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TITLE:

Investigation of the Transcriptional Role of a *RUNX1* Intronic Silencer by CRISPR/Cas9 Ribonucleoprotein in Acute Myeloid Leukemia Cells

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SUMMARY:

Direct delivery of preassembled Cas9/guide RNA ribonucleoprotein complexes is a fast and efficient means for genome editing in hematopoietic cells. Here, we utilize this approach to delete a *RUNX1* intronic silencer and examine the transcriptional responses in OCI-AML3 leukemic cells.

ABSTRACT:

The bulk of the human genome (~98%) is comprised of non-coding sequences. *Cis*-regulatory elements (CREs) are non-coding DNA sequences that contain binding sites for transcriptional regulators to modulate gene expression. Alterations of CREs have been implicated in various diseases including cancer. While promoters and enhancers have been the primary CREs for studying gene regulation, very little is known about the role of silencer, which is another type of CRE that mediates gene repression. Originally identified as an adaptive immunity system in prokaryotes, CRISPR/Cas9 has been exploited to be a powerful tool for eukaryotic genome editing. Here, we present the use of this technique to delete an intronic silencer in the human *RUNX1* gene and investigate the impacts on gene expression in OCI-AML3 leukemic cells. Our approach relies on electroporation-mediated delivery of two preassembled Cas9/guide RNA (gRNA) ribonucleoprotein (RNP) complexes to create two double-strand breaks (DSBs) that flank the silencer. Deletions can be readily screened by fragment analysis. Expression analyses of different mRNAs transcribed from alternative promoters help evaluate promoter-dependent

effects. This strategy can be used to study other CREs and is particularly suitable for hematopoietic cells, which are often difficult to transfect with plasmid-based methods. The use of a plasmid- and virus-free strategy allows simple and fast assessments of gene regulatory functions.

INTRODUCTION:

Cis-regulatory elements (CREs) are non-coding DNA sequences that contain binding sites for transcriptional regulators to control gene expression[1,2]. These elements are typically 100 to 1,000 base pairs (bp) long. Promoters and enhancers are the two most characterized types of CREs. Promoters are present in close proximity to the transcription start sites and constitute the basic unit of transcription. Many genes have more than one promoter and their alternative use contributes to transcriptome diversity and tissue specificity[3,4]. On the other hand, enhancers activate transcription and can be located upstream, downstream or within introns of the target genes. Enhancers can act from far distance (over a megabase) and independent of orientation[1,2]. CREs also include silencers and insulators[5,6]. The former acts oppositely to enhancers to inhibit gene expression by binding to transcriptional repressors, whereas the latter partitions the genome into discrete topologically domains to insulate genes from other CREs from neighboring domains. These elements act in concert with each other through short- and/or long-range chromatin interactions and are organized into regulatory hubs to direct proper spatiotemporal gene expression. Recent advancements in high-throughput sequencing techniques have accelerated the identification and functional annotation of many CREs that have greatly facilitated our understandings of the transcriptional networks that dictate lineage-specific gene expression in different cell and tissue types[7-12].

Given the fundamental roles of CREs in regulating transcription, their alterations can lead to aberrant gene expression. It has been shown that CREs are frequently disrupted by genetic and epigenetic changes in different types of human cancers, thereby contributing to tumor initiation, progression and aggressiveness[13,14]. In addition, CRE-binding factors are often mutated and/or misexpressed in various cancer types, further highlighting the significance of CRE deregulation in oncogenesis[15]. CREs can also be affected by structural aberrations, as exemplified by frequent chromosomal rearrangements of the immunoglobulin heavy (IgH) gene enhancer that result in abnormal activation of the neighboring oncogenes in B-cell lymphomas[16]. In acute myeloid leukemia (AML), repositioning of a single enhancer by chromosome 3q rearrangements causes concomitant *GATA2* downregulation and *EVI1* activation, which can be potentially targeted by BET inhibition of enhancer functions[17]. Recently, we characterized a novel chromosomal translocation involving perturbation of a *RUNX1* intronic silencer that might contribute to AML progression in a pediatric patient[18]. Thus, deciphering the non-coding cancer genome provides fruitful avenues for elucidating disease pathogenesis, biomarker discovery and therapeutic interventions, which ultimately improve patient outcomes.

The CRISPR/Cas9 nuclease pathway, originally identified as an adaptive immune system in prokaryotic cells, has been exploited as a rapid and cost-effective means for site-specific genomic editing in living cells and organisms[19-22]. The CRISPR/Cas9 system involves two principal components: the gRNA and *Streptococcus pyogenes*-derived Cas9 nuclease. The gRNA contains a

specific sequence called protospacer that recognizes the target region and directs Cas9 for editing. The gRNA is composed of two parts: CRISPR RNA (crRNA), typically a 20-mer nucleotide sequence complementary to the target DNA, and a trans-activating crRNA (tracrRNA), which serves as a binding scaffold for the nuclease. A protospacer adjacent motif (PAM) (5'-NGG) immediately adjacent to the target site is required for Cas9 cleavage and the cleavage site is located 3 nucleotides upstream of the PAM. CRISPR/Cas9-mediated gene editing is commonly performed by transfecting cells with a plasmid that encodes Cas9 and the cloned gRNA[23]. However, this approach is challenging for hematopoietic cells, which are often hard to transfect and require lengthy virus-based transduction methods. An alternative approach is direct cellular delivery of preassembled Cas9/gRNA RNP complexes[24]. A common method for the RNP delivery is electroporation, which generates temporary pores in the cell membrane, thus allowing entry of the RNP complexes into the cells[25,26]. The advantages of this approach include ease of use, reduced off-target effects and stability of the RNP complexes. Here, we describe a protocol of using the RNP delivery method to investigate the transcriptional role of a *RUNX1* intronic silencer in the OCI-AML3 leukemia cell line[18], which was established from the peripheral blood of an AML patient diagnosed with the French-American-British M4 subtype[27]. The protocol includes the design of crRNA, preparation of RNP complexes, electroporation as well as screening and subsequent characterization of the desired clones.

