Supplementary Information

Cell-type-specific genome editing with a microRNA-responsive CRISPR-Cas9 switch

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This file includes:

Supplementary Text

Engineering miR-Cas9 switch and sgRNA that target EGFP

We noticed that Cas9 activity from control *Cas9* mRNA was originally lower in hiPSCs than HeLa cells, although we observed a difference in Cas9 activity using control *Cas9* mRNA and miR-302-Cas9 switches in hiPSCs (Supplementary Figure S4A). Our initial $sgRNA_{GFP}$ contained an additional GG sequence at the 5' region to facilitate *in vitro* transcription by T7 RNA polymerase. A previous report showed that the extra nucleotides at the 5' end could affect mutation frequencies at on-target and off-target sites (1). To check the effect of the GG sequences in $sgRNA_{GFP}$, we removed them from $sgRNA_{GFP}$ ($sgRNA2_{GFP}$) and found that $sgRNA2_{GFP}$ with *Cas9* mRNA showed higher Cas9 activity compared with the original $sgRNA_{GFP}$ (Supplementary Figure S4A *vs* Supplementary Figure S4B). However, we also observed leaky expression of Cas9 in cells transfected with miR-302-Cas9 switch, $sgRNA2_{GFP}$, and negative miRNA inhibitor (Supplementary Figure S4B).

We therefore aimed to improve the sensitivity of miR-Cas9 switches to avoid leaky Cas9 expression in the target cells (Supplementary Figure S4B). We inserted four copies of the anti-miR sequence in the 5'-UTR region of the switch (4x miR-302-Cas9 switch) and found that this switch improved sensitivity to the target miR-302 and reduced leaky Cas9 expression in iPSCs (Figure 3A).

We also tuned the amount of miR-Cas9 switch and sgRNA. Consequently, we optimized conditions and improved Cas9 activity with lower leaky expression in the presence of miR-302 in hiPSCs (Supplementary Figure S4C).

Supplementary Methods

Evaluation of miRNA expression level

Relative miRNA expression level was quantified using TaqMan® MicroRNA Cells-to-C_T™ Kit (Ambion). Cell lysate containing total RNA was reverse transcribed using has-miR-21-5p (Assay ID: 000397), 302a-5p (Assay ID: 002381) and RNU6B (Assay ID: 001093) TaqMan probes (Applied Biosystems). Quantitative PCR (qPCR) was performed with the TaqMan probes, and samples were analyzed using a StepOne Plus Real-Time PCR System (Applied Biosystems). Target miRNA was normalized to the the small RNA RNU6B and analyzed by $\Delta\Delta$Ct method.

Evaluation of Cas9 protein expression level

After PBS wash, cells were lysed by adding 50 μL M-PER cocktail (contains M-PER® Mammalian Protein Extraction Reagent (ThermoFisher), protease inhibitor, PMSF). After shaking for 5 min, lysate was collected into a 1.5 mL tube and centrifuged at 12400 rpm at 4˚C for 10 min. The supernatant was collected and used in a BCA assay using Pierce™ BCA Protein Assay Kit (ThermoFisher) following the manufacturer's protocol. The protein in the cell lysates were diluted to 0.5 mg/mL and analyzed using automated capillary electrophoresis-based immunoblotting with the *Wes* (ProteinSimple) following the manufacturer's protocol. Cas9 antibody (Active Motif) and GAPDH antibody (Santa Cruz) were

diluted 1 : 50 and 1 : 100, respectively. To detect primary antibody, anti-mouse and anti-rabbit antibodies (ProteinSimple) were used.

Cell killing assay by targeting Alu1 element

We collected all cell samples at 48 h after RNA transfection. After washing with PBS, normal HeLa cells were treated with 100 μL 0.25% trypsin-EDTA, incubated at 37˚C with 5% CO_2 for 5 min, and then 100 μL cultured medium was added. After the cell suspension was collected, it was centrifuged at 1000 rpm at room temperature for 5 min. The supernatant was discarded and the remaining cell pellet was washed with PBS and centrifuged (1000 rpm) at room temperature for 5 min. After aspirating supernatant, cell samples were stained with 2.5 μL annexin V, Alexa Fluor 488 conjugate (Life Technologies) and 0.5 μL SYTOX Red dead-cell stain in 50 μL Annexin-binding buffer 5x (Life Technologies). Samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences).

T7E1 assay

For the T7E1 assay, 100 ng *Cas9* mRNA and 300 ng sgRNA were used. Genomic DNA was extracted before PCR. The target site was amplified by nested PCR using the appropriate primers (Supplementary Table S2). The first reaction condition was 94˚C for 2 min, 20 cycles of 98˚C for 10 sec, 60˚C for 30 sec, and 68˚C for 30 sec, and 72˚C for 3 min and finally 4˚C for ∞. The second reaction condition was 94˚C for 2 min, 35 cycles of 98˚C for 10 sec, 60˚C for 30 sec, and

68˚C for 15 sec, and 72˚C for 3 min and 4˚C for ∞. The PCR amplicon was purified by MinElute PCR purification Kit (QIAGEN). Then, denaturing and re-annealing reactions were conducted at 95˚C for 5 min, 95˚C to 85˚C at -2.0˚C/sec, 85˚C to 25˚C at -0.1˚C/sec and finally 4˚C for ∞). This fragment was then digested by T7 Endonuclease I. The resulting products were analyzed by electrophoresis in a 5% polyacrylamide gel and were visualized by staining with SYBR Green mixture (I+II =1:1).

Insertions and deletions (Indels) were determined by the formula, ***100 × (1 – sqrt(1 – (b + c)/(a + b + c)))***, where ***a*** is the integrated intensity of the undigested PCR product, and ***b*** and ***c*** are the integrated intensities of each cleavage product.

Sequencing

For phosphorylation of PCR amplicon (T7E1 second PCR product), primers (T7E1_EGFP Fwd_ORF and T7E1_EGFP Rev_Univ., sequences are shown in Supplementary Table S2) were phosphorylated by T4PNK (TAKARA) and ATP (Ambion). Amplicon was phosphorylated by PCR reaction using the phosphorylated primers and cloned in pUC19 vector at the SmaI site. The purified plasmids were sequenced by using M13 FwdNew primer, T7E1 Fwd primer or T7E1 Rev primer as the sequence primer. Sequencing was conducted by using Applied Biosystems 3500xL Genetic analyzer (Applied Biosystems).

Evaluation of gene expression level

To collect total RNA, cells were treated with Trizol (ThermoFisher) following the manufacturer's protocol. To eliminate genomic DNA, samples were treated with TURBO DNase Inactivation Kit (Ambion) following the manufacturer's protocol. To measure the mRNA level, 250 or 300 ng of total RNA were reverse-transcribed by ReverTra Acr®qPCR RT Master Mix (TOYOBO) following the manufacturer's protocol. Quantitative PCR (qPCR) was performed using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) following the manufacturer's protocol, and the samples were analyzed using a StepOne Plus Real-Time PCR System (Applied Biosystems). Primers for qPCR are listed in Supplementary Table S3. Target mRNA was normalized to GAPDH and analyzed by the ΔΔCt method.

Cy5-labelling of sgRNA

Cy5 labelled sgRNA were prepared by ligation using T4 RNA ligase (Ambion) and pCp-Cy5 (Jane Bioscience). Cy5 labelling was performed with 200 pmol purified sgRNA using 40 U T4 RNA ligase, 4 nmol pCp-Cy5 and 10% DMSO in 40 μL at 16˚C for 48 h. Cy5-labelled sgRNA was purified using an RNeasy MinElute Cleanup Kit (QIAGEN).

Evaluation of transfection efficiency

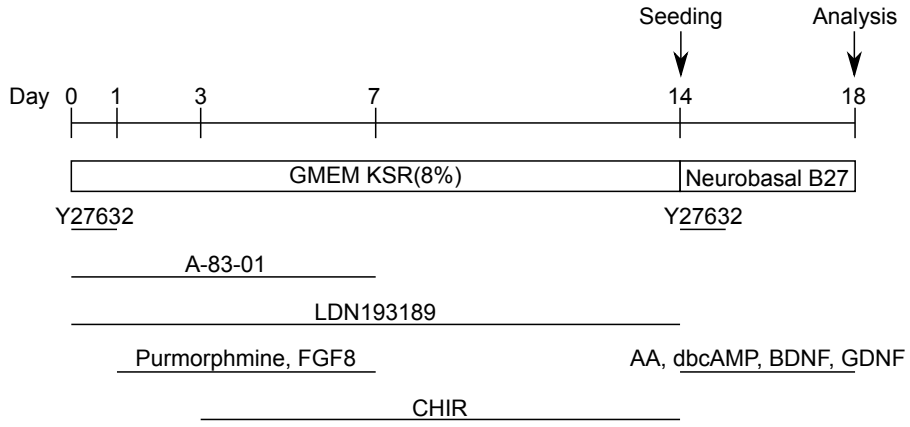
To measure the relative transfection efficiency, 100 ng *BFP* mRNA and 300 ng Cy5-labeled sgRNA were transfected into HeLa-EGFP cells or iPS-EGFP cells. At 24 h after RNA transfection, cells were washed with PBS three times. These

cells were harvested as described in Cas9 activity assay and analyzed on a LSR flow cytometer.

