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# Letter to the Editor

# Validation of CRISPR targeting for proliferation and cytarabine resistance control genes in the acute myeloid leukemia cell line MOLM-13

**Subhash C Prajapati[‡,1], Nicholas Dunham[‡,1], Hao Fan[1] & Francine E Garrett-Bakelman*[,‡,1,2,3]**

[1]*Department of Biochemistry & Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA 22908, USA;* [2]*Department of Medicine, University of Virginia School of Medicine, Charlottesville, VA 22908, USA;* [3]*University of Virginia Cancer Center, Charlottesville, VA 22903, USA; *Author for correspondence: Tel.: +1 434 924 9245; fg5q@virginia.edu;* [‡]*Authors contributed equally*

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### ABSTRACT

Acute myeloid leukemia patients with FMS-like tyrosine kinase 3–internal tandem duplications and mixed lineage leukemia–protein AF9 fusion proteins suffer from poor clinical outcomes. The MOLM-13 acute myeloid leukemia cell line harbors both of these abnormalities and is used in CRISPR experiments to identify disease drivers. However, experimental observations may be biased or inconclusive in the absence of experimentally validated positive control genes. We validated sgRNAs for knockdown of *TP53* for cell proliferation and for *DCK* knockdown and *CDA* upregulation for cytarabine resistance control genes in MOLM-13 cells. We have provided a detailed CRISPR protocol applicable to both gene knockdown or activation experiments and downstream leukemic phenotype analyses. Inclusion of these controls in CRISPR experiments will enhance the capacity to identify novel myeloid leukemia drivers in MOLM-13 cells.

### METHODSUMMARY

CRISPR-Cas9 knockdown and upregulation approaches were used for changing the expression of control genes *TP53*, *DCK*, and *CDA* in the acute myeloid leukemia cell line MOLM-13. Lentivirus transduced cells were selected (sorted for green fluorescent protein positive cells or selected by antibiotic treatment), and knockdown or upregulation of genes in the stable cells was confirmed by real-time quantitative PCR and western blot analyses. EdU incorporation assay was used to measure cell proliferation. Colorimetric proliferation/survival assay and flow cytometric cell counting were used to measure cell growth and survival. Results from the experimental and control groups were compared using Student's *t*-test.

### KEYWORDS:

acute myeloid leukemia • *CDA* • CRISPR-Cas9 • cytarabine • *DCK* • MOLM-13 • p53 • proliferation • resistance

Acute myeloid leukemia (AML) is the most frequent acute leukemia in adults [1]. Despite significant scientific and clinical advances in the field, AML remains a life-threatening cancer for most patients [2]. To improve upon therapeutic options for AML patients, there is a continued need for a better understanding of disease biology and molecular events amenable to therapeutic targeting [3]. Cell lines derived from AML patients or associated antecedent disorders are frequently used as cell models to study AML disease mechanisms [4]. The MOLM-13 cell line is a widely used cell model in AML research [5,6]. MOLM-13 was derived from a patient who was diagnosed with myelodysplastic syndrome that evolved to AML and experienced disease relapse after treatment. It harbors the recurrent cytogenetic abnormality mixed lineage leukemia–protein AF9 fusion [7] and an *FLT* internal tandem duplication [8], which are associated with poor clinical outcomes in AML patients [3]. MOLM-13 cells have been used in studies identifying AML drivers that promote cell proliferation, survival, self-renewal and chemoresistance [9–11]. These cells have also been used to assess therapeutic drugs for effective targeting in AML cells [5,10,12].

Gene activation and knockout experiments using CRISPR-Cas9 approaches in MOLM-13 cells are informative about potential disease mechanisms in AML [5,6,13]. However, experimental observations may be biased or inconclusive in the absence of validated positive control genes for AML cell proliferation and chemoresistance. We assessed the use of sgRNA-mediated gene downregulation (*TP53* and *DCK*) or upregulation (*CDA*) as a positive control in CRISPR-Cas9 experiments in MOLM-13 cells. These genes have been implicated in AML pathogenesis [13–15] and chemotherapy resistance [13,16]. *TP53* (protein p53) is a tumor suppressor involved in the regulation of the cell cycle, apoptosis and DNA damage repair [15]. DCK phosphorylates endogenous deoxynucleosides during DNA synthesis [17]. DCK also phosphorylates the deoxynucleoside analogue cytarabine (Ara-C) [13,18]. Ara-C is a major component of standard AML chemotherapy that requires phosphorylation for its anti-tumor activity [3,18]. CDA is a pyrimidine salvage pathway enzyme that catalyzes the hydrolytic deamination of cytidine and deoxycytidine to their corresponding uracil nucleosides [19]. CDA has been implicated in Ara-C deamination, leading to inactivation and chemotherapy resistance [19]. We validated the functional targeting of these genes for