PROTOCOL:

1. Design of crRNA

1.1. Design two crRNAs, one 5' and the other 3' of the target CRE using a web-based CRISPR design tool[28-31]. Ensure that a PAM of NGG is located immediately downstream of the target sequence for Cas9 recognition. The design of the two crRNAs (crRNA-1 and crRNA-2) for the deletion of the *RUNX1* silencer[18] is shown in **Figure 1**.

NOTE: A crRNA typically contains a 20-mer protospacer that is complementary to the target sequence.

1.2. Check the presence of single nucleotide polymorphisms (SNPs)/indels in the target and adjacent PAM sequences by entering the genomic locations of the sequences into the search box of an online genome browser (e.g., NCBI 1000 Genomes or UCSC Genome Browser).

NOTE: Common SNPs/indels have a minor allele frequency of at least 1% in the general population.

1.3. Submit the selected crRNA sequences for synthesis with a commercial vendor. Also, purchase the tracrRNA for gRNA duplex formation.

2. Design of deletion screening primers

2.1. Design a pair of primers that flank the intended deletion region. Ensure that the primers are at least 50 bp from the Cas9 cleavage sites so that PCR amplification is minimally affected by indels formed at the cleavage sites.

2.2. Ensure that the amplicon is smaller than 1,200 bp (preferably smaller than 600 bp for better size resolution). Also, label one of the primers with a fluorescent dye (e.g., 6-carboxyfluorescein (6-FAM)) at the 5'-end for the detection. The primers used for screening of the *RUNX1* silencer deletion[18] are shown in **Figure 1**.

3. Preparation of Cas9/gRNA RNP complexes

3.1. Resuspend the crRNAs and tracrRNA in 1x TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5) to a final concentration of 200 µM.

3.2. For each crRNA, mix 2.2 µL of 200 µM crRNA, 2.2 µL of 200 µM tracrRNA and 5.6 µL of 1x TE buffer (total 10 µL) in a 0.2 mL tube to obtain a final duplex concentration of 44 µM.

3.3. Incubate at 95 °C for 5 min in a thermocycler. Allow the tubes to cool to room temperature for gRNA complex formation.

3.4. Dilute 10.4 µL of 62 µM recombinant Cas9 nuclease with 7.6 µL of 1x phosphate-buffered saline (PBS) to obtain a final nuclease concentration of 36 µM.

NOTE: This amount is sufficient for the preparation of two Cas9/gRNA complexes.

3.5. Mix equal volumes (8.5 µL) of the diluted nuclease with each of the gRNA duplexes obtained from step 3.3.

3.6. Incubate at room temperature for 20 min to allow RNP complex formation. Keep the mixtures on ice until electroporation.

4. Electroporation of the RNP complexes into OCI-AML3 cells

4.1. Culture OCI-AML3 cells in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1x GlutaMAX, 100 units/mL of penicillin and 100 µg/mL of streptomycin (hereafter referred to as complete RPMI 1640 medium) at 37 °C with 5 % CO_2.

4.2. Count cells using trypan blue staining. Ensure that the cell viability at the time of electroporation is over 90%.

4.3. Centrifuge 2.5×10^6 cells in a 1.5 mL tube at 500 x *g* for 5 min at room temperature. Remove the supernatant and wash the cells with 1 mL of 1x PBS. Spin down the cells again and remove all residual supernatant.

4.4. Resuspend the cells in 163 µL of RPMI 1640 medium without phenol red. Add 16.7 µL of each RNP complex (from step 3.6) and 3.6 µL of 100 µM Electroporation Enhancer to the cells (total volume=200 µL). Mix gently by pipetting.

NOTE: The Electroporation Enhancer is a carrier DNA for enhancing the editing efficiency.

4.5. Transfer the mixture to a 0.2 cm-gap electroporation cuvette without any bubbles. Perform electroporation with an electroporation system (Mode: exponential; Voltage: 150 V; Capacitance: 700 µF; Resistance: 50 Ω).

4.6. Transfer the cells to a T-25 tissue culture flask containing 6 mL of complete RPMI 1640 medium and incubate at 37 °C with 5 % CO_2.

5. Screening and selection of cell clones with biallelic deletions

5.1. Dilute the cells to 5×10^3/mL in complete RPMI 1640 medium one day after electroporation. Add 100 µL of the diluted cell suspension into each well of 96-well tissue culture plates and allow the cells to grow for 7-14 days.

5.2. Extract genomic DNA from the cells using a high-throughput purification system.

5.3. Add 100 µL of plate binding and lysis buffer to each well of a 96-well extraction plate. Add 5×10^4 cells resuspended in 10 µL of 1x PBS to the buffer and mix them by pipetting.

5.4. Incubate at room temperature for 30 min to allow binding of genomic DNA to the wells.

5.5. Aspirate the solution from the wells without scraping the well surfaces. Wash the wells with 120 µL of wash buffer.

5.6. Air dry the wells containing the bound DNA.

5.7. Prepare 20 µL of PCR mix (2 µL of 10x High Fidelity buffer, 0.8 µL of 50 mM $MgSO_4$, 0.4 µL of 10 mM dNTPs, 0.4 µL of 10 µM FAM-labeled forward primer (step 2.2), 0.4 µL of 10 µM unlabeled reverse primer and 0.4 U of *Taq* DNA polymerase for each sample).

NOTE: Prepare a master mix to ensure the addition of standardized amounts of reagents in each sample.