Neuron differentiation assay

Differentiated mDA-EGFP cells were derived from iPS-EGFP cells. Briefly, differentiation was started from a single cell suspension of iPS-EGFP cells in day 0 differentiation medium with ROCK inhibitor and seeded at $5x10^6$ cells/well in laminin-coated 6-well plates. The differentiating cells were cultured at 37˚C with 5% CO_2. At least 24 h later, the medium was changed to day 1 medium, and the medium was changed daily. For the assays, cells were passaged at day 14 in 24-well plates at a density of $2.0x10^5$ cells/well.

Schematic of mDA neuron differentiation assay



8GMK

Glasgow's MEM (Thermo FisherScientific)
1X Non-essential amino acids (Thermo FisherScientific)
8% knockout-serum (Thermo FisherScientific)
100 μM sodium pyruvate (Thermo FisherScientific)
100 μM β-mercaptoethanol (Wako)

Day 0

8GMK
10 μM Y26732 (Wako)
100 nM LDN-193189 (Stemgent)
500 nM A-83-01 (Wako)

Day 1-2

8GMK
100 nM LDN-193189
500 nM A-83-01
2 μM Purmorphamine (Calbiochen)
100 ng/mL Human reconbinant FGF-8 (Wako)

Day 3-6

8GMK
100 nM LDN-193189
500 nM A-83-01
2 μM Purmorphamine
100 ng/mL Human reconbinant FGF-8
30 μM CHIR99021 (Stemgent)

Day 7-13

8GMK
100 nM LDN-193189
30 μM CHIR99021

Neurobasal B27

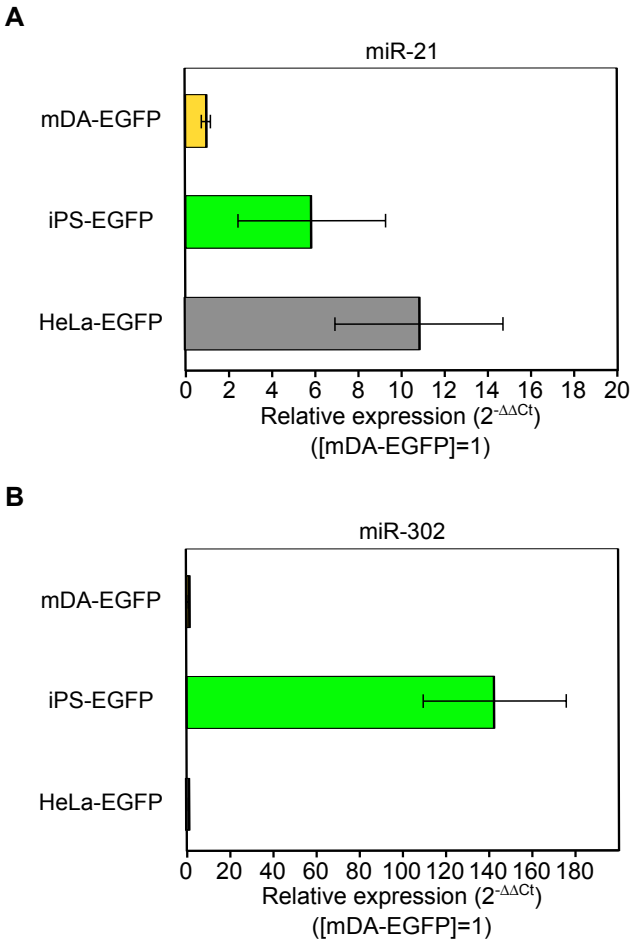
Neurobasal medium (Thermo FisherScientific)
2% B27 supplement (Thermo FisherScientific)
2 mM L-glutamine

Day 14

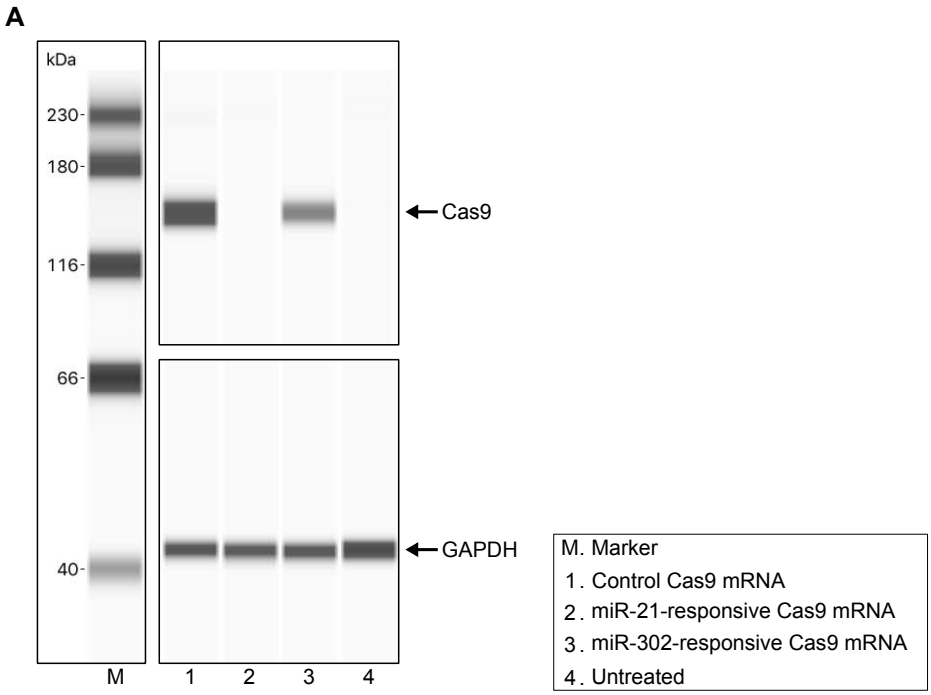
Neurobasal B27
10 μM Y26732
200 μM Abscorbic acid (Sigma Aldrich)
400 μM dbcAMP (Sigma Aldrich)
20 ng/mL Human reconbinant BDNF (R&D)
10 ng/mL Human reconbinant GDNF (R&D)