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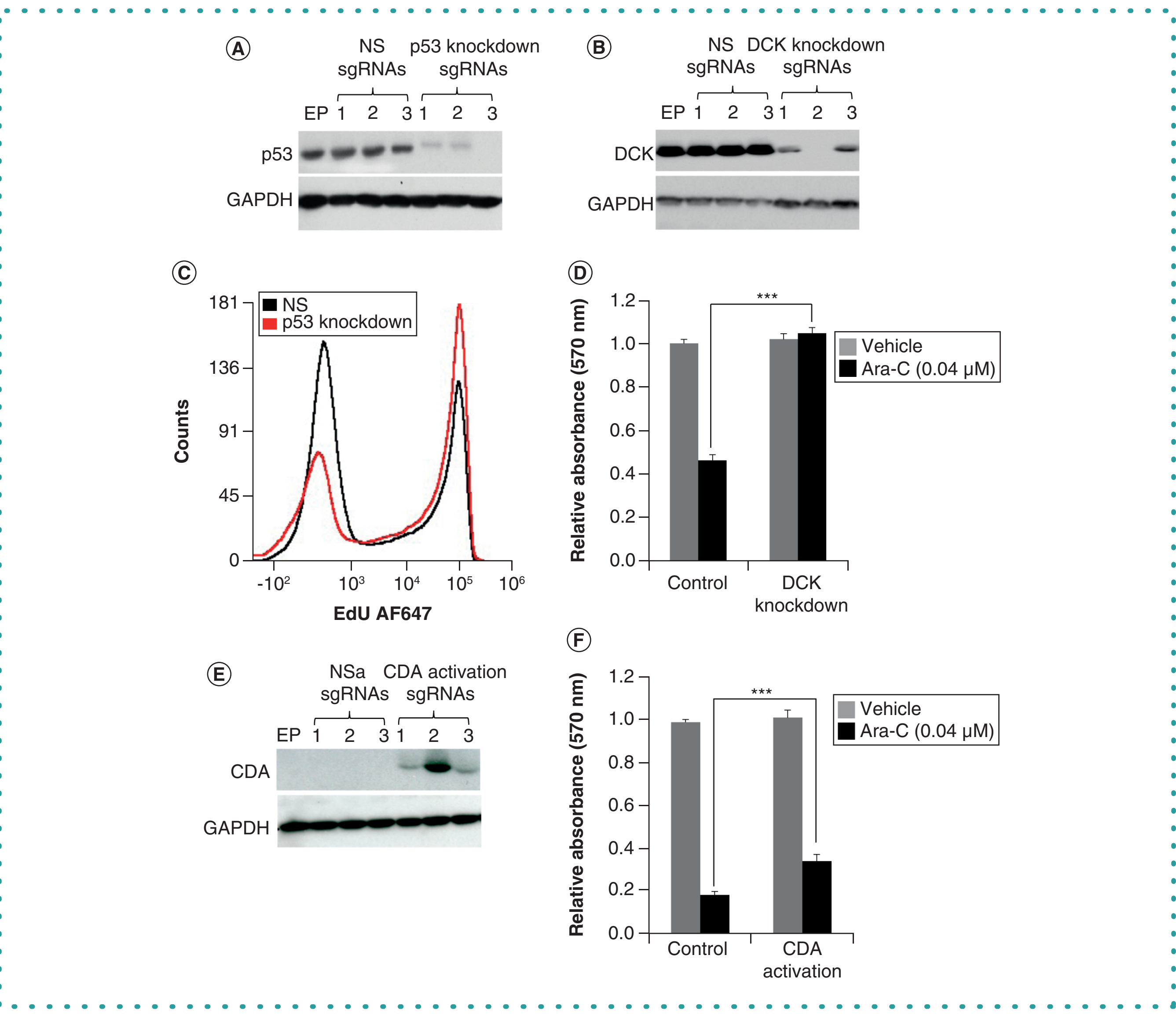