5.8. Add the PCR mix to each well of the extraction plate and run the reactions in a thermocycler (Conditions: initial 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s and 68 °C for 1 min).

5.9. Estimate the amount of the products by measuring their concentrations in a selected number of samples using a fluorometer. Dilute all samples with nuclease-free H_2O to 0.5 ng/µL.

5.10. Mix 1 µL of the diluted PCR products with 8.5 µL of deionized formamide and 0.5 µL of fluorescent dye-labeled size standard in a 96-well plate compatible with the genetic analyzer.

NOTE: Prepare a master mix containing deionized formamide and the size standard.

5.11. Cover the plate with a plate septa and denature the samples at 95 °C for 3 min in a thermocycler. Do not close the lid of the machine.

5.12. Perform capillary gel electrophoresis to separate the labeled PCR products as previously described[32].

5.13. After electrophoresis, open the analysis software to analyze the results.

5.14. Click **New Project** and select **Microsatellite**. Then click **OK**.

5.15. Click **Add Samples to Project** and select the result files (contain the .fsa extension). Then click **Add to list** to import the files.

5.16. In the table showing the selected result files, choose **Microsatellite Default** in the **Analysis Method** column. Also, select the size standard used in the **Size Standard** column. Then click the **Analyze** icon, enter the experiment name and save the experiment.

5.17. Click **Display Plots** to view the results and choose **Fragment Analysis** in the plot setting.

5.18. Choose the appropriate colored channels for analysis. Check the orange icon to view the labeled fragments in the size standard to assess the quality of size calling.

5.19. Check the blue icon to view the labeled PCR products. Identify the peaks that correspond to the wild-type and mutant (i.e., bearing the expected deletions) products. Estimate the mutant level in each sample by dividing the area under the mutant peak by the sum of the area under the wild-type and mutant peaks.

5.20. Select multiple cell pools with high levels of the expected deletions for further serial dilutions.

5.21. Repeat the DNA extraction, fluorescent PCR and capillary electrophoresis steps. Select cell clones with mutant levels >95% representing biallelic deletions for subsequent analyses.

5.22. Verify the identity of the deletions in the selected clones by Sanger sequencing.

6. Functional analyses of the silencer deletion by real-time quantitative RT-PCR analysis

6.1. Extract total RNA from the selected clones and perform complementary DNA (cDNA) synthesis.

6.2. Mix 1 µg of RNA with 1 µL of 50 µM oligo(dT)$_{20}$ primer and 1 µL of 10 mM dNTP mix in a total volume of 10 µL. Incubate at 65 °C for 5 min and then place on ice for at least 1 min.

6.3. Add 10 µL of cDNA synthesis mix containing 2 µL of 10x RT buffer, 4 µL of 25 mM $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNase inhibitor (40 U/µL) and 1 µL of reverse transcriptase (200 U/µL). Incubate at 50 °C for 50 min and then 85 °C for 5 min in a thermocycler.

NOTE: Prepare a master mix for the reverse transcription.

6.4. Chill the samples on ice. Add 1 µL of RNase H and incubate at 37 °C for 20 min. Store the cDNA at -20 °C.

6.5. Design primers and TaqMan probes that specifically recognize individual transcript variants generated from alternative promoters.

NOTE: Pre-designed transcript-specific primer/probe sets are commercially available.

6.6. Clone DNA fragments containing the specific transcript sequences into plasmid DNA. Prepare a 10-fold dilution series (10^6 to 10 copies) of the recombinant plasmids as standard curves for transcript quantification.

6.7. Prepare 20 µL of PCR mix (0.5 µL of DNA template, 1 µL of 20x pre-designed TaqMan probe/primer assay and 10 µL of 2x TaqMan PCR Master Mix) for each sample (both cDNA and plasmid standards). Measure each sample in triplicate.

NOTE: Prepare a master mix for the real-time PCR.

6.8. Run the reactions in a real-time PCR machine (Conditions: initial 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min).

6.9. After the amplification, click the **Analyze** icon in the software to analyze the data. Check the slope and the correlation coefficient of the standard curves to evaluate the efficiency and linearity of the reactions. Ensure that the slopes are between -3.1 and -3.6 and correlation coefficients are greater than 0.99.

6.10. Normalize the copy number of the target transcripts in each sample with a housekeeping gene (e.g., *GAPDH*).

REPRESENTATIVE RESULTS:

The aim of this experiment is to delete an intronic silencer in the *RUNX1* gene and examine the impacts on *RUNX1* transcription in OCI-AML3 cells. The silencer was identified by a combinatorial molecular approach and found to contain a 209-bp core element[18]. To enable more accurate evaluation of this core element in controlling *RUNX1* expression, the crRNAs (crRNA-1 and crRNA-2) were designed to target closely to this region[18] (**Figure 1**). The predicted Cas9 cleavage sites brought by crRNA-1 and crRNA-2 were 29 bp and 35 bp from the core element, respectively (**Figure 1**). It should be noted that while the PAM sites of the two crRNAs reside on opposite strands, DSBs will occur independently of PAM sequence location. Thus, the concomitant introduction of two Cas9/gRNA RNP complexes guided by crRNA-1 and crRNA-2 is expected to excise the silencer element from the *RUNX1* locus.