Day 15-

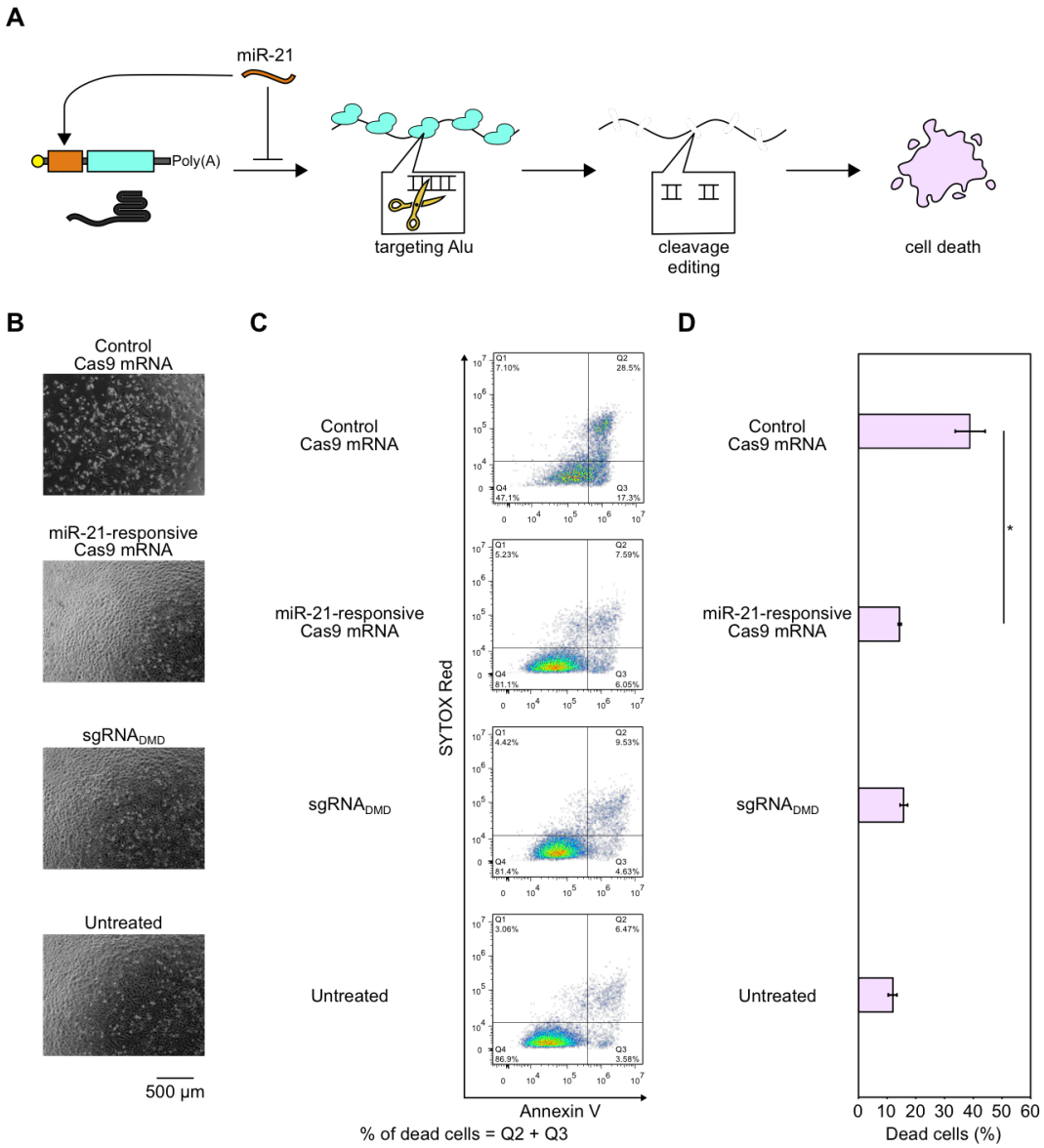
Neurobasal B27
200 μM Abscorbic acid (Sigma Aldrich)
400 μM dbcAMP (Sigma Aldrich)
20 ng/mL Human reconbinant BDNF (R&D)
10 ng/mL Human reconbinant GDNF (R&D)



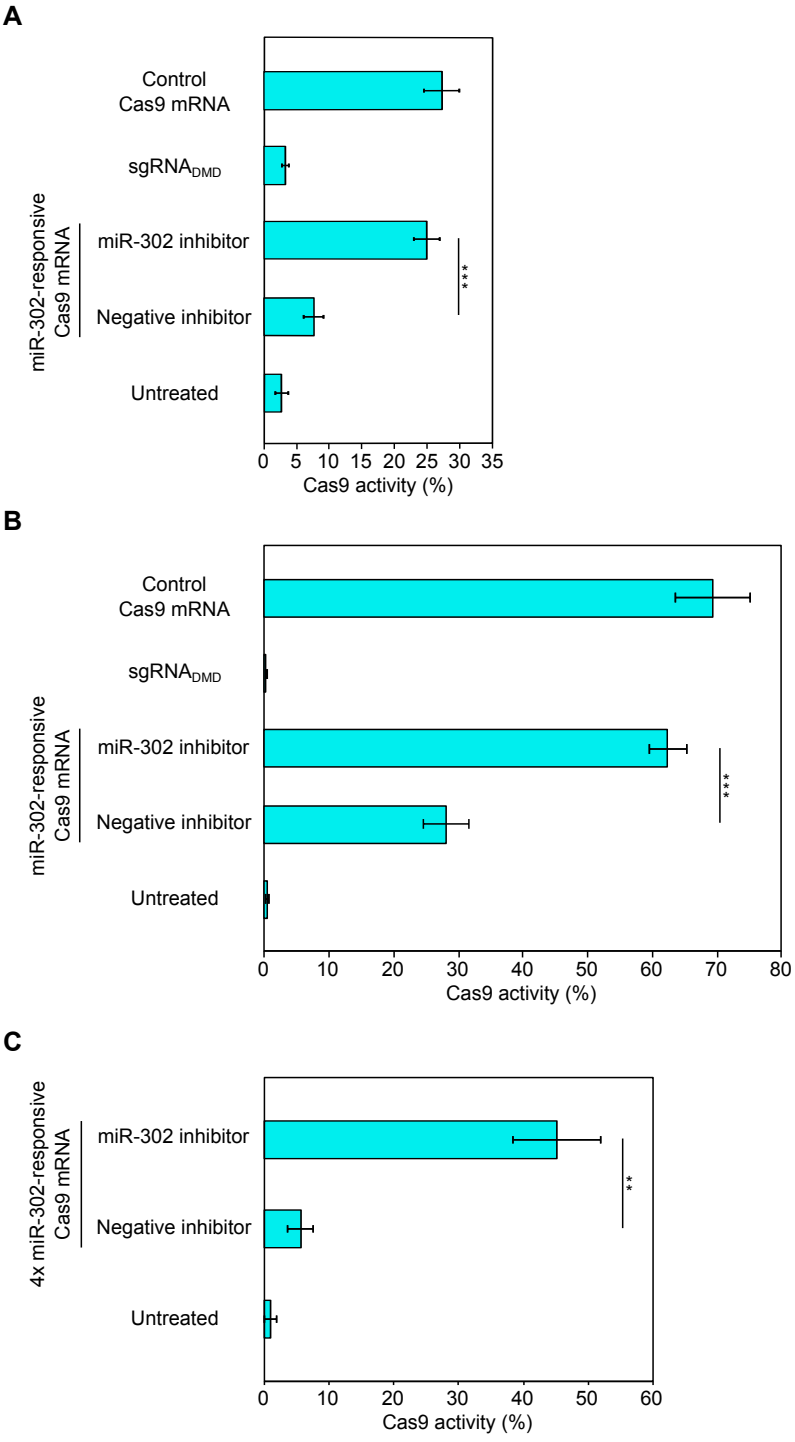
Supplementary Figure S1. Analysis of endogenous miRNA expression by qPCR. (A) Representative bar graph of miR-21 expression in HeLa cells, iPS cells and mDA neurons (Day 14). (**B**) Representative bar graph of miR-302 expression levels in HeLa cells, iPS cells and mDA neurons (Day 14). Each miRNA expression was normalized with RNU6B expression ($2^{-\Delta\Delta Ct}$) and then each normalized miRNA expression ($2^{-\Delta\Delta Ct}$) was normalized again with mDA neurons (*i.e.,* $2^{-\Delta\Delta Ct}{}_{\_mDA\ neurons}$ =1). Error bars indicate the mean ± SD (n = 3).



Supplementary Figure S2. Cas9 protein expression levels in HeLa cells. Representative virtual blot of Simple Western analysis at 24 h after RNA transfection. Cas9 protein expression levels were detected by virtual blot-like images of the capillary electrophoresis. GAPDH was used as a loading control. Lanes: M, molecular weight marker; 1, control *Cas9* mRNA; 2, miR-21-responsive *Cas9* mRNA; 3, miR-302-responsive *Cas9* mRNA; 4, untreated cells. The experiment was repeated three times. A representative image is shown.

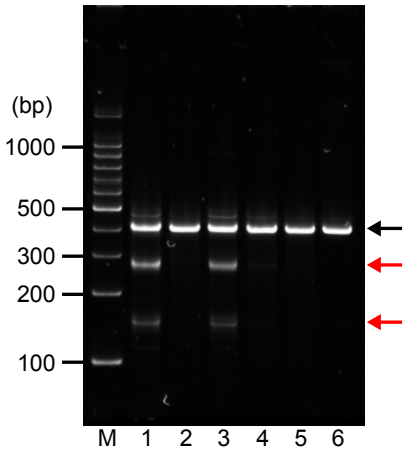


Supplementary Figure S3. miR-Cas9 switch targeting Alu. (A) Schematic illustration of the system targeting Alu. The Cas9-sgRNA complex targeting a common Alu sequence cleaves genomic DNA at multiple locations and induces cell death. **(B)** Representative microscopic images at 48 h after RNA transfection. The scale bar represents 500 μm. **(C)** Representative dot plots of the cells at 48 h after RNA transfection. Positive populations of Annexin V (x-axis) and SYTOX Red (y-axis) indicate dead cells. Representative images are shown from three independent experiments. **(D)** Dead cells ware calculated by counting Q2 + Q3 cells shown in **(C)**. Negative control targets the *DMD* gene. Error bars indicate the mean ± SD (n = 3).


