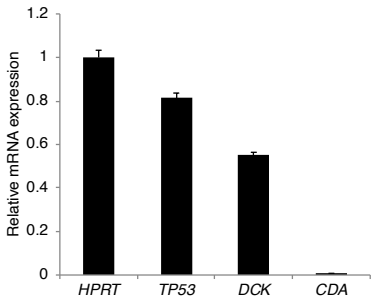
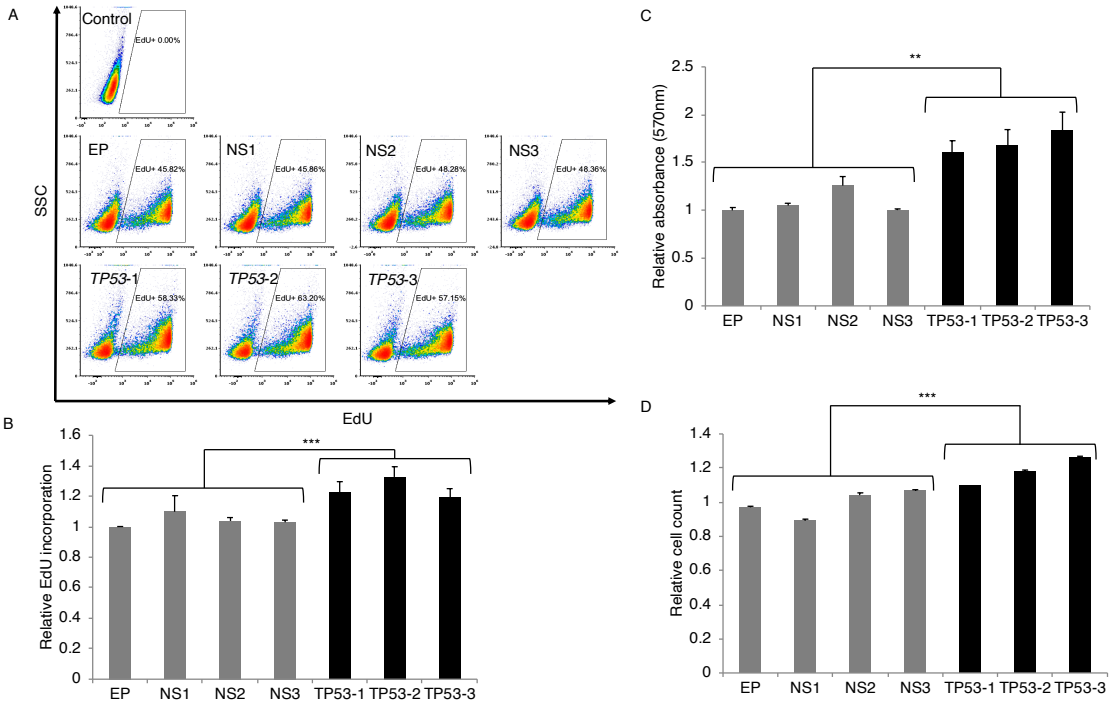
Validation of CRISPR targeting for proliferation and cytarabine resistance control genes in the AML cell line MOLM-13

