contrast to studies using transfection (which results in a high copy number per cell) and strong viral promoters. Variance of efficacy between spacers as seen in our study has been observed before and may be attributed to recently published criteria on optimal spacer design.[3, 25] Beside variances detected, efficient cleavage for all pre-tested spacers was detected *in-vivo* (Supplementary Figure 6,8,9,12,16,17,18). Analysis of mutations from mouse samples showed that NHEJ in murine hematopoietic cells primarily causes deletions. Deletion size was variable ranging from 1 bp to 251 bp. The majority of mutations were between 1-40 bp. The cell type used and its particular DNA repair preferences may however influence the spectrum of mutations that occur.
Larger deletions than the ones detected may occur but detection is limited with PCR based approaches.
Subsequent analysis of cleavage at off-target sites did not detect mutations at these sites in line with former publications showing abrogation of Cas9 cleavage when spacers have three or more mismatches to the genomic site.
One concern when using integrating viral vectors is the accidental activation of nearby oncogens, which may cause leukemia or contribute to leukemia development.[26] In our study a self-inactivating lentiviral vector with a eukaryotic EFS promoter was used. Both the use of self-inactivating vectors with internal promoters and the use of physiological promoters strongly reduce the risk of insertional mutagenesis.[27] It is therefore unlikely that vector integration has contributed to leukemia development in our study.

Materials&Methods

Vector construction and viral particle production

Lentiviral vectors were based on the pLKO_TRC005 lentiviral backbone. The codon optimized *Streptococcus pyogenes* Cas9 (SpCas9) cDNA and small guide RNA (sgRNA) have been described before.[28] The sgRNA was amplified from the pLX330 backbone and placed behind the human U6 promoter of the pLKO_TRC005. Vectors expressing only Cas9 and no sgRNA were generated by deleting the hU6 promoter. BbsI sites were replaced by BsmBI sites for insertion of the spacer sequence. The hPGK promoter of the pLKO_TRC005 was replaced by a minimal size hEF1a promoter (EFS).[28] To generate vectors expressing Cas9 only, the hU6 promoter was deleted from the pLKO_TRC005 backbone by cutting PpuMI/EcoRI followed by Klenow fragment fill-in reaction and ligation. SpCas9 cDNA was PCR amplified (Phusion DNA Polymerase, NEB) and placed behind the EFS promoter followed by addition of P2A-eGFP, P2A-tagRFP, P2A-PAC or P2A-BSD. Optimized picorna virus 2A sites have been described before[29] and were fused to respective cDNAs via PCR. The sgRNA-only vectors were generated by replacing the SpCas9 and respective marker gene with a fluorescent protein marker, or selection marker only. The RFP657 fluorescent protein was described before.[30]

Suitable protospacer sequences were identified as described before with unique 13bp seed sequence and a minimum of 3 mismatches to the mouse genome.[23]

Lentiviral reporter vectors were generated by replacing the eGFP fluorescent reporter gene in the RRL.PPT.SFFV.IRES.eGFP.pre* (kindly provided by Christopher Baum

and Axel Schambach, Hannover Medical School, Hannover, Germany) with PAC, followed by insertion of the RFP657 fluorescent reporter gene 5' of the IRES. CRISPR/Cas target sites were introduced behind the start codon of the RFP657.

Lentiviral particles were produced by transient transfection of 293T cells using the calcium-phosphate transfection method. Viral constructs were co-transfected with pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) (both kindly provided by Didier Trono., EPFL, Lausanne, Switzerland). Lentiviral particles were concentrated using ultracentrifugation.

Protospacer sequences used for the cloning of sgRNAs used in this study are shown in Supplementary Table 1.

Reporter based efficacy assessment of CRISPR/Cas spacer sequences

Fluorescent reporter vectors were constructed and produced as described above. HEL cells (ATCC) were cultured in RPMI with 10% FBS and 100 U/ml Penicillin/Streptomycin. HEL cells were transduced with CRISPR/Cas reporter vectors in the presence of 4ug/ml hexadimethrine bromide (Polybrene (Life Technologies)) and transduced cells were selected with 2ug/ml Puromycin (Life Technologies) for 72h, starting 48h post transduction. Selection was verified by flow cytometry. Selected reporter cell lines were transduced with CRISPR/Cas vectors targeting the respective target sites and non-targeting controls. RFP657 reporter fluorescence was assessed by flow cytometry at 6 days post transduction in comparison to non-targeting controls and untransduced cells.

T7-endonuclease assay

Ba/F3 cells (DSMZ) were cultured in RPMI with 10% FBS, 100 U/ml Penicillin/Streptomycin and 5 ng/ml Il3 (Peprotech). Ba/F3 cells were transduced with CRISPR/Cas vectors, and transduced cells were purified by FACS 7 days post-infection. Genomic DNA was isolated according to manufacturers instruction with a DNA Micro Kit (Quiagen). Targeted loci were amplified by PCR (Phusion DNA Polymerase, NEB). CRISPR/Cas efficacy at the endogenous locus was assessed using the Surveyor Mutation Detection Kit (Transgenomic).