A
Control
Cas9 mRNA
sgRNA$_{DMD}$
miR-302 inhibitor
Negative inhibitor
Untreated
miR-302-responsive
Cas9 mRNA
***
0 5 10 15 20 25 30 35
Cas9 activity (%)
B
Control
Cas9 mRNA
sgRNA$_{DMD}$
miR-302 inhibitor
Negative inhibitor
Untreated
miR-302-responsive
Cas9 mRNA
***
0 10 20 30 40 50 60 70 80
Cas9 activity (%)
C
miR-302 inhibitor
Negative inhibitor
Untreated
4x miR-302-responsive
Cas9 mRNA
**
0 10 20 30 40 50 60
Cas9 activity (%)

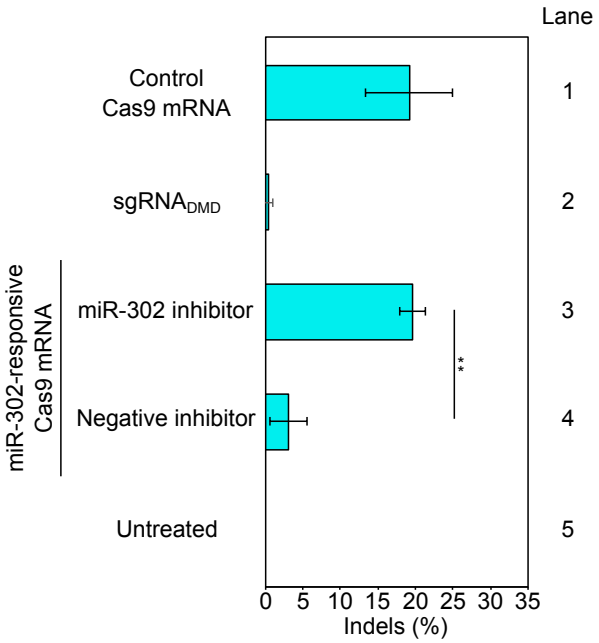
Supplementary Figure S4. Improvement of Cas9 activity in hiPSCs by engineering sgRNA and miR-302-Cas9 switch. (**A**) First evaluation of miR-302-responsive *Cas9* mRNA in hiPSCs. A miR-302 inhibitor rescued Cas9 activity expressed from miR-302-responsive *Cas9* mRNA, whereas a negative miRNA inhibitor did not. However, the Cas9 activity was low (25 - 30%, Control *Cas9* mRNA). **(B)** Improvement of Cas9 activity by changing sgRNA (removal of GG from 5' terminal, $sgRNA2_{GFP}$) in hiPS-EGFP cells. In comparison with data using the original sgRNA ($sgRNA_{GFP}$) shown in **(A)**, Cas9 activity increased about 40-50%, though leaky expression of Cas9 was observed (Negative inhibitor). **(C)** Optimization of 4x miR-302-responsive *Cas9* mRNA and $sgRNA2_{GFP}$. Final condition was 50 ng 4x miR-302-responsive *Cas9* mRNA and 300 ng $sgRNA2_{GFP}$. High Cas9 activity (45%, miR-302 inhibitor) and reduced leaky expression (8%, Negative inhibitor) were observed. Error bars indicate the mean ± SD (n = 3).

A



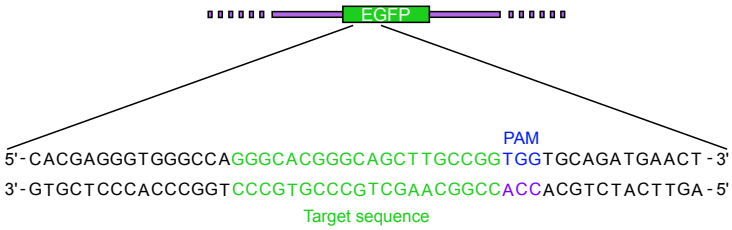
lane no.	1	2	3	4	5	6
Cas9 mRNA	control	control	miR-302	miR-302	-	-
sgRNA	EGFP	DMD	EGFP	EGFP	-	-
miR-302 inhibitor	-	-	+	-	-	-
Negative inhibitor	-	-	-	+	-	-
T7E1 treatment	+	+	+	+	+	-

B



Supplementary Figure S5. T7E1 assay for hiPSCs with miR-302-Cas9 switch. **(A)** Representative gel image of T7E1 assay. Top band (black arrow, 436 bp) indicates the target amplicon. The lower two bands (red arrows, 286 bp and 150 bp) indicate digested fragments by T7 endonuclease I. Lanes: M, molecular weight marker (100-bp ladder); 1, control *Cas9* mRNA; 2, negative control (targeting DMD); 3, miR-302-responsive *Cas9* mRNA + miR-302 inhibitor; 4, miR-302-responsive *Cas9* mRNA + negative miRNA inhibitor; 5, untreated cells with T7 endonuclease I; and 6, untreated cells without T7 endonuclease I. The samples are summarized in the table to the right. **(B)** Quantification of Cas9 activity (indels) from **(A).** No digestion products were detected in the untreated sample. Error bars indicate the mean ± SD (n = 3).

A



B

Control Cas9 mRNA

5'-AGTTCATCTGCACCACCG GCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	WT	15/18
5'-AGTTCATC--C------- GCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	Δ9	1/18
5'-AGTTCATCTGCACCACCGTGCAGATGACCTGAAGTTCATCTGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	+23	1/18
5'-AGTTCATCTGCACCACCGTGCCCTGGCCCACCCTC GCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	+17	1/18

miR-302-responsive Cas9 mRNA + miR-302 inhibitor

5'-AGTTCATCTGCACCACCGG CAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	WT	16/18
5'-AGTTCATCTGCACCACCGGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	+1	1/18
5'-AGTTCATCTGCACCACC-G CAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	Δ1	1/18

miR-302-responsive Cas9 mRNA + Negative inhibitor

5'-AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	WT	19/19

$sgRNA_{DMD}$

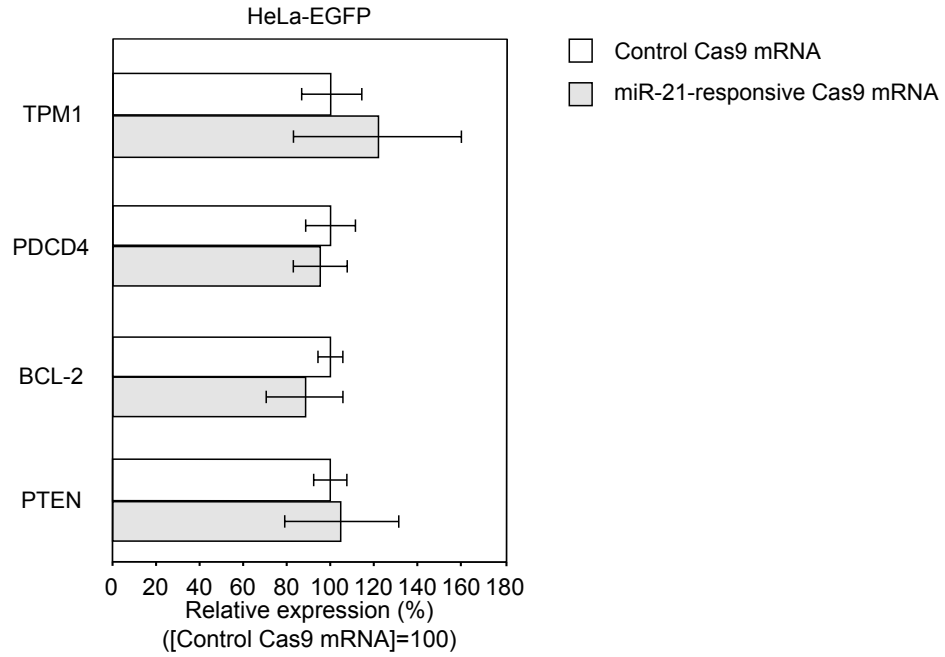
5'-AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	WT	10/10