Supplementary Information

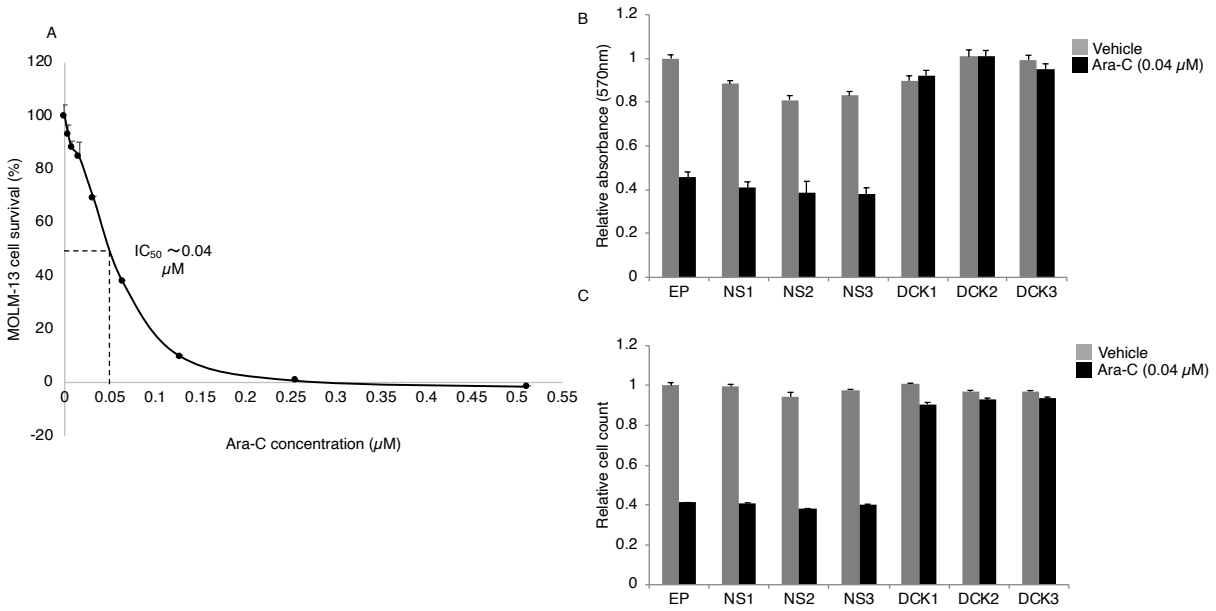
Supplementary Figures



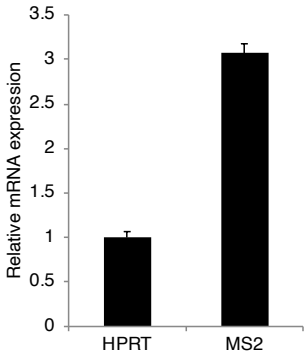
Supplementary figure 1. Control genes' mRNA expression relative to HPRT in MOLM-13 cells. GAPDH was used as reference gene for data normalization. Data represents mean ± SEM (n = 3).



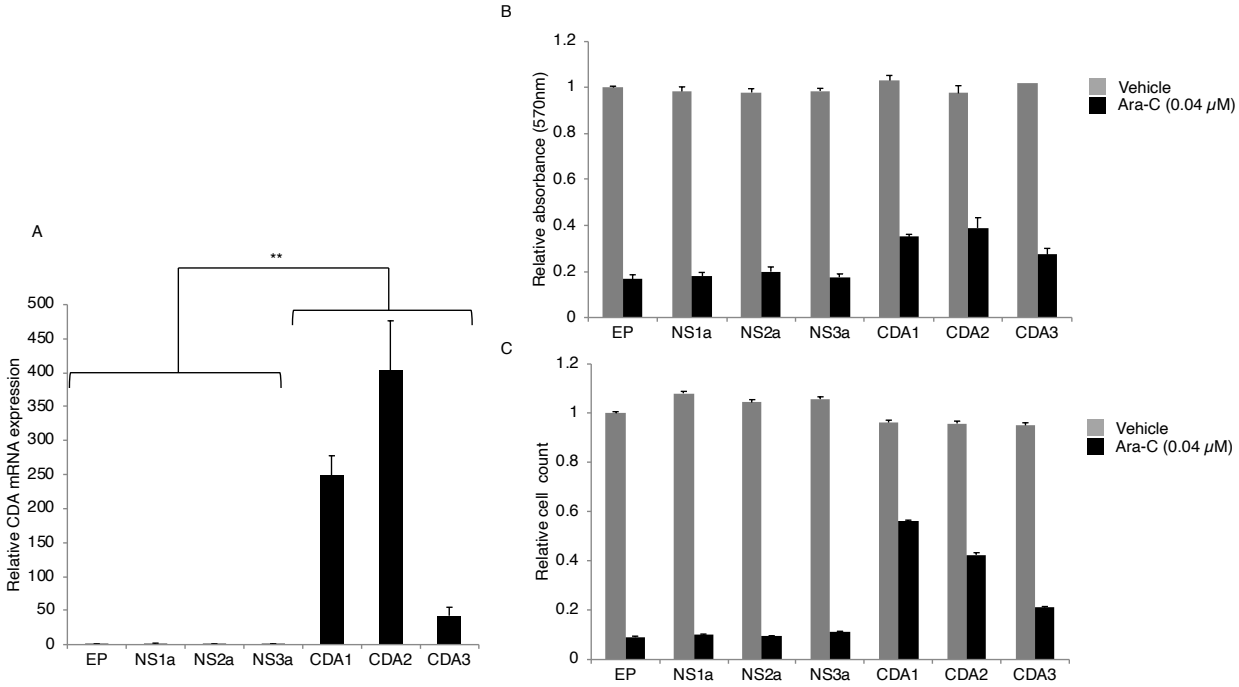
Supplementary figure 2. p53 depletion induces MOLM-13 cells' proliferation. (A) Density plot of EdU incorporation analysis of p53 (p53-1, -2 and -3) depleted MOLM-13 cells. LentiCRISPRv2-GFP (EP) and three independent NS sgRNA lentiCRISPRv2-GFP (NS1-3) constructs transduced cells served as controls. EdU untreated MOLM-13 cell sample was used as control to gate EdU positive cells. (B) Bar graph shows relative quantitation of EdU incorporation. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. (C) Colorimetric proliferation analysis of p53 depleted cells compared to control cells. EP and three independent NS sgRNA constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. (D) Flow cytometric cell count analysis of control versus p53 depleted MOLM-13 cells. EP and NS sgRNA constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. Significance testing was performed using student's t-test **p<0.01, ***p<0.001. EP = empty plasmid; NS= non-specific; EP = empty plasmid.