To screen for the desired deletions in a large number of samples, a 96-well format of genomic DNA extraction kit was used for high-throughput purification. Also, DNA bound on the wells of the extraction plates can be subjected to direct amplification, thus minimizing errors or contamination due to repeated sample transfer. The primers used for screening of the deletions[18] are shown in **Figure 1**. The expected size of the wild-type PCR product is about 500 bp. Since the intended deletion spans 273 bp, the mutant product is expected to be about 230 bp. This size range enables simple and rapid fragment analysis by capillary gel electrophoresis. A total of 160 initial cell pools were screened and 14 were found to carry the expected deletions with mutant levels of at least 70%. Five pools were then selected for further serial dilutions to identify clones bearing biallelic deletions. Representative electropherograms from cell clones with different levels of mutant products are shown in **Figure 2A**. The identity of the deletions was verified by Sanger sequencing (**Figure 2B**). As expected, indels formed by non-homologous end joining repair of DSBs[33] were observed at the predicted cleavage sites in the deletion clones. These resulted in the amplification of mutant products of varying sizes, which could also be detected by capillary electrophoresis (**Figure 2A**).

The *RUNX1* gene contains two promoters namely the distal P1 and proximal P2, which are separated by a large intron harboring the silencer element[34]. Three major mRNA transcripts are produced by these promoters: *RUNX1c* by P1 and *RUNX1a* and *RUNX1b* by P2[34]. The nucleotide sequence of *RUNX1c* and *RUNX1b* are identical except the former has a unique N-terminus, from which a specific TaqMan gene expression assay can be designed (**Figure 3A**). To measure *RUNX1b*, a TaqMan assay recognizing both *RUNX1b* and *RUNX1c* was used (**Figure 3A**). *RUNX1b* levels were then determined by subtracting total *RUNX1b*/*RUNX1c* from *RUNX1c*. *RUNX1a* is a distinctly shorter isoform due to alternative splicing and a specific TaqMan assay is available for this variant (**Figure 3A**). Thus, the activity of the P1 and P2 promoters can be determined individually. Real-time quantitative RT-PCR showed that deletion of the silencer element significantly upregulated the expression levels of both P1- and P2-derived transcripts (**Figure 3B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Strategy to delete the *RUNX1* intronic silencer. The silencer (red box) is located in the first intron of the *RUNX1* gene separating the two promoters P1 and P2 (hg19 coordinates are shown). Two crRNAs (crRNA-1 and crRNA-2) were designed to introduce DSBs flanking the silencer element. The predicted Cas9 cleavage sites are indicated by vertical red lines and the

PAM sites (NGG) are in purple. The two primers used for screening of the deletions are represented by open arrows.

Figure 2: Identification of the deletion clones. (A) Representative electropherograms of cell clones showing different levels of mutant PCR products (MUT). The size of the mutant products varies among the clones because of indels formed at the cleavage sites. WT, wild-type. **(B)** Verification of the deletions from representative clones by Sanger sequencing.

Figure 3: Functional consequences of the silencer deletion. (A) The three major RUNX1 isoforms (RUNX1a, RUNX1b and RUNX1c) are shown. These variants contain the same Runt DNA binding domain but different N- (orange) or C-terminus (blue). The location of the TaqMan probe/primer pairs is indicated by red lines. Numbers indicate the amino acid residues. **(B)** Real-time quantitative RT-PCR analysis of *RUNX1* P1- and P2-derived transcripts in cell populations with (DEL) or without (WT) the biallelic deletions[18]. *GAPDH* was used for normalization. * and ** indicate $P<0.05$ and $P<0.01$, respectively by the Mann-Whitney test. This figure has been modified from Cheng et al.[18].

DISCUSSION:

The CRISPR/Cas9 system has been used in a wide range of genome editing applications such as gene knockout and knock-in studies[35,36], transcriptional regulation[37,38], genetic engineering of various model organisms[39-44] and gene therapy[45,46]. Here, we demonstrate the use of CRISPR/Cas9 to investigate the functional consequences of deleting an intronic silencer on the *RUNX1* gene. The delivery of the CRISPR components in our approach did not rely on plasmid DNA, cloning of gRNA or virus but electroporation of preassembled Cas9/gRNA RNP complexes. It has been shown that the use of exogenous DNA can be associated with undesirable integration of foreign vector sequences into the host genome, increased toxicity and low efficiency[25,47,48], whereas virus transduction methods are time-consuming. In addition, prolonged expression of Cas9 from plasmid DNA can augment off-target effects[48]. On the contrary, the direct RNP-based delivery approach has been established as the preferred method as it is fast and straightforward with improved editing efficiency, selectivity and cell viability. Indeed, a variety of methods such as lipofection[49,50], electroporation[25,51], nanoparticles[52], cell-penetrating peptides[53], iTOP[54] and TRIAMF[55] have been developed for efficient CRISPR/Cas9 delivery into diverse cell types as well as animal and plant species[24-26,56-63]. Since non-coding DNA sequences are hotspots of genetic variations[64], checking the presence of common SNPs/indels in the target and neighboring PAM sequences is particularly relevant when designing gRNA that targets regulatory elements.

A bottleneck in CRISPR/Cas9 genome editing involves screening of desired mutant clones in a large number of samples. We employed fluorescent PCR coupled with capillary gel electrophoresis for the screening as the target mutation is a small genomic deletion of about 300 bp. This method is rapid and sensitive and can be performed in a high-throughput fashion. Also, this method allows accurate estimation of mutant levels and deletion sizes simultaneously. In addition, multiplex analysis of PCR fragments labeled with different fluorescent dyes is supported. We have been routinely using this technique to genotype small insertions/deletions in myeloid neoplasms[65,66]. In our experience, we can consistently detect fragment sizes that are

differ by 4 bp with high precision and mutant burden down to ~3 %. However, it should be noted that this method has a fragment size limit of 1,200 bp, and thus it is not suitable for screening of large deletions. Also, base substitutions (resulting in unchanged fragment size) and potential off-target events in other genomic regions cannot be detected. For the latter, the costly whole genome sequencing is required to comprehensively profile global undesirable changes in the target clones. To adopt our current approach for investigation of large non-coding regulatory sequences (>1,000 bp), a detailed deletion and mutagenesis analyses of putative transcription factor binding sites using *in vitro* reporter gene assays can be performed beforehand to delineate the minimal functional region for CRISPR/Cas9 editing[18].