Untreated

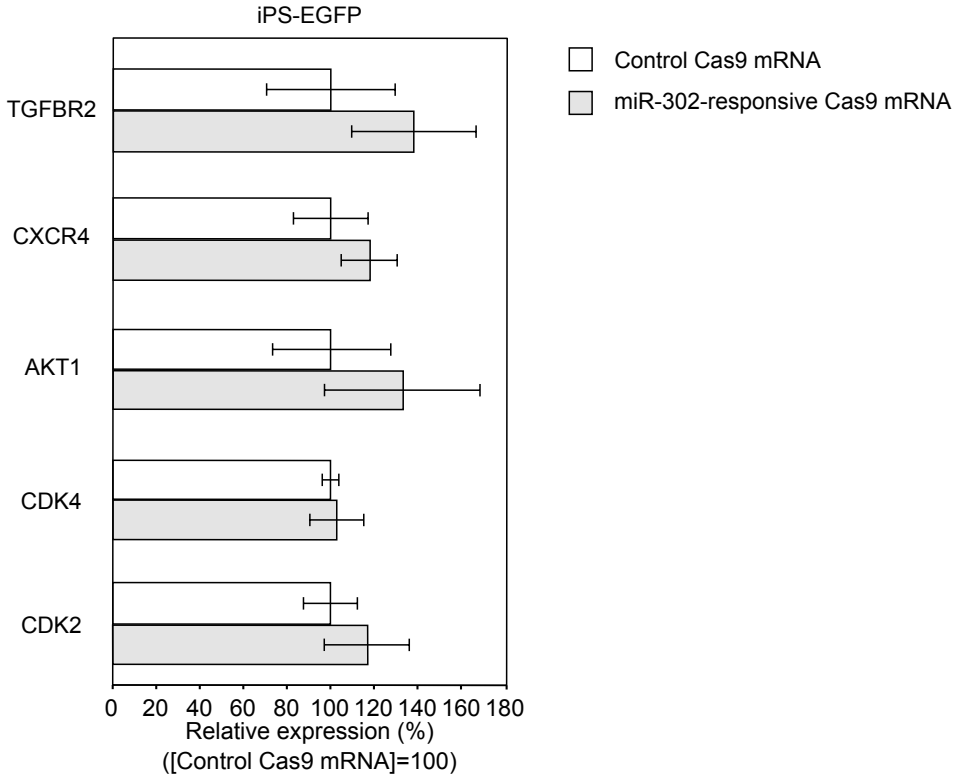
5'-AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG-3'	WT	9/9

Supplementary Figure S6. Confirmation of DNA sequencing in T7E1 assays. (**A**) Illustration of the target sequence. Target sequence is green letters. Protospacer adjacent motif (PAM) sequence and complimentary PAM sequence are written as blue and purple letters, respectively. (**B**) Observed sequences in Supplementary Figure S5. Red letters and dashes indicate the identified mutations. The sgRNA targeting sequence and complimentary PAM sequence are written as green and purple letters, respectively. The size of the insertions and deletions are shown to the right of each mutated sequence (+, insertions; Δ, deletions). Numbers on the right indicate the numbers of mutated clones and all analyzed clones. Each sequence was read by forward and reverse primers.

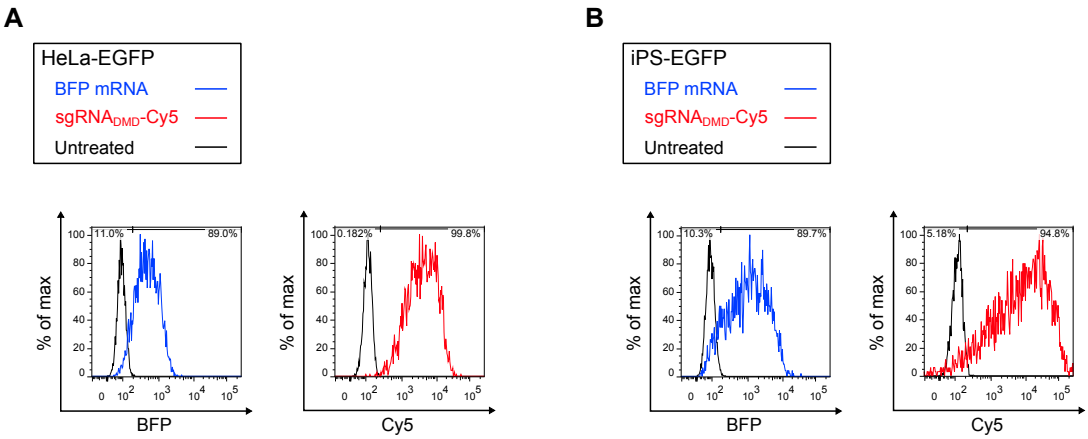
A


HeLa-EGFP
TPM1
PDCD4
BCL-2
PTEN
0 20 40 60 80 100 120 140 160 180
Relative expression (%)
([Control Cas9 mRNA]=100)
Control Cas9 mRNA
miR-21-responsive Cas9 mRNA

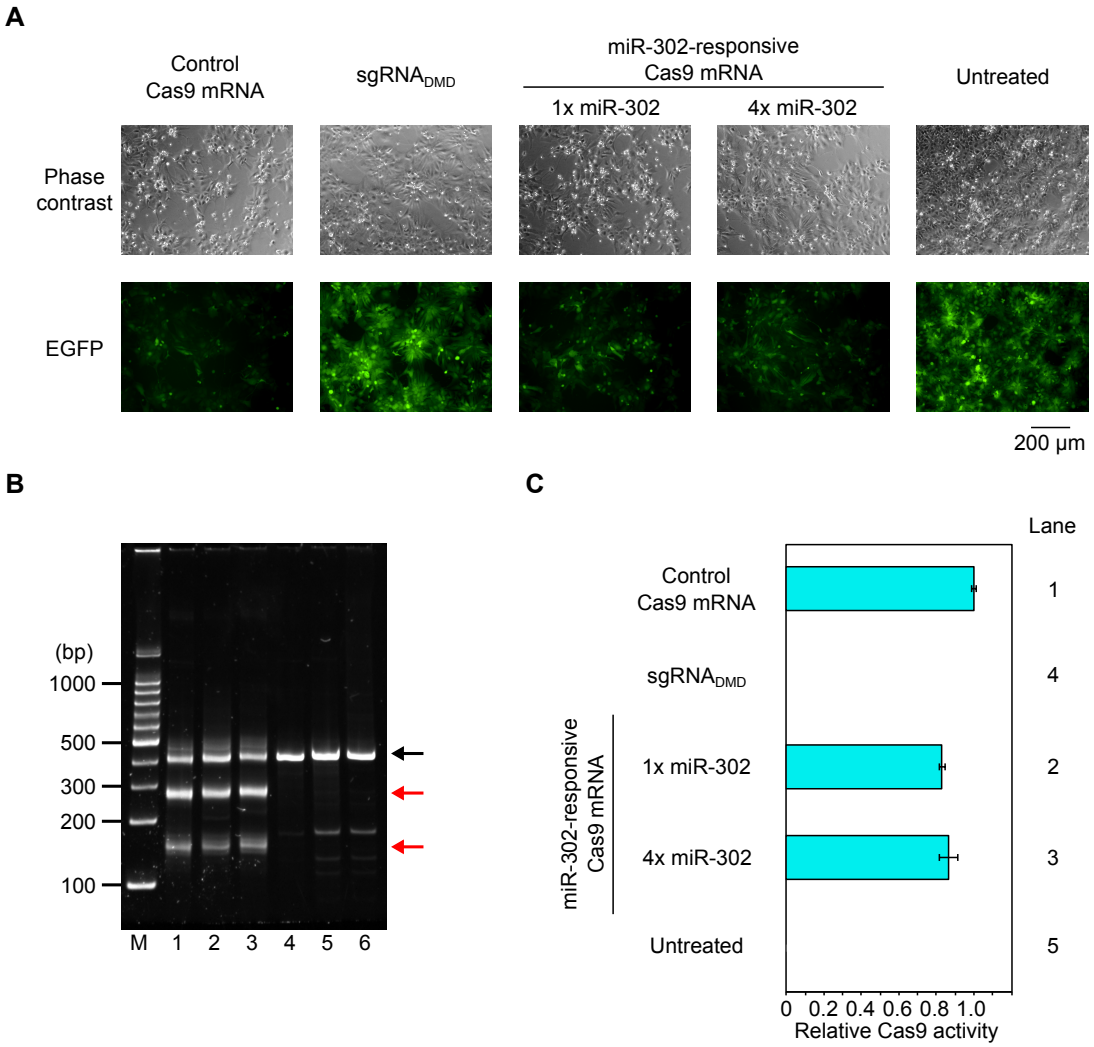
B


iPS-EGFP
TGFBR2
CXCR4
AKT1
CDK4
CDK2
0 20 40 60 80 100 120 140 160 180
Relative expression (%)
([Control Cas9 mRNA]=100)
Control Cas9 mRNA
miR-302-responsive Cas9 mRNA

Supplementary Figure S7. Effect of miRNA-responsive *Cas9* mRNA on endogenous gene expression in HeLa and iPS cells. (**A**) The effect of miR-21-responsive *Cas9* mRNA in HeLa cells was evaluated by qPCR. (**B**) The effect of miR-302-responsive *Cas9* mRNA in iPS cells was evaluated by qPCR. Target mRNA levels were normalized with *GAPDH* mRNA (target gene expression) and then each target gene expression was normalized with each control *Cas9* mRNAs. Error bars indicate the mean ± SD (n = 6).