Supplementary figure 3. DCK depletion induces MOLM-13 cells' resistance to Ara-C. (A) Ara-C kill curve of MOLM-13 cells. Dashed lines' intersection indicates half maximal inhibitory concentration (IC_{50}) of Ara-C for MOLM-13 cells. (B) Colorimetric survival analysis of DCK depleted MOLM-13 cells treated with Ara-C. lentiCRISPRv2-GFP (EP) and NS sgRNA lentiCRISPRv2-GFP constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. (C) Flow cytometric cell count analysis of control (NS) versus DCK depleted MOLM-13 cells subjected to Ara-C treatment. EP and NS sgRNA constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. ***$p<0.001$ Ara-C treated DCK group was compared with Ara-C treated control group. EP = empty plasmid; NS= non-specific sgRNAs.



Supplementary figure 4. MS2 expression in lentiMPHv2 stably transduced MOLM-13 cells. Shown is relative mRNA transcript level of MS2 relative to HPRT in a representative MOLM-13 sample transduced with lentiMPHv2. GAPDH was used as the reference gene for data normalization. Data represents mean ± SEM (n = 3).



Supplementary figure 5. CDA upregulation induces MOLM-13 cells' resistance to Ara-C. (A) CDA mRNA expression (q-PCR) in activation constructs transduced lentiMPHv2 MOLM-13 cells. LentiSAMv2 (EP) and three independent NSa sgRNA LentiSAMv2 constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. (B) Colorimetric survival analysis of activation constructs transduced lentiMPHv2 MOLM-13 cells treated with Ara-C. EP and three NSa sgRNA constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. (C) Flow cytometric cell count analysis of control versus CDA upregulated MOLM-13 cells subjected to Ara-C treatment. EP and NSa sgRNA constructs transduced cells served as controls. Data represents mean ± SEM (n = 6) and are from two independent triplicate experiments. ***$p<0.0001$ Ara-C treated CDA group was compared with Ara-C treated control group. Comparison testing was performed using student's t-test. EP = empty plasmid; NSa = non-specific sgRNAs.