As many genes contain more than one promoters[3,4], it is important to be aware of the existence of alternative promoters in the target gene locus as manipulating regulatory elements may affect the promoters differentially. Thus, transcript variants derived from different promoters need to be measured individually to evaluate any promoter-specific responses. The use of TaqMan probe-based assays is preferred over SYBR Green because of better specificity and reproducibility. If the more advanced digital PCR system is available, transcript quantification can be performed more precisely without the need of standard curve construction.

An important consideration in performing CRISPR/Cas9 experiments in cancer cell lines is the ploidy and target gene copy number in the cells used as virtually all cancer cell lines harbor genetic alterations including structural and copy number variations. In our case, OCI-AML3 has a hyperdiploid karyotype with 45 to 50 chromosomes. Also, the cell line was found to carry a normal *RUNX1* copy number as revealed from the Cancer Cell Line Encyclopedia[67] and fluorescence *in situ* hybridization studies[18]. When targeting a gene with copy number gain, the delivery method may need to be optimized to provide sufficient levels of the CRISPR components for the editing. Also, more clones may need to be screened in order to identify the complete knockouts. Importantly, it has been shown that targeting at amplified genomic regions, particularly those caused by structural rearrangements, can trigger gene-independent antiproliferative responses in cancer cells, leading to false-positive results in gene functional studies[68-70]. In this regard, alternative approaches like RNA interference (RNAi) knockdown and/or cDNA overexpression should be employed to verify the CRISPR findings. Also, multiple cell lines should be used to avoid misinterpretation of cell line-specific but gene-independent CRISPR editing effects.

The CRISPR/Cas9 system has revolutionized basic and translational research by providing a simple and efficient means to genome editing. Here we demonstrate the ease of using CRISPR/Cas9 to disrupt an intronic silencer for transcriptional studies in a cancer cell line. This technique allows for the study of CREs at the DNA level and offers the opportunities to examine CRE functions in the endogenous context rather than the traditional heterologous reporter genes. Recently, a CRISPR-based RNA editing system has also been identified[71] and may serve as a novel tool to study CREs by targeting RNA transcribed from the regulatory elements. By combining with chromosome conformation capture techniques, CRISPR/Cas9 will certainly help decipher the involvements of CREs in altered genome organization and gene expression linked to various health problems.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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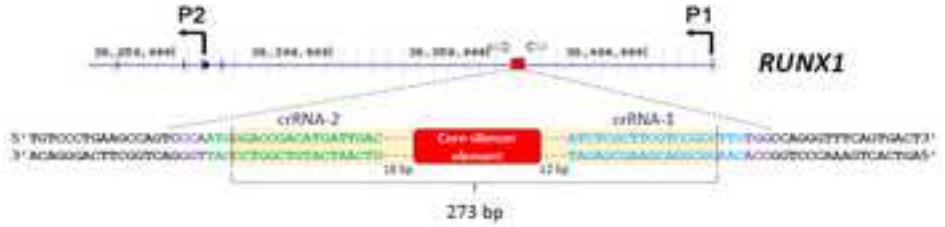
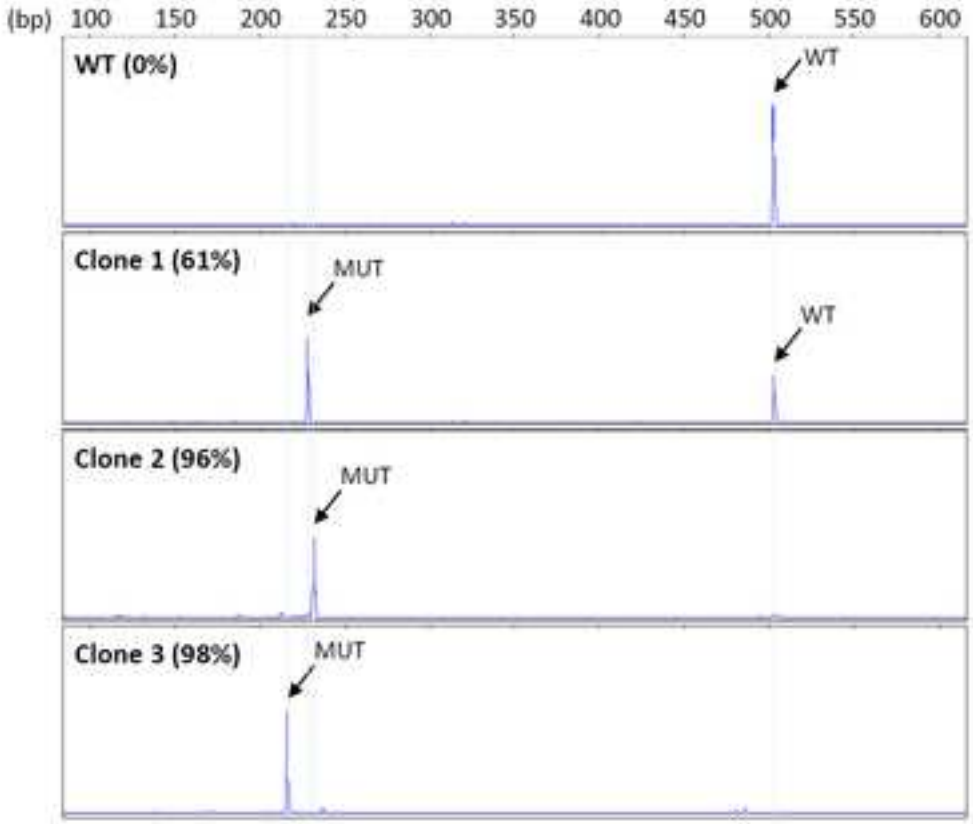