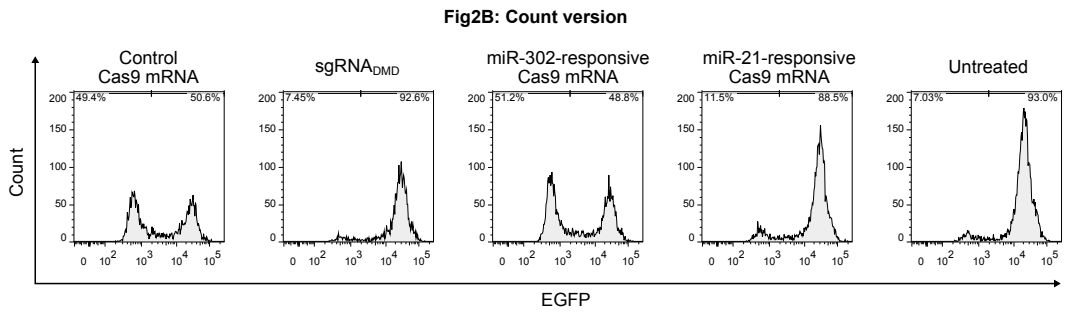
Supplementary Figure S8. Relative transfection efficiencies in HeLa and iPS cells. (**A**) Relative transfection efficiencies in HeLa cells. Representative flow cytometry histograms of *BFP* mRNA (blue) and Cy5-labeled sgRNA (red). (**B**) Relative transfection efficiencies in iPS cells. Representative flow cytometry histograms of *BFP* mRNA (blue) and Cy5-labeled sgRNA (red). x- and y-axes indicate BFP or Cy5 intensity and % of max, respectively. These experiments were repeated three times.



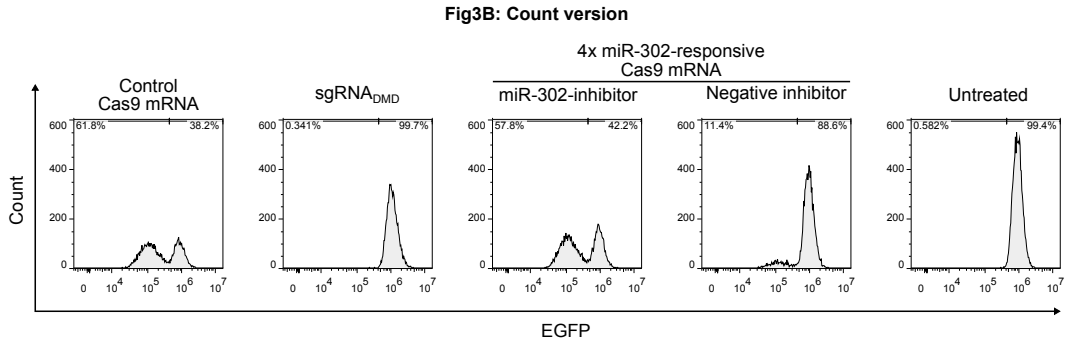
lane no.	1	2	3	4	5	6
Cas9 mRNA	control	1x miR-302	4x miR-302	control	-	-
sgRNA	EGFP	EGFP	EGFP	DMD	-	-
T7E1 treatment	+	+	+	+	+	-

Supplementary Figure S9. miR-302-Cas9 switch in mDA neuron. (A) Representative fluorescent microscopic images at 72 h after RNA transfection. Phase contrast and EGFP indicate phase contrast and EGFP fluorescent images, respectively. Scale bar represents 200 μm. **(B)** Representative gel image from a T7E1 assay using mDA-EGFP cells derived from hiPS-EGFP cells. Top band (black arrow, 436 bp) indicates the target amplicon. The below two bands (red arrows, 286 bp and 150 bp) indicate digested fragments by T7 endonuclease I. Lanes: M, molecular weight marker (100-bp ladder); 1, control *Cas9* mRNA; 2, miR-302-responsive *Cas9* mRNA; 3, 4x miR-302-responsive *Cas9* mRNA; 4, negative control (targeting DMD); 5, untreated cells with T7 endonuclease I; and 6, untreated cells without T7 endonuclease I. The samples are summarized in the table below. **(C)** Quantification of relative Cas9 activity from **(B)**. Each Cas9 activity was normalized by control *Cas9* mRNA (lane no.1). Error bars indicate the mean ± SD (n = 3).

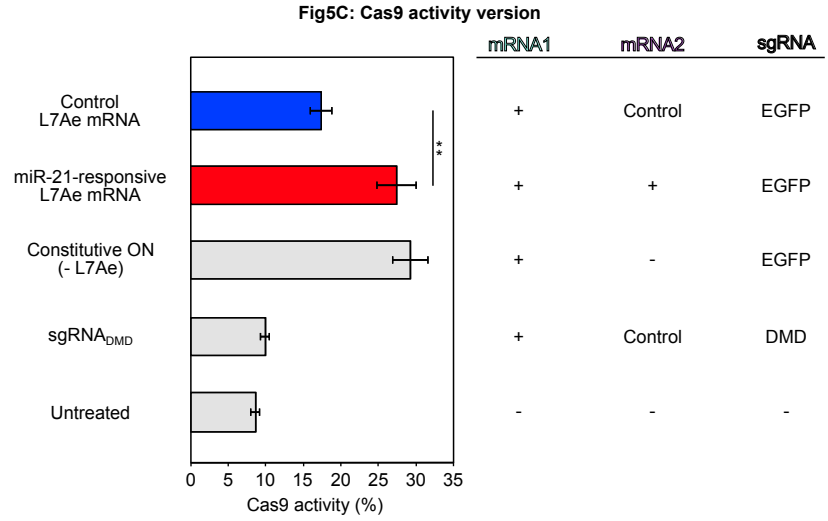
A



B



C



Supplementary Figure S10. Unnormalized raw data. (A) and **(B)** Cell counts from Figure 2B and 3B. x- and y-axes indicate EGFP intensity and cell number, respectively. (**C**) Quantification of Cas9 activity from Figure 5B.

Supplementary Table S1: Primers and oligo DNAs for RNAs

1. miRNA target sites and RNA motifs are written as orange and red letters, respectively.
2. sgRNA target sequences are written as green letters.
3. All primers were designed manually.

Primer/Oligo DNA name	Sequence (5' -> 3')
TAP_T7_G3C fwd primer	CAGTGAATTGTAATACGACTCACTATAGGGC
IVT_5prime_UTR primer	CAGTGAATTGTAATACGACTCACTATAGGGCGAATTAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGACACCGGTCGCCACCATG
Rev5UTR primer	CATGGTGGCGACCGGTGTCTTATATTTCTTCTTACTC
SphcCas9 ORF fwd primer	CACCGGTCGCCACCATGGATAAGAAATACAGCATTGGAC
SphcCas9 ORF rev primer	GCCCCGCAGAAGGTCTAGACTATCACACCTTCCTCTTCTTCTTGG
L7Ae_IVTfwd	CACCGGTCGCCACCATGTACGTGAGATTTGAGGTTCCTG
Tag BFP Fwd	CACCGGTCGCCACCATGGGATCCAGCGAG
TAP_IVTrev	GCCCCGCAGAAGGTCTAGACTATCACTCGAGATGCATATGAGATC
IVT_3prime_UTR primer	TCTAGACCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTTCTTCTCTCCCTTGCACCTGTACCTCTTGGTCTTTGAATAAAGCCTGAGTAGG
Fwd3UTR primer	TCTAGACCTTCTGCGGGGC
Rev3UTR2T20	TTTTTTTTTTTTTTTTTTTTCCTACTCAGGCTTTATTCAAAGACCAAG
GCT7pro_5UTR2	GCTAATACGACTCACTATAGGTTCCTTAATCGCGGATCC
5UTRtemp_T302a-5p	CGACTCACTATAGGTTCCGCGATCGCGGATCCAGCAAGTACATCCACGTTTAAGTAGATCCACCGGTCGCCACCATG
5UTRtemp_T21-5p	CGACTCACTATAGGTTCCGCGATCGCGGATCCTCAACATCAGTCTGATAAGCTAAGATCACACCGGTCGCCACCATG