Experiments	Experimental group mean	Experimental group standard deviation	Control group mean	Control group standard deviation	p-value	t statistic
DCK Ara-C resistance by colorimetric assay (Figure 1D & Supp. Figure 3B)	0.960772137	0.045286253	0.407885724	0.036056774	9.436E-05	-17.40
CDA AraC resistance by colorimetric assay (Figure 1F & Supp. Figure 5B)	0.33804251	0.09634933	0.18074227	0.04768599	2.59E-08	-4.53
TP53 cell proliferation by EdU assay (Supp. Figure 2A-B)	1.248442849	0.132343332	1.041872577	0.103890461	0.0002256	-4.55
TP53 cell proliferation by colorimetric assay (Supp. Figure 2C)	1.708231818	0.120253546	1.078010736	0.122654256	0.0015627	-6.80
TP53 cell proliferation by EdU assay (Figure 1C)	1.179209145	0.071551546	0.992656317	0.072541008	1.618E-05	-3.034
TP53 cell proliferation by flow cytometric cell count (Figure 2D)	1.1792	0.07155	0.9926	0.0725	0.038	-3.03
DCK Ara-C resistance by flow cytometric cell count (Supp. Figure 3C)	0.923582993	0.015850018	0.401145521	0.014419599	9.084E-07	-44.84
CDA mRNA upregulation (Supp. Figure 5A)	223.810182	214.842428	1.152112792	0.736753068	0.0028389	-4.98
CDA AraC resistance by flow cytometric cell count (Supp. Figure 5C)	0.399935899	0.153890438	0.100439944	0.008986713	0.0003836	-2.91

Supplementary Table 1. Results from experimental comparison testing. Statistics of experimental results and results from paired T-tests performed. Shown are experimental and control group means and standard deviation as well as results from comparative testing performed (t statistic and p-value from paired t-tests).

Supplementary Table 2. Reagents, supplies and equipment table. Information on reagents, supplies and equipment used in described protocol with annotation to respective protocol step.

Protocol

Materials and equipment used in this protocol are detailed in Supplementary Table 2.

1. **MOLM-13 cell culture:** MOLM-13 cells were cultured in growth media (RPMI 1640 media containing 10% heat inactivated fetal bovine serum and 1% Penicillin-Streptomycin (Pen-Strep)). Cells were maintained at 0.5 million cells/mL and split every 2-3 days. MOLM-13 cells were authenticated using STR Profiling 300 (Supp. Table 2).

2. Reverse transcription quantitative PCR (RT-qPCR) using PrimeTime qPCR primers was performed to assess relative expression of following genes.

Target Gene	**PrimeTime qPCR primer assay ID**
TP53	Hs.PT.58.39489752.g
DCK	Hs.PT.58.26817851
CDA	Hs.PT.58.40649952
HPRT1	Hs.PT.58v.45621572
GAPDH	Hs.PT.39a.22214836

RT-qPCR reactions were performed as follows:

Component	Amount per reaction (µL)	Final concentration
Hot start Taq 2X Master Mix	10	1x
EvaGreen 20X	1	1x
Fwd primer 10 µM	1	0.5 µM
Revprimer 10 µM	1	0.5 µM
cDNA	4	1 ng/µL
Ultrapure water	3	
Total	20	

Real-Time PCR cycling conditions were as follows:

Cycle/condition	Hold	Denature	Anneal/Extend	Melt

Denaturation/Enzyme activation	95°C, 10 min			
Cycles 1-40		98°C, 15 sec	63°C, 30 sec	55°C, 1 min
Hold	4°C			

3. **Lentiviral plasmids:** Lentiviral plasmids (LentiCRISPRv2-GFP, LentiMPHv2, LentiSAMv2, psPAX2, and pMD2.G) were purchased from Addgene as bacterial glycerol stocks. Glycerol stocks were streaked onto LB agar plates with ampicillin (100 µg/mL) and incubated for 16 hours at 37°C. Single bacterial colonies were inoculated into LB media and cultured in a shaker incubator at 37°C at 200 rpm. Overnight grown bacterial cultures were used for plasmid isolation using QIAprep Spin Miniprep Kit per manufacturer's protocol.