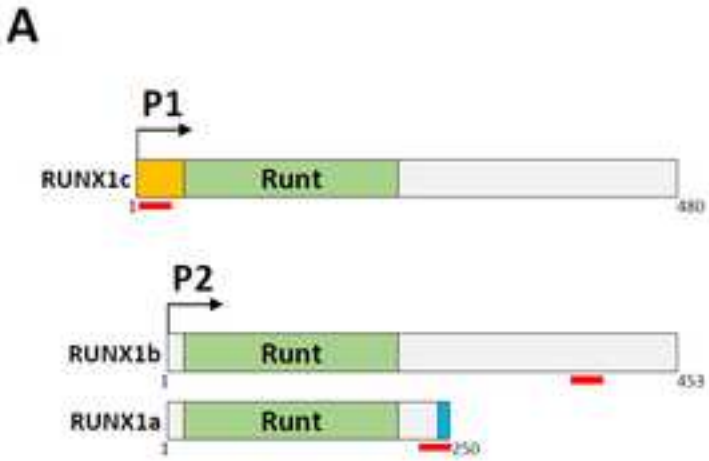
Figure 2

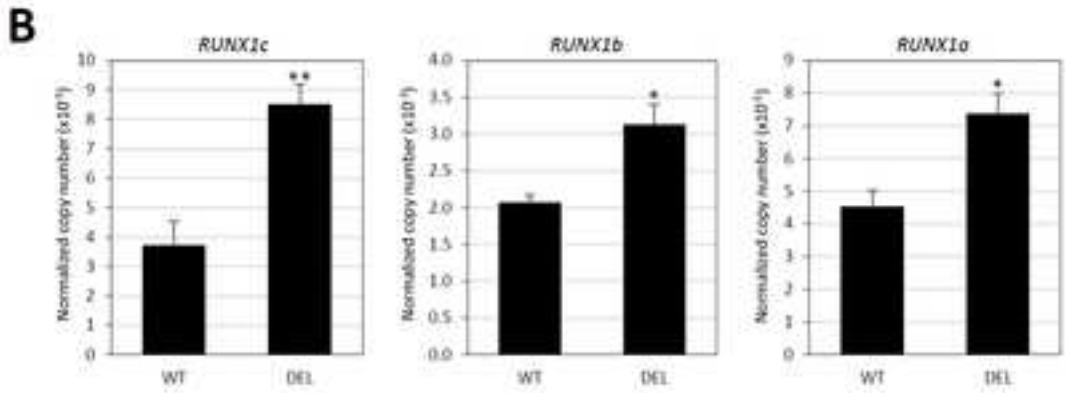
A



B

Figure 3





Name of Material/ Equipment	Company	Catalog Number
0.2 cm-gap electroporation cuvette	Bio-Rad	1652086
1×TE buffer, pH7.5	Integrated DNA Technologies	11-01-02-02
10mM dNTP mix	Thermo Fisher Scientific	18427013
3500 Genetic Analyzer	Thermo Fisher Scientific	4405673
6-FAM-labeled fluorescent PCR forward primer	Thermo Fisher Scientific	None
7300 Real-Time PCR System	Thermo Fisher Scientific	None
7300 System SDS Software	Thermo Fisher Scientific	None
Bio-Rad Gene Pulser Xcell system	Bio-Rad	1652660
ChargeSwitch Direct gDNA Purification Kit, 96-well	Thermo Fisher Scientific	CS11205
crRNA-1	Integrated DNA Technologies	Alt-R CRISPR-Cas9 crRNA
crRNA-2	Integrated DNA Technologies	Alt-R CRISPR-Cas9 crRNA
Deionized formamide	Thermo Fisher Scientific	4311320
Electroporation Enhancer	Integrated DNA Technologies	1075915
fetal bovine serum	Thermo Fisher Scientific	10270098
Fluorometer	Thermo Fisher Scientific	Q32857
GeneMapper Software 5	Thermo Fisher Scientific	4475073
GeneScan 600 LIZ Size Standard	Thermo Fisher Scientific	4408399
GlutaMAX	Thermo Fisher Scientific	35050061
PBS, 10X Solution, pH7.4	Affymetrix	75889
Penicillin and streptomycin	Thermo Fisher Scientific	15140122
Platinum Taq DNA Polymerase High Fidelity	Thermo Fisher Scientific	11304029
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854
Recombinant S. pyogenes Cas9 nuclease	Integrated DNA Technologies	1081058
RPMI 1640 medium	Thermo Fisher Scientific	31800-022
RPMI 1640 medium without phenol red	Thermo Fisher Scientific	11835030
RUNX1a TaqMan gene expression assays	Thermo Fisher Scientific	4331182
RUNX1b/c TaqMan gene expression assays	Thermo Fisher Scientific	4331182
RUNX1c TaqMan gene expression assays	Thermo Fisher Scientific	4331182
SuperScript III First-Strand Synthesis System	Thermo Fisher Scientific	18080051
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific	4304437
tracrRNA	Integrated DNA Technologies	1072533
TRIzol Reagent	Thermo Fisher Scientific	15596018

Unlabeled PCR reverse primer	Thermo Fisher Scientific	None

Comments/Description

Version 1.3.1

Components of the Cas9/gRNA complex
Components of the Cas9/gRNA complex

Components of the Cas9/gRNA complex

Hs04186042_m1
Hs00231079_m1
Hs01021966_m1

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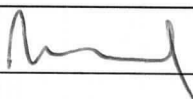
Signature:	
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   5.3. Add 100 µL of plate binding and lysis buffer to each well of a 96-well extraction plate. Add 5×10^4 cells resuspended in 10 µL of 1×PBS to the buffer and mix them by pipetting.
   5.4. Incubate at room temperature for 30 min to allow binding of genomic DNA to the

wells.