**Figure 1. Perturbation of control genes' expression induces enhanced acute myeloid leukemia phenotypes. (A & B)** Western blot (WB) of p53 **(A)** and DCK **(B)** expression in MOLM-13 cells transduced with three independent CRISPR knockout sgRNA constructs. LentiCRISPRv2-GFP (EP) and three independent non-specific (NS) sgRNA LentiCRISPRv2-GFP constructs served as controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. **(C)** Representative results from an EdU incorporation experiment using Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay in p53 depleted (p53 knockdown) and control MOLM-13 cells. Shown is a histogram plot from MOLM-13 cells transduced with NS1 (black) and TP53-2 (red) sgRNAs. **(D)** Colorimetric survival analysis of DCK depleted (knockdown) MOLM-13 cells compared with control cells subjected to cytarabine (Ara-C) treatment. EP and three independent NS sgRNAs construct transduced cells served as controls. Data represent mean ± standard error of the mean (SEM) (n = 6) and are from two independent triplicate experiments. **(E)** WB of CDA expression in MOLM-13 cells transduced with three independent CRISPR activation sgRNA constructs. LentiSAMv2 (EP) and three independent non-specific activation (NSa) sgRNA LentiSAMv2 constructs served as controls. GAPDH was used as a loading control. **(F)** Colorimetric survival analysis of MOLM-13 cells with upregulated CDA compared with control cells subjected to Ara-C treatment. EP and three independent NSa sgRNAs construct transduced cells served as controls. Data represent mean ± SEM (n = 6) and are from two independent triplicate experiments. Significance testing was performed using Student's *t*-test. *p $<$ 0.001.

use as MOLM-13 cell proliferation (*TP53*) or chemoresistance (*DCK* and *CDA*) positive controls using gene knockdown and activation experiments.

First, we analyzed the basal expression of these genes in MOLM-13 cells. Real-time quantitative PCR (RT-qPCR) expression analyses confirmed *TP53* and *DCK* transcription, while *CDA* was not detected (Supplementary Figure 1 & materials listed in Supplementary Table 2). We assessed p53 and DCK knockdown as positive control genes for proliferation and Ara-C resistance experiments in MOLM-13 cells, respectively, and depleted their expression using the CRISPR-Cas9 knockout approach as described in their protocol (supplementary

materials), a modified protocol from a previous study [20]. Specifically, we used a lentiviral plasmid lentiCRISPRv2-GFP that expresses Cas9, an sgRNA and green fluorescent protein (GFP) for the selection of stably transduced cells. Briefly, *TP53* and *DCK* CRISPR knockout sgRNA constructs were prepared and transduced into MOLM-13 cells. Stably transduced cells ($GFP^+$) were selected using flow sorting and cultured as described in our protocol. Western blot (WB) analyses of $GFP^+$ cell protein lysates using protein-specific antibodies confirmed p53 and DCK depletion. Cell lysates from cells transduced with empty plasmid (EP) lentiCRISPRv2-GFP or non-specific (NS) sgRNA constructs were used as controls (Figure 1A & B). Cell proliferation analyses were conducted in p53 depleted $GFP^+$ cells. Higher EdU incorporation was detected in p53 depleted cells compared with control cells, consistent with enhanced proliferation after p53 knockdown (Figure 1C & Supplementary Figure 2A & B & Supplementary Table 1). Independent colorimetric proliferation/survival and flow cytometric cell counting assays corroborated EdU cell proliferation assay results and confirmed enhanced survival of p53 depleted cells (Supplementary Figure 2C & D; Supplementary Table 1). Ara-C resistance analyses were performed in DCK depleted $GFP^+$ cells. Cells were treated with half maximal inhibitory concentration ($IC_{50}$; 0.04 $\mu$m) of Ara-C daily for 5 days and changes in cell number were measured by the colorimetric proliferation/survival assay. Ara-C $IC_{50}$ was determined from a kill curve generated by treating MOLM-13 cells with a range of Ara-C concentrations (0–0.25 $\mu$m) daily for 5 days (Supplementary Figure 3A). DCK depleted cells exhibited enhanced survival compared with control cells subject to Ara-C treatment (Figure 1D & Supplementary Figure 3B & Supplementary Table 1). Flow cytometric cell counting assay corroborated the results (Supplementary Figure 3C & Supplementary Table 1). These results validated sgRNAs targeting *TP53* and *DCK* for use as controls in CRISPR-Cas9 knockdown experiments assessing MOLM-13 proliferation and Ara-C resistance, respectively.