4. **Single guide RNAs (sgRNAs):** Non-specific (NS) and gene specific (*TP53*, *DCK*, and *CDA*) sgRNA sequences were obtained from previously published libraries as described in the table. Standard desalted sgRNA oligos were ordered from IDT (https://www.idtdna.com)

Gene editing	**sgRNA ID**	**Target sequence**	**Source**
Knockout	NS sgRNA1	5' GACAATCATGGTGAAAGCGG 3'	Human GeCKOv2 library (1)
Knockout	NS sgRNA2	5' CTGAGTGAAAAATAAAAGTT 3'	Human GeCKOv2 library (1)
Knockout	NS sgRNA3	5' TTTCCCATGATCATTTAGTG 3'	Human GeCKOv2 library (1)
Knockout	*TP53* sgRNA1	5' CCCCGGACGATATTGAACAA 3'	Human GeCKOv2 library (1)
Knockout	*TP53* sgRNA2	5' CCCCGGACGATATTGAACAA 3'	Human GeCKOv2 library (1)
Knockout	*TP53* sgRNA3	5' CCCCTTGCCGTCCCAAGCAA 3'	Human GeCKOv2 library (1)
Knockout	*DCK* sgRNA1	5' AAGGTAAAAGACCATCGTTC 3'	Human GeCKOv2 library (1)
Knockout	*DCK* sgRNA2	5' AGCTCTTATTCGACTGAGAC 3'	Human GeCKOv2 library (1)

Knockout	*DCK* sgRNA3	5’ ACTTTGAACATTGCACCATC 3’	Human GeCKOv2 library (1)
Activation	NSa sgRNA1	5' CTGAGTGAAAAATAAAAGTT 3'	Human GeCKOv2 library (1)
Activation	NSa sgRNA2	5' ATCGTTTCCGCTTAACGGCG 3'	Human GeCKOv2 library (1)
Activation	NSa sgRNA3	5' ACGGAGGCTAAGCGTCGCAA 3'	Human GeCKOv2 library (1)
Activation	*CDA* sgRNA1	5’ GACTGTCAGCCCTTGGAGCT 3’	Human SAM library (2)
Activation	*CDA* sgRNA2	5’ GGGGCAGTGTGAGATCTCAA 3’	Human SAM library (2)
Activation	*CDA* sgRNA3	5’ GGTGGTGCCACGCTACTGGA 3’	Human SAM library (2)

5. **CRISPR knockout and activation sgRNA constructs preparation:** CRISPR knockout and activation sgRNA constructs were prepared by cloning sgRNAs into lentiCRISPRv2-GFP (Addgene #82416) and lentiSAMv2 (Addgene #75112) plasmid, respectively by following a previously published cloning protocol (2).

(A) sgRNA and plasmid preparation for cloning: Individual sgRNAs were commercially synthesized as single-stranded 60 bp DNA oligonucleotides with 17-23 bp flanking sequences overlapping the ends of the BsmBI digested lentiCRISPRv2-GFP or lentiSAMv2. Oligonucleotides were PCR amplified using Q5 Hot Start High Fidelity 2X Master Mix previously published primers and protocol (2). NS control and sgRNA oligonucleotides targeting *TP53* and *DCK* genes were amplified using Oligo-Fwd and Oligo-Knockout-Rev primers. NS control and sgRNA oligonucleotides targeting the *CDA* gene were amplified using Oligo-Fwd and Oligo-Activation-Rev primers.

Oligo-Fwd	5’ GTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC 3’
Oligo-Knockout-Rev	5’ ACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC 3’
Oligo-Activation-Rev	5’ ATTTTAACTTGCTAGGCCCTGCAGACATGGGTGATCCTCATGTTGGCCTAGCTCTAAAAC 3’

PCR amplified sgRNAs were resolved on a 1.5% agarose gel and purified using QIAprep Gel Extraction Kit per manufacturer's protocol (Supplementary table). LentiCRISPRv2-GFP and

lentiSAMv2 plasmids were digested with BsmBI by following a previously published protocol (2). Digested plasmids were resolved on a 1% agarose gel, purified using QIAprep Gel Extraction Kit, and used for cloning. DNA was quantified using NanDrop spectrophotometer (Table 1).