5.5. Aspirate the solution from the wells without scraping the well surfaces. Wash the wells with 120 µL of wash buffer.

5.6. Air dry the wells containing the bound DNA.

6. 6.2-6.5: Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed?
Response: More details have been added to the protocol as below:

6.2. Mix 1 µg of RNA with 1 µL of 50 µM oligo(dT)$_{20}$ primer and 1 µL of 10 mM dNTP mix in a total volume of 10 µL. Incubate at 65 °C for 5 min and then place on ice for at least 1 min.

6.3. Add 10 µL of cDNA synthesis mix containing 2 µL of 10×RT buffer, 4 µL of 25 mM $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNase inhibitor (40 U/µL) and 1 µL of reverse transcriptase (200 U/µL). Incubate at 50 °C for 50 min and then 85 °C for 5 min in a thermocycler. Note: Prepare a master mix for the reverse transcription.

6.4. Chill the samples on ice. Add 1 µL of RNase H and incubate at 37 °C for 20 min. Store the cDNA at -20 °C.

6.5. Design primers and TaqMan probes that specifically recognize individual transcript variants generated from alternative promoters. Note: Pre-designed transcript-specific primer/probe sets are commercially available.

6.6. Clone DNA fragments containing the specific transcript sequences into plasmid DNA. Prepare a 10-fold dilution series (10^6 to 10 copies) of the recombinant plasmids as standard curves for transcript quantification.

6.7. Prepare a 20 µL PCR mix containing 0.5 µL of DNA template, 1 µL of 20×pre-designed TaqMan probe/primer assay and 10 µL of 2×TaqMan PCR Master Mix for each sample (both cDNA and plasmid standards). Measure each sample in triplicate. Note: Prepare a master mix for the real-time PCR.

6.8. Run the reactions in a real-time PCR machine (Conditions: initial 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 1 min).

6.9. After the amplification, click the "Analyze" icon in the software to analyze the data. Check the slope and the correlation coefficient of the standard curves to evaluate the efficiency and linearity of the reactions. Ensure that the slopes are between -3.1 and -3.6 and correlation coefficients are greater than 0.99.

6.10. Normalize the copy number of the target transcripts in each sample with a housekeeping gene (e.g. *GAPDH*).

6. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
Response: We have highlighted the steps as suggested.

7. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
   Response: We have highlighted all relevant details as suggested.

8. References: Please do not abbreviate journal titles; use full journal name.
   Response: Full journal names have been provided.

9. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
   Response: The information in the Table of Materials has been checked and items have been sorted alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors interestingly describe a comprehensive CRISPR/Cas9 protocol that use the in vitro generation of gRNAs and CAS9 protein to delete an intronic enhancer of RUNX1. As a proof-of principle, they use an electroporation method to transfer the so called RNP complex (ie. consisting of gRNAs and CAS9 protein) and they further use capillary gel electrophoresis to detect for positive mutant (deletion) clones. The method they use here is simple and can be applied instantly in any basic laboratory of molecular biology.

Major Concerns:

There are no major concerns that I would like to discuss.

Minor Concerns:

The minor concern that I would like to discuss is written in one sentence (line 393-395). In contrast to what the authors have written, RNAi is successfully used to study CREs by targeting active transcripts generated from so-called enhancer RNAs. I suggest that the statement would be written differently and take into consideration that, as an alternative to RNAi, one can utilise the RNA targeting Cas13. Therefore, studying CRE function can be achieved either by targeting DNA (cas9) or RNA (cas13).

Response: As suggested by the reviewer, we have modified the sentences to "This technique allows for the study of CREs at the DNA level and offers the opportunities to examine CRE functions in the endogenous context rather than the traditional heterologous reporter genes. Recently, a CRISPR-based RNA editing system has also been identified[71] and may serve as a novel tool to study CREs by targeting RNA transcribed from the regulatory elements." in lines 425-429.

Reviewer #2:

The protocol describes how CRISPR-Cas9 ribonucleoproteins can be used to delete silencers in one AML cell line (OCI-AML3). The technique becomes more and more important and will soon become the standard for the investigation of genomic regions.
The protocol does not really exceeds what is provided by most vendors, who distribute the system. Several passages are too vague and undetailed for a protocol (see below). Moreover, as only one cell line was used, the protocol does no go beyond previously published protocols (e.g. Bak et al. Nat Protocols 2018), which even used primary hematopoietic stem/progenitor cells.

Major concerns:

1. The description of limiting-dilution clonogenicity assay is not clear. At 5x10e3/mL cell density it is hard to believe that you get many clean clones. Why the author did not use the starndard limiting-dilution assay (1 cell in 300 µl; 3 wells 100 µl each). Most preferred, why didn't the authors use single cell sorting?
   Response: In our approach, we seeded 100 µL of cells at 5x10^3/ml density 24 hours post electroporation (mentioned in step 5.1). After sufficient cell proliferation, genomic DNA was extracted and fragment analysis was performed. This initial screen served to identify cell pools (heterogeneous populations) carrying the desired deletions. Then, multiple pools with high levels of the desired deletions were selected for subsequent serial dilutions to identify clones with mutant levels >95 % representing biallelic deletions for downstream *RUNX1* expression studies (mentioned in step 5.20). We reason that this stepwise screening approach is less laborious and time-consuming than the traditional limiting dilution. Also, this approach can be applied to cells that cannot be propagated from single cells. To make our screening approach clearer, we have re-named the initial cell populations as "cell pools" (line 251).
   We agree with the reviewer that single cell sorting is a powerful technique for single cell isolation. However, we did not have the sophisticated instruments and expertise for performing the experiments. Also, since our approach does not introduce exogenous marker protein (e.g. GFP) and that it is uncertain if the mutant cells exhibit aberrant physical properties/surface antigen expression, single cell sorting may not be applicable in our case.