Primer/Oligo DNA name	Sequence (5' -> 3')
5UTRtemp_4x302a-5p	CGACTCACTATAGGTTCCGCGATCGCGGATCCAGCAAGTACAT CCACGTTTAAGTAGCAAGTACATCCACGTTTAAGTAGCAAGTAC ATCCACGTTTAAGTAGCAAGTACATCCACGTTTAAGTAGATCAC ACCGGTCGCCACCATG
CGCT7G2	CGCTAATACGACTCACTATAGG
5UTR4ntmoxCDr0sp	CGCTAATACGACTCACTATAGGATCCGTGATCGGAAACGTGAG ATCCACCTCAGATCCGCTAGGACACCCGCAGATCGAG
Spacer5UTRFwd	ACACCCGCAGATCGAGAAGAAGGCGAATTAAGAGAGAAAAGA AGAGTAAGAAGAAATATAAGACACCGGTCGCCACCATG
3UTR120A	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTCCTACTCAGGCTTTATTCA
T7-sgRNA fwd primer (DMD)	GAAATTAATACGACTCACTATAGGTATCTTACAGGAACTCCGTT TTAGAGCTAGAAATAGCAAG
T7-sgRNA fwd primer (EGFP)	GAAATTAATACGACTCACTATAGGGGCACGGGCAGCTTGCC GGGTTTTAGAGCTAGAAATAGCAAG
T7-sgRNA fwd primer (EGFP ΔGG)	GAAATTAATACGACTCACTATAGGGCACGGGCAGCTTGCCGG GTTTTAGAGCTAGAAATAGCAAG
T7-sgRNA fwd primer (Alu1)	GAAATTAATACGACTCACTATAGGGCCTGTAATCCCAGCACTTT GTTTTAGAGCTAGAAATAGCAAG
sgRNA+85 rev primer	AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACT AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Supplementary Table S2: Primers for T7E1 assay

1. T7E1_EGFP Fwd_L and T7E1_EGFP Rev_univ. were designed using Primer3Plus*.
2. Other primers were designed manually.

Primer/Oligo DNA name	Sequence (5' -> 3')
T7E1_EGFP Fwd_L	CAGCCATTGCCTTTTATGG
T7E1_EGFP Rev_Univ.2	GTGTTCTGCTGGTAGTGGTCGGCGAG
T7E1_EGFP Fwd_ORF	TGAGCAAGGGCGAGGAGCTGTTCAC
T7E1_EGFP Rev_Univ.	TTGTAGTTGTACTCCAGCTTGTGC
M13 FwdNew primer	GTTTTCCCAGTCACGAC

*Primer3Plus: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

Supplementary Table S3: qPCR primers used in this study

1. Primers were designed according to the given reference (except for CXCR4 Fwd & PDCD4 Rev).
2. CXCR4 Fwd was redesigned using Primer3Plus* to minimize amplicon size.
3. PDCD4 Rev's 3' terminal base was replaced (C to G).

Target gene		Sequence (5' -> 3')	Length (nt)	Tm (˚C)	GC%	Amplicon (bp)	Ref.
PTEN	Fwd	CAAGATGATGTTTGAAACTATTCCAATG	28	55	32	77	(2)
	Rev	CCTTTAGCTGGCAGACCACAA	21	59	52		
BCL2	Fwd	TCCGCATCAGGAAGGCTAGA	20	59	55	113	(3)
	Rev	AGGACCAGGCCTCCAAGCT	19	62	63		
PDCD4	Fwd	TATGATGTGGAGGAGGTGGATGTGA	25	61	48	96	(4)
	Rev	CCTTTCATCCAAAGGCAAAACTACAG	26	58	42		
TPM1	Fwd	GCCGACGTAGCTTCTCTGAAC	21	59	57	160	(5)
	Rev	TTTGGGCTCGACTCTCAATGA	21	58	48		
CDK2	Fwd	GCTAGCAGACTTTGGACTAGCCAG	24	61	54	85	(3)
	Rev	AGCTCGGTACCACAGGGTCA	20	61	60		
CDK4	Fwd	CTGGTGTTTGAGCATGTAGACC	22	57	50	102	(3)
	Rev	AAACTGGCGCATCAGATCCTT	21	59	48		

Target gene		Sequence (5' -> 3')	Length (nt)	Tm (˚C)	GC%	Amplicon (bp)	Ref.
AKT1	Fwd	GCACAAACGAGGGGAGTACAT	21	58	52	113	(3)
	Rev	CCTCACGTTGGTCCACATC	19	56	58		
CXCR4	Fwd	TCATCCTGGCCTTCATCAGT	20	57	50	112	(6)
	Rev	ATCCAGACGCCAACATAGAC	20	55	50		
TGFBR2	Fwd	GTAGCTCTGATGAGTGCAATGAC	23	57	48	132	(7)
	Rev	CAGATATGGCAACTCCCAGTG	21	56	52		

*Primer3Plus: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

Supplementary Table S4: List of PCR reactions

No.	Name	Type	Templates	Forward primer	Reverse primer	Additional oligos
1	5'-UTR (control)	UTR	IVT_5prime_UTR primer	TAP_T7_G3C fwd primer	Rev5UTR primer	
2	3'-UTR	UTR	IVT_3prime_UTR primer	Fwd3UTR primer	Rev3UTR2T20	
3	ORF PCR (Cas9)	ORF	pHL-EF1a-SphcCas9-iC-A	SphcCas9 ORF fwd primer	SphcCas9 ORF rev primer	
4	ORF PCR (L7Ae)	ORF	pAM_L7Ae	L7Ae_IVTfwd	TAP_IVTrev	
5	ORF PCR (BFP)	ORF	pA9-tagBFP	Tag BFP Fwd	TAP_IVTrev	
6	Control Cas9 mRNA Template	IVT template	ORF PCR (Cas9), 5'-UTR (control), 3'-UTR	TAP_T7_G3C fwd primer	3UTR120A	
7	miR-21-responsive Cas9 mRNA Template	IVT template	ORF PCR (Cas9), 5UTRtemp_T21-5p, 3'-UTR	GCT7pro_5UTR2	3UTR120A	
8	miR-302-responsive Cas9 mRNA Template	IVT template	ORF PCR (Cas9), 5UTRtemp_T302a-5p, 3'-UTR	GCT7pro_5UTR2	3UTR120A	
9	4x miR-302-responsive Cas9 mRNA Template	IVT template	ORF PCR (Cas9), 5UTRtemp_4x302a-5p, 3'-UTR	GCT7pro_5UTR2	3UTR120A	
10	Kt-Cas9 mRNA Template	IVT template	ORF PCR (Cas9), 5UTR4ntmoxCDr0sp, 3'-UTR	CGCT7G2	3UTR120A	Spacer5UTRFwd
11	Control L7Ae mRNA Template	IVT template	ORF PCR (L7Ae), 5'-UTR (control), 3'-UTR	TAP_T7_G3C fwd primer	3UTR120A	
12	miR-21-responsive L7Ae mRNA Template	IVT template	ORF PCR (L7Ae), 5UTRtemp_T21-5p, 3'-UTR	GCT7pro_5UTR2	3UTR120A	
13	BFP mRNA Template	IVT template	ORF PCR (BFP), 5'-UTR (control), 3'-UTR	TAP_T7_G3C fwd primer	3UTR120A	
14	$sgRNA_{DMD}$ Template	IVT template		T7-sgRNA fwd primer (DMD)	sgRNA+85 rev primer	
15	$sgRNA_{GFP}$ Template	IVT template		T7-sgRNA fwd primer (EGFP)	sgRNA+85 rev primer	
16	$sgRNA2_{GFP}$ Template	IVT template		T7-sgRNA fwd primer (EGFP ΔGG)	sgRNA+85 rev primer	
17	$sgRNA_{Alu1}$ Template	IVT template		T7-sgRNA fwd primer (Alu1)	sgRNA+85 rev primer	
18	T7E1 assay First PCR product	T7E1 PCR template	Genomic DNA	T7E1_EGFP Fwd_L	T7E1_EGFP Rev_Univ.2	
19	T7E1 assay Second PCR product	For T7E1 assay and sequencing	T7E1 assay First PCR product	T7E1_EGFP Fwd_ORF	T7E1_EGFP Rev_Univ.	

Supplementary Table S5: RNA sequences used in this study

1. The 5’ terminus of the mRNA is capped with ARCA.
2. The protein coding regions are shown as bold letters.
3. The start and stop codons are underlined.
4. miRNA target sites and RNA motifs are written as orange and red letters, respectively.
5. sgRNA target sequences are written as green letters.