(B) *TP53* and *DCK* sgRNA cloning: Gel purified *TP53* and *DCK* sgRNAs were cloned into lentiCRISPRv2-GFP to prepare CRISPR knockout sgRNA constructs. NS sgRNAs were cloned into lentiCRISPRv2-GFP to prepare control sgRNA constructs. Briefly, 33 ng of BsmBI digested gel purified lentiCRISPRv2-GFP and 5 ng of gel purified *TP53* or *DCK* sgRNA were mixed with 1 µL of 2X NEBuilder HiFi DNA Assembly Master Mix in a 2 µL total reaction mixture. The reaction mixture was incubated at 50°C in a thermal cycler for 1 hour and used to transform chemically competent Stbl3 cells.

(C) *CDA* sgRNA cloning: Gel purified *CDA* sgRNAs were cloned into lentiSAMv2 to prepare CRISPR activation sgRNA constructs. NSa sgRNAs were cloned into lentiSAMv2 to prepare control sgRNA constructs. Briefly, 33 ng of BsmBI digested gel purified lentiSAMv2 and 5 ng of gel purified *CDA* sgRNA were mixed with 1 µL of 2X NEBuilder HiFi DNA Assembly Master Mix in a 2 µL total reaction mixture. The reaction mixture was incubated at 50°C in a thermal cycler for 1 hour and used to transform chemically competent Stbl3 cells.

(D) Bacterial transformation: HiFi DNA Assembly reaction mixture was mixed with 50 µL One Shot Stbl3 chemically competent cells and incubated on ice. After 30 minutes, cells were transferred to a 42°C water bath for 45 seconds and then immediately incubated on ice. After 5 minutes, 200 µL SOC media was added and cells were cultured in a 37°C shaking incubator. After 1 hour, 50 µL cell culture was spread on Ampicillin (100 µg/mL) LB agar plate and incubated in a 37°C incubator for 16 hours.

(E) **CRISPR sgRNA construct isolation and sequence validation:** Single bacterial colonies were inoculated in LB media with Ampicillin (100 µg/mL) and cultured in a shaker incubator at 37°C at 200 rpm. Overnight grown bacterial cultures were used for plasmid constructs isolation using QIAprep Spin Miniprep Kit per manufacturer's protocol. Cloned sgRNA sequences in CRISPR constructs were verified by Sanger sequencing (Genewiz) using a primer targeting the U6 promoter manually. Sequences were read using Geneious Software and verified constructs were used to prepare lentiviral media.

6. CRISPR sgRNA constructs lentiviral media preparation:

A. **HEK293FT cell culture:** HEK293FT cells were cultured in HEK293FT cell media (DMEM media containing 10% non-heat inactivated fetal bovine serum (FBS), 0.1 mM MEM non-essential amino acids (MEM-NEAA), 6 mM L-glutamine, 1 mM sodium pyruvate, geneticin (500 µg/mL), and 1% Pen-Strep). Cells were cultured in T75 flasks and passaged at 80-90% confluency.

B. **HEK293FT cell transfection:** CRISPR constructs lentiviral media was prepared by transfecting individual CRISPR sgRNA constructs with packaging plasmids psPAX2 and pMD2.G in a ratio 2: 1.5: 1, respectively, into HEK293FT cells using Lipofectamine 2000 per manufacturer's protocol. Briefly, HEK293FT cells were seeded at 1 million cells per

well in a 6-well plate in 2mL Pen-Strep free media. On the next day, a transfection reaction mixture containing lentiCRISPRv2 sgRNA construct (1.22 µg) or lentiMPHv2 (1.22 µg), packaging plasmids (0.93 µg psPAX2 & 0.61 µg pMD2.G), Plus Reagent, and Lipofectamine 2000 in Opti-MEM media was added to HEK293FT cells (360 µL). Cells were incubated in a cell culture incubator at 37°C (5% CO2). After 4-6 hours, transfection media was replaced with fresh HEK293FT cell media. Cell media was harvested 48 and 72 hours post transfection, pooled, and filtered through a 0.45 µM sterile filter. Media was either directly used as lentiviral media for transduction or snap frozen in Eppendorf tubes in liquid nitrogen and stored at -80°C for future use.