2. Line 302 ; How many clone were screened and how many were positive for the deletion?
   Response: A total of 160 initial cell pools were screened and 14 were found to carry the expected deletions with mutant levels of at least 70%. Five pools were then selected for further serial dilutions to identify clones bearing biallelic deletions. This information has been provided in the Results (lines 318-321).

3. What is the viability of the cells 24 hours post electroporation?
   Response: The viability of the cells was found to be 70% 24 hours post electroporation.

4. What is the source of OCI-AML3 cell line? The author presents a different culture medium for the cell line from what DSMZ states. If the cell line is obtained from another source then is the cell line authenticated?

Response: As indicated in the acknowledgements, we obtained OCI-AML3 from Prof. M.D. Minden (Princess Margaret Cancer Centre, University Health Network, Toronto, Canada). This cell line has been reported to be cultured in RPMI-1640-based media in different labs who obtained the cells from the same source (Walter et al. Blood. 2005; Konopleva et al. Cancer Res. 2008; Faderl et al. Cancer Res. 2009; Yi et al. Oncol Lett. 2012).
It has been reported that among 79 myeloid cell lines tested, OCI-AML3 was the only cell line carrying the AML-specific *NPM1* mutation (Quentmeier et al. Leukemia. 2005). We have been routinely using the cell line from Prof. Minden as the positive control for *NPM1* mutation screening in our diagnostic workup for AML patients. Also, we confirmed that the cell line harbored the *DNMT3A* R882C mutation as reported. Moreover, our fluorescence in situ hybridization studies showed that the line carried a normal copy number of *RUNX1* (mentioned in lines 410-412) but an extra copy of *RUNX1T1*. These findings are consistent with those reported in the Broad Institute Cancer Cell Line Encyclopedia. Together, these molecular characterizations strongly indicate the authenticity of the cell line used.

5. Line 141; TE buffer recipe; why 0,1 mM EDTA was used instead of 1 mM EDTA?
   Response: We obtained the crRNAs and tracrRNA from a commercial vendor (Integrated DNA Technologies). It is recommended that the RNAs should be resuspended in IDTE buffer (1×TE solution) containing 10 mM Tris and 0.1 mM EDTA for the best stability.

6. Line 144-156; The preparation of the RNPs is not explained with enough detail as I would expect it from a method paper. Most importantly, please provide volumes (each component and final) and required amounts for the reaction.
   Response: As suggested by the reviewer, we have provided more details on the preparation of the RNPs as below:
   3.2. For each crRNA, mix 2.2 μL of 200 μM crRNA, 2.2 μL of 200 μM tracrRNA and 5.6 μL of 1×TE buffer (total 10 μL) in a 0.2 mL tube to obtain a final duplex concentration of 44 μM.
   3.3. Incubate at 95 °C for 5 min in a thermocycler. Allow the tubes to cool to room temperature for gRNA complex formation.
   3.4. Dilute 10.4 μL of 62 μM recombinant Cas9 nuclease with 7.6 μL of 1×phosphate-buffered saline (PBS) to obtain a final nuclease concentration of 36 μM. Note: This amount is sufficient for the preparation of two Cas9/gRNA complexes.
   3.5. Mix equal volumes (8.5 μL) of the diluted nuclease with each of the gRNA duplexes obtained from step 3.3.

7. Figure 3B: Please show results per isoform. Is there any change in RUNX1 protein level?
   Response: As suggested by the reviewer, we have presented the results per isoform in the revised figure 3B.
   Since no Western blot studies were performed, we could not ascertain if RUNX1 protein level was also upregulated in OCI-AML3 cells following the silencer deletion. However, in our previous immunohistochemical studies on bone marrow biopsy from an AML patient carrying a novel t(5;21) translocation that disrupts the *RUNX1* silencer (Cheng et al. Mol

Cancer. 2018), we did observe prominent RUNX1 protein expression in the leukemic blasts in association with increased *RUNX1* mRNA levels.

8. Can the author comment on the consequences of deleting RUNX1 silencer? Why P1 activity is affected if the silencer element is downstream P1 ?
   Response: As discussed in our previous publication (Cheng et al. Mol Cancer. 2018), the *RUNX1* silencer was found to interact with and repress the P2 promoter through long-range chromatin interactions. Thus, deleting the silencer is expected to upregulate P2 transcription. However, since RUNX1 can bind to two RUNX motifs in the P1 promoter to activate its transcription (Martinez et al. PLoS One. 2016), it is possible that the increased P2-isoform expression may activate P1 through a positive feedback loop, leading to the concomitant increase in P1-derived *RUNX1c*.

9. There are several grammar and typos. For example : Line 29; mean not means. Line 31; leukemic not leukemia. Line 68; understanding of the not on the; Line 91; ''directs Cas9 there for editing'' no need for there. Line 92; CRISPR not crispr
   Response: As suggested by the reviewer, the following mistakes have been addressed: leukemia to leukemic, understanding on to understanding of, directs Cas9 there to directs Cas9 and crispr to CRISPR.

10. The text contains some scientific generalisations without referencing. For example : Line 48; not all hematopoietic cells are difficulties to transduce. Line 50; ''limited'' the off-target is dependent on the gRNA sequence but delivery methods may influence the off-target effect.
    Response: As suggested by the reviewer, we have modified the sentence in line 48 from "which are difficult to transfect with plasmid-based methods." to "which are often difficult to transfect with plasmid-based methods." Also, we have deleted the sentence "with limiting off-target effects." in line 50. The off-target issue was discussed in the first paragraph of the Discussion with references.

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