We next assessed *CDA* upregulation using sgRNAs in a CRISPR activation approach as a positive control gene for Ara-C resistance experiments in MOLM-13 cells. We upregulated *CDA*'s expression using a two-plasmid CRISPR activation approach [20]. In the two-plasmid approach, the lentiMPHv2 plasmid expressing transcriptional effectors (MS2, p65 and HSF1) and an sgRNA cloning plasmid lentiSAMv2 expressing dCas9-VP64 are transduced to upregulate a target gene [20]. First, we generated a clonal lentiMPHv2 stable MOLM-13 cell line by stably transducing lentiMPHv2 lentiviruses into MOLM-13 cells (protocol described in Supplementary Materials). The generation of stable lentiMPHv2 cells was confirmed by RNA expression of MS2 (Supplementary Figure 4). To upregulate *CDA*, we prepared *CDA* CRISPR activation constructs and transduced them into clonal lentiMPHv2 stably transduced MOLM-13 cells. Cells were treated with blasticidin for 14 days to select stably transduced cells. WB and RT-qPCR analyses confirmed CDA upregulation in *CDA* activation construct transduced cells compared with control cells transduced with EP lentiSAMv2 or non-specific activation (NSa) sgRNA constructs (Figure 1E, Supplementary Figure 5A and materials listed in Supplementary Table 2). Ara-C resistance analyses were implemented in *CDA* activation construct transduced lentiMPHv2 MOLM-13 cells with *CDA* upregulation as described in our protocol. Cells with upregulated *CDA* expression were treated with 0.04 $\mu$m Ara-C every day for 5 days and changes in cell survival were measured using the colorimetric proliferation/survival assay. Cells with upregulated *CDA* expression showed enhanced survival compared with control cells with undetectable CDA expression (Figure 1F & Supplementary Figure 5B & Supplementary Table 1). Flow cytometric cell counting assay corroborated the colorimetric assay results (Supplementary Figure 5C & Supplementary Table 1). These results validate sgRNAs targeting *CDA* upregulation for use as controls in MOLM-13 Ara-C resistance experiments.

In summary, we validated a set of sgRNAs targeting genes for use as MOLM-13 cell line proliferation and Ara-C resistance positive controls in CRISPR-Cas9 experiments. We have provided a detailed, step-by-step protocol for knockdown or activation of these control genes. This protocol is applicable to gene knockdown or activation experiments of target genes and downstream leukemic phenotype analyses. Inclusion of these controls in CRISPR-Cas9 experiments will enhance the capacity to identify novel AML drivers in MOLM-13 cells. Further assessments are needed; however, these sgRNAs and their targets may also be applicable as functional controls for experimentation in additional AML cell lines.

The use of CRISPR-Cas9 to modulate gene expression in cancer cell models has been widely implemented due to the platform's high specificity, targetability and ease of use. CRISPR-Cas9 experiments have been used to identify actionable targets in AML models [6]. Additionally, CRISPR-Cas9 shows promise for therapeutic avenues in AML. In addition to chimeric antigen Receptor T-cell approaches to treating AML patients (anti-CD33 chimeric antigen receptor-expressing T cells; NCT03971799), currently CRISPR-Cas9 technology is also being used for a clinical trial utilizing a novel therapeutic approach for CD33 positive AML cases (NCT04849910). We anticipate that the uses of CRISPR-Cas9 experimentally for disease biology interrogation will continue to evolve. Furthermore, we predict that the use of CRISPR-Cas9 techniques in novel therapeutic approaches will offer future personalized medicine options to manage this disease.

## Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2021-0089

## Author contributions

Conceived the study: SC Prajapati; Performed experiments: SC Prajapati, N Dunham and H Fan; generated figures: SC Prajapati; performed data interpretation and wrote manuscript: SC Prajapati, N Dunham and FE Garrett-Bakelman.

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## Financial & competing interests disclosure

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