Control *Cas9* mRNA

GGGCGAAUUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAAGACACCGGUCGCCACC**AU**

GGAUAAGAAAUACAGCAUUGGACUGGACAUUGGGACAAACUCCGUGGGAUGGGCCGU

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AGCUGAAGCAACUCGGCUGAAAAGAACUGCUCGGAGAAGGUAUACCCGCCGAAAGAA

UAGGAUCUGCUACCUGCAGGAGAUUUUCAGCAACGAAAUGGCCAAGGUGGACGAUAG

UUUCUUUCACCGCCUGGAGGAAUCAUUCCUGGUCGAGGAAGAUAAGAAACACGAGCG

GCAUCCCAUCUUUGGCAACAUUGUGGACGAGGUCGCUUAUCACGAAAAGUACCCUAC

CAUCUAUCAUCUGAGGAAGAAACUGGUGGACUCCACAGAUAAAGCAGACCUGCGCCU

GAUCUAUCUGGCCCUGGCUCACAUGAUUAAGUUCCGGGGCCAUUUUCUGAUCGAGGG

GGAUCUGAACCCAGACAAUUCUGAUGUGGACAAGCUGUUCAUCCAGCUGGUCCAGAC

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CAUCCUGAGUGCCAGACUGUCUAAGAGUCGGAGACUGGAGAACCUGAUCGCUCAGCU

GCCAGGGGAAAAGAAAAACGGCCUGUUUGGGAAUCUGAUUGCACUGUCACUGGGACU

GACUCCCAACUUCAAGAGCAAUUUUGAUCUGGCCGAGGACGCUAAACUGCAGCUGUC

CAAGGACACCUAUGACGAUGACCUGGAUAACCUGCUGGCUCAGAUCGGGGAUCAGUA

CGCAGACCUGUUCCUGGCCGCUAAGAAUCUGUCUGACGCCAUCCUGCUGAGUGAUAU

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GCCUGAGUAGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAA

miR-21-responsive *Cas9* mRNA

GGUUCCUUAAUCGCGGAUCCUCAACAUCAGUCUGAUAAGCUAAGAUCCACCGGUCGCCA

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AAAAAAAAAA

miR-302-responsive *Cas9* mRNA

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4x miR-302-responsive *Cas9* mRNA

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Kt-*Cas9* mRNA

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L7Ae mRNA

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miR-21-responsive *L7Ae* mRNA

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Tag *BFP* mRNA

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sgRNA$_{GFP}$

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sgRNA2$_{GFP}$

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sgRNA$_{DMD}$

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sgRNA$_{Alu1}$

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Supplementary Table S6: Plasmid sequences used in this study

1. The protein coding regions (Cas9, L7Ae or BFP) are shown as bold letters.
2. The start and stop codons are underlined.
3. L7Ae was cloned from L7Ae plasmid provided by A. Huttenhofer (Innsbruck Medical University, Biocenter) and T.S. Rozhdestvensky (University of Muenster).
4. BFP was cloned from pTagBFP-actin (Evrogen).

pHL-EF1a-SphcCas9 (Addgene, Plasmid #60599)

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Supplementary Table S7: Transfection tables for all experiments in this study

Figure 2A-C					
	Control Cas9 mRNA	sgRNA$_{DMD}$ (Negative control)	miR-302-responsive Cas9 mRNA	miR-21-responsive Cas9 mRNA	Untreated
Control Cas9 mRNA	100 ng	100 ng			
miR-302-responsive Cas9 mRNA			100 ng		
miR-21-responsive Cas9 mRNA				100 ng	
sgRNA$_{GFP}$	300 ng		300 ng	300 ng	
sgRNA$_{DMD}$		300 ng			

Figure 2D					
	Control Cas9 mRNA	sgRNA$_{DMD}$ (Negative control)	miR-21-responsive Cas9 mRNA + miR-21 inhibitor	miR-21-responsive Cas9 mRNA + negative inhibitor	Untreated
Control Cas9 mRNA	100 ng	100 ng			
miR-21-responsive Cas9 mRNA			100 ng	100 ng	
sgRNA$_{GFP}$	300 ng		300 ng	300 ng	
sgRNA$_{DMD}$		300 ng			
miR-21 inhibitor			5 pmol		
negative inhibitor				5 pmol	

Figure 3					
	Control Cas9 mRNA	sgRNA$_{DMD}$ (Negative control)	4x miR-302-responsive Cas9 mRNA + miR-302 inhibitor	4x miR-302-responsive Cas9 mRNA + negative inhibitor	Untreated
Control Cas9 mRNA	100 ng	100 ng			
4x miR-302-responsive Cas9 mRNA			100 ng	100 ng	
sgRNA2$_{GFP}$	300 ng		300 ng	300 ng	
sgRNA$_{DMD}$		300 ng			
miR-302 inhibitor			5 pmol		
negative inhibitor				5 pmol	

Figure 4B-D				
	Control Cas9 mRNA	sgRNA$_{DMD}$ (Negative control)	4x miR-302-responsive Cas9 mRNA	Untreated
Control Cas9 mRNA	50 ng	50 ng		
4x miR-302-responsive Cas9 mRNA			50 ng	
sgRNA2$_{GFP}$	150 ng		150 ng	
sgRNA$_{DMD}$		150 ng		

Figure 5B, C					
	Control L7Ae mRNA	miR-21-responsive L7Ae mRNA	Constitutive ON (-L7Ae)	sgRNA$_{DMD}$ (Negative control)	Untreated
Kt-Cas9 mRNA	5 ng	5 ng	5 ng	5 ng	
sgRNA2$_{GFP}$	150 ng	150 ng	150 ng		
sgRNA$_{DMD}$				150 ng	
Control L7Ae mRNA	15 ng			15 ng	
miR-21-responsive L7Ae mRNA		15 ng			

Supplementary Figure S2				
	Control Cas9 mRNA	miR-21-responsive Cas9 mRNA	miR-302-responsive Cas9 mRNA	Untreated
Control Cas9 mRNA	100 ng			
miR-21-responsive Cas9 mRNA		100 ng		
miR-302-responsive Cas9 mRNA			100 ng	
$sgRNA_{GFP}$	300 ng	300 ng	300 ng	

Supplementary Figure S3B-D				
	Control Cas9 mRNA	miR-21-responsive Cas9 mRNA	$sgRNA_{DMD}$ (Negative control)	Untreated
Control Cas9 mRNA	10 ng		10 ng	
miR-21-responsive Cas9 mRNA		10 ng		
$sgRNA_{Alu1}$	300 ng	300 ng		
$sgRNA_{DMD}$			300 ng	

Supplementary Figure S4A, S5, S6B					
	Control Cas9 mRNA	$sgRNA_{DMD}$ (Negative control)	miR-302-responsive Cas9 mRNA + miR-302 inhibitor	miR-302-responsive Cas9 mRNA + negative inhibitor	Untreated
Control Cas9 mRNA	100 ng	100 ng			
miR-302-responsive Cas9 mRNA			100 ng	100 ng	
$sgRNA_{GFP}$	300 ng		300 ng	300 ng	
$sgRNA_{DMD}$		300 ng			
miR-302 inhibitor			5 pmol		
negative inhibitor				5 pmol	

Supplementary Figure S4B					
	Control Cas9 mRNA	$sgRNA_{DMD}$ (Negative control)	miR-302-responsive Cas9 mRNA + miR-302 inhibitor	miR-302-responsive Cas9 mRNA + negative inhibitor	Untreated
Control Cas9 mRNA	100 ng	100 ng			
miR-302-responsive Cas9 mRNA			100 ng	100 ng	
$sgRNA2_{GFP}$	300 ng		300 ng	300 ng	
$sgRNA_{DMD}$		300 ng			
miR-302 inhibitor			5 pmol		
negative inhibitor				5 pmol	

Supplementary Figure S4C			
	4x miR-302-responsive Cas9 mRNA + miR-302 inhibitor	4x miR-302-responsive Cas9 mRNA + negative inhibitor	Untreated
4x miR-302-responsive Cas9 mRNA	50 ng	50 ng	
$sgRNA2_{GFP}$	300 ng	300 ng	
miR-302 inhibitor	5 pmol		
negative inhibitor		5 pmol	

Supplementary Figure S7A			
	Control Cas9 mRNA	miR-21-responsive Cas9 mRNA	Untreated
Control Cas9 mRNA	100 ng		
miR-21-responsive Cas9 mRNA		100 ng	
$sgRNA_{GFP}$	300 ng	300 ng	

Supplementary Figure S7B			
	Control Cas9 mRNA	miR-302-responsive Cas9 mRNA	Untreated
Control Cas9 mRNA	100 ng		
miR-302-responsive Cas9 mRNA		100 ng	
$sgRNA_{GFP}$	300 ng	300 ng	

Supplementary Figure S8		
	BFP + Cy5	Untreated
BFP mRNA	100 ng	
$sgRNA_{DMD}$ -Cy5	300 ng	

Supplementary Figure S9					
	Control Cas9 mRNA	$sgRNA_{DMD}$ (Negative control)	1x miR-302-responsive Cas9 mRNA	4x miR-302-responsive Cas9 mRNA	Untreated
Control Cas9 mRNA	100 ng	100 ng			
miR-302-responsive Cas9 mRNA			100 ng		
4x miR-302-responsive Cas9 mRNA				100 ng	
$sgRNA2_{GFP}$	100 ng		100 ng	100 ng	
$sgRNA_{DMD}$		100 ng			

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