7. CRISPR knockout sgRNA construct transduction and selection:

A. CRISPR knockout construct transduction into MOLM-13 cells was performed by spinfection. Briefly, lentiviral media of CRISPR knockout sgRNA construct (200 µL) was mixed with 0.5 x 10^6 MOLM-13 cells (800 µL of growth media); total of 1 mL) cell suspension containing polybrene (8 µg/mL) in 12-well plate. The cell suspension in the plate was centrifuged at 32°C and 900 x g for 90 minutes and incubated in a CO_2 incubator at 37°C overnight. The following day lentiviral media was replaced with growth media and cultured in a CO_2 incubator at 37°C. After 3 days, cells were processed for selection of stably transduced cells.

B. *TP53* and *DCK* knockout sgRNA construct transduced MOLM-13 cells were selected by GFP+ cell sorting. Polyclonal sorted cells were cultured and used in downstream gene knockdown and AML phenotype analyses.

8. CRISPR activation sgRNA construct transduction and selection:

A. We followed a two-step staged transduction protocol. CRISPR activation sgRNA constructs (*CDA* and NSa) were transduced into lentiMPHv2 stable MOLM-13 cells expressing transcriptional effectors MS2, p65, and HSF1 by following the above spinfection protocol. To generate lentiMPHv2 stable MOLM-13 cell line, MOLM-13 cells were transduced with lentiMPHv2 lentiviral media (Step 5B) and following the above spinfection protocol (step 7) to generate lentiMPHv2 stable cells. Three days after spinfection, cells were treated with 250 µg/mL hygromycin for selection of lentiMPHv2 stable cells. After 14 days, selected live cells were single cell sorted and cultured for generation of clonal lentiMPHv2 stable MOLM-13 cell line. Sorted cells were cultured in 100 µL growth media. After 7 days, fresh 100 µL growth media was added and cells cultured for additional 7 days. After 14 days, monoclonal cell cultures were transferred to 1 mL growth media and used in downstream analyses. Establishment of lentiMPHv2 stable cell line was confirmed by analyzing MS2 RNA expression by RT-qPCR using MS2-Fwd (5' ATT TCG CTA ATG GGG TGG CA 3') and MS2-Rev (5' ACG CTG CAT GTC ACC TTG TA 3') primers (step 2). MS2 expression relative to housekeeping gene HPRT was calculated by $2^{-\Delta\Delta CT}$ method (3).

B. Activation constructs transduced lentiMPHv2 stable MOLM-13 cells were treated with 10 µg/mL Blasticidin for selection of activation sgRNA expressing stable cells. After 14 days, selected cells were used in downstream gene upregulation and AML phenotype analyses.

9. **Gene knockdown and activation analyses:** Gene knockdown (*TP53* & *DCK*) and activation (*CDA*) were confirmed by Western Blot (WB) analyses. Briefly, GFP+ sorted or Blasticidin selected MOLM-13 cells (1 million) were lysed in RIPA buffer (100 µL) containing protease inhibitor cocktail. Lysed cells were centrifuged at 4°C at 14000 rpm for 10 minutes. Supernatant was transferred to an Eppendorf tube and used as cell lysate. Equal amounts of CRISPR knockout or activation and control sgRNA construct transduced cell lysates (30 µg) were resolved in 4% stacking and 15% resolving Tris-glycine polyacrylamide gel (50V for 30 minutes and 100V until dye reaches the bottom of gel at room temperature) and transferred to a 0.2 µM PVDF membrane (1X Tris/Glycine buffer with 200 ml methanol/Liter). PVDF membrane was blocked with 5% nonfat dry milk in PBS for 1 hour, incubated overnight at 4°C with optimized concentration of primary antibodies. On the next day, membranes were washed and incubated with a secondary antibody in 5% non-fat dry milk in PBS and developed with enhanced chemiluminescence substrate. CDA upregulation was also confirmed by RT-qPCR analysis as described in step 2.