Mouse bone marrow transplant and analysis

Total BM was isolated from femurs and tibias from 6-10 week old female C57Bl/6 mice or heterozygous 6-12 week old Flt3-ITD knock-in mice.[35] BM was subjected to erythrocyte lysis (BD PharmLyse, BD Bioscience), followed by magnetic bead selection of cKit (CD117) positive cells using CD117 MicroBeads (Milteny Biotech). CD117 selected cells were stained with APC labeled cKit (eBioscience), PE labeled Sca1 (BioLegend) PacificBlue labeled Gr1, CD11b, B220(CD45R), Ter119, and CD3 (all BioLegend) and then flow sorted on a BD FACS Aria II. LSK cells were cultured in StemSpan SFEM (StemCell Technologies) supplemented with 50ng/ml murine Thpo and 50ng murine Scf (both Peprotech) for 48h and then transduced with concentrated lentiviral supernatant in presence of 2ug/ml Polybrene. 24h post transduction cells were collected and intravenously injected into lethally irradiated (950 cGy) 6-10 week old female C57Bl/6 mice. C57Bl/6 mice were obtained from Taconic. Flt3-ITD mice were bred in local facilities.

All transplant experiments were performed with 4-5 mice per group. Statistical significance between groups was assessed using an unpaired t-test.
No blinding and no randomization were carried out.

All experiments and procedures were performed in the Children's Hospital Boston animal facility and were approved by the Children's Hospital Boston Institutional Animal Care and Use Committee.

Peripheral blood was sampled by retro-orbital bleeding. Bone marrow was harvested from femurs and tibias. Nucleated cells were obtained by erythrocyte lysis.
To detect mature lineage markers, cells were stained with Gr1-APC-eFluor780 (eBioscience), CD11b-APC (BioLegend), B220(CD45R)-PE-Cy7 (BioLegend) and CD3e-PE (eBioscience). HSPCs were stained with PacificBlue labeled Gr1, CD11b, B220(CD45R), Ter119, and CD3 (all BioLegend), Sca1-PE (BioLegend), CD150-PE-Cy7 (BioLegend), cKit-APC (eBioscience), CD48-PerCP-Cy5.5 (eBioscience), and CD34-AlexaFluor700 (eBioscience). Flow cytometry was performed on a BD LSR II flow cytometer. Cells expressing both the eGFP and RFP657 reporter gene were stained with Gr1-PE-Cy7 (eBioscience) and CD11b-PE (BioLegend) or B220(CD45R)-PE-Cy7 and CD3e-PE for mature lineage detection.

To assess mutations at targeted loci, peripheral blood nucleated cells were purified by FACS. Genomic DNA was isolated according to manufacturers instruction with a DNA Micro Kit (Quiagen). Targeted loci were amplified by PCR (Phusion DNA Polymerase, NEB). PCR products were cloned into pJet1.2 PCR cloning vector (Life Technologies). Plasmid DNA was isolated from individual bacterial clones with a DNA Plasmid Mini Kit (Quiagen) and sequences were assessed by Sanger sequencing.

Secondary transplant of leukemic mice was performed by transplanting 5x10^6 primary BM cells into sub-lethally (650cGy) irradiated C57Bl/6 mice.

Peripheral blood semars were stained with May-Gruenwald/Giemsa stain. BM histology was assessed from paraffin embedded tissue sections stained with Hematoxilin and Eosin.

Clonogenic colony assays were performed in cytokine-supplemented methylcellulose (M3434; StemCell Technologies). 25,000 C57Bl/6 BM wildtype BM cells or 25,000 cells from leukemic mice were plated. Colony numbers were assessed 7 days post seeding. For re-plating experiments, cells were washed with PBS, counted, and plated as described above.

Immunoblots

Nucleated C57Bl/6 wildtype BM cells or leukemic BM cells were lysed with RIPA buffer (Pierce) supplemented with Halt Protease Inhibitor (Pierce). Protein concentrations were determined using Bradford protein assay (Bio-Rad). Equal protein amounts were separated by SDS-PAGE and blotted on nitrocellulose membrane, blocked with fat free milk and incubated with primary antibodies (Cell Signaling; Ezh2 clone D2C9; Smc3 clone D47B5; phospho-Erk1/2 (phospho-p44/42) clone D13.14.4E; Erk1/2 (p44/42) clone 137F5). Mouse beta actin was obtained from Santa Cruz (clone C4). After washing blots were incubated with HRP-conjugated secondary antibody and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (Graphpad Software). Data are shown as the mean with standard error of the mean. Groups were compared using an unpaired, two sided t-test. Normal distribution was assumed. Differences in variance were analyzed using an F-test. Welch correction for t-test was applied for unequal variances as indicated.

Next Generation Sequencing to Assess Mutational Repertoire

gDNA was isolated using a DNA Blood Mini kit (Quiagen) according to manufacturers instructions. The genomic region flanking the CRISPR target site for each region was amplified by a nested PCR to attach the Illumina adapters in the first round and to add barcodes unique for each individual mouse in the second PCR. All libraries were pooled and purified using Agencourt AMPure XP system (Beckman Coulter). Single end, 300 cycle sequencing was performed using MiSeq (Illumina). Reads were adapter trimmed and aligned using BLAT to reference amplicon sequences. Indels were surveyed using Integrative Genomics Viewer.