10. Cell proliferation analyses:

(A) Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay: MOLM-13 cells transduced with control or *TP53* sgRNAs were assessed for proliferation using Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay per manufacturer's protocol. Briefly, equal numbers of control and p53 depleted MOLM-13 cells (0.5 million) were plated in 1 mL media containing 10 µM EdU and incubated in a CO2 incubator at 37°C. After 3 hours, cells were harvested and washed with 3 mL 1% BSA in PBS. Cells were fixed in 100 µL of Click-iT fixative for 15 minutes protected from light at room temperature. Fixed cells were washed with 3 mL 1% BSA in PBS and incubated with 100 µL of 1X Click-iT saponin-based permeabilization solution at room temperature. After 15 minutes, 500 µL Click-iT Plus reaction cocktail containing Alexa Fluor 647 picolyl azide was mixed with cells and incubated at room temperature protected from light. After 30 minutes, cells were washed with 3 mL 1X Click-iT saponin-based permeabilization solution. Washed cells were resuspended in 300 µL of 1X Click-iT saponin-based permeabilization solution and analyzed for EdU incorporation. Attune NxT Flow Cytometer was used for data collection and analysis was performed using FCS Express 7 software. Control cells (EdU untreated) were used to gate EdU positive cells. Results from experimental and control groups were compared using student's t-test in Microsoft Excel.

(B) CellTiter Non-radioactive Proliferation Assay: MOLM-13 cells transduced with experimental or control sgRNAs were assessed for proliferation using CellTiter Non-radioactive Proliferation Assay per manufacturer's protocol. Briefly, equal numbers of viable control and experimental MOLM-13 cells (3000) were plated in 100 µL growth media into two separate 96 well plates - one plate for baseline and the other plate for endpoint reads. Baseline plate was read on the same day of cell plating. Endpoint plate cells were cultured in a CO2 incubator at 37°C and read on day 5. Both baseline and endpoint plates were read colorimetrically at 570/670 nm using BioTek Plate Reader. Baseline reads were subtracted from the endpoint reads to calculate net absorbance and changes in cell proliferation. Results from experimental and control groups were compared using student's t-test in Microsoft Excel.

11. **Ara-C resistance analyses:** MOLM-13 cells transduced with *DCK*, *CDA* or control sgRNAs were assessed for Ara-C resistance. First, an Ara-C kill curve was generated by treating parent MOLM-13 cells ($0.5x10^6$/mL) with a range of Ara-C (0-0.25 μM) every day for 5 days. The curve was used to determine Ara-C half maximal inhibitory concentration ($IC_{50,}$ 0.04 μM) which reduced MOLM-13 cell viability by 50%. To measure Ara-C resistance, equal numbers of MOLM-13 cells (50000) transduced with relevant control sgRNAs, *DCK* or *CDA* sgRNAs were plated in 100 μL media in 96 well plates and treated with 0.04 μM Ara-C every day. After 5 days, cell survival was measured by CellTiter Non-radioactive Proliferation Assay as described in Step 10B.

12. **Flow cytometric cell counting assay:** Proliferation of *TP53* and survival of *DCK* and *CDA* sgRNA constructs transduced MOLM-13 cells were also assessed by flow cytometric cell counting. Briefly, equal numbers of viable control and *TP53* sgRNA constructs transduced MOLM-13 cells (30000) were plated in 1 mL growth media in a 12 well plate and cultured in CO_2 incubator at 37°C. After 5 days, live cells were counted in equal volume (20 μL) of cell suspension using Attune NxT flow cytometer. DAPI was added to the cell suspension at a final concentration of 5 μg/mL to gate out dead cells. Similarly, equal numbers of viable control and *DCK* or *CDA* sgRNA constructs transduced MOLM-13 cells (500000) were plated in 1 mL growth media in 12 well plate, cultured in CO_2 incubator at 37°C, and treated with 0.04 μM Ara-C every day. After 5 days, live cells were counted in equal volume (20 μL) of cell suspension. DAPI was added to the cells suspension at a final concentration of 5 μg/mL to gate out dead cells.

References

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3. Livak KJ and Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25(4):402-08