Supplemental Information for "**CRISPRs for optimal targeting: Delivery of CRISPR components as DNA, RNA and protein into cultured cells and single-cell embryos**"

Table S1 sgRNA and primer sequences

Target Name	Target site sequence	PCR forward primer	PCR reverse primer
ApoE #1	GGCTCTGTGGGCCCTGCTGTTGG	CACCCCGGGGTGCTGAGATAGAGAT	TTCCACCATGTTGGGCTCCG
ApoE #3	TCCCATTGCTGACAGGTATGGGG	CACCCCGGGGTGCTGAGATAGAGAT	TTCCACCATGTTGGGCTCCG
ApoE #7	GGGATGATCATTGATTCTCCAGG	GTGCGCTCCAAGATGGAGGA	GGCCATGGAATGTGTGCTATGTC
ApoE C2	CGCAACATCCATGACCAGCCAGG	GTGCGCTCCAAGATGGAGGA	GGCCATGGAATGTGTGCTATGTC
mRosa26	CGCCCATCTTCTAGAAAGACTGG	AAGGGAGCTGCAGTGGAGTA	CAAAACCGAAAATCTGTGGG
rRosa26	ACTCCAGTTGCAGATCACGAGGG	GGCGGATCACAAGCAATAAT	CAGTGGAGTAGGCGGAGAAA
rRosa26 (in vitro)	ACTCCAGTTGCAGATCACGAGGG	GGTGGGCATGTCTTCAATCTA	AAGGGAGCTGCAGTGGAGTAG
mPtsg1	CACAGCTGTGCTGCTGCCCATGG	ACAGCTCCTCCAACATGGAC	GTCCTCCTCCATAAAACAGTGG
mFfar1	TGGGGGTATGCACAGCTCAGTGG	AGGGAAGATTGCAGACCCTT	AACAGCAAAGGATCTCTTAGTGG
ApoE OT1-1	GTCACTGTGAGCCCTGCTGTAAG	CTAACAAAAGTCTGCCAGGCG	TCCCTTTCTGAGACGTTCCC
ApoE OT1-2	GGGTCTGGGAGCCCTGCTGTAGG	GTTGTGGGAATGGGCATGAG	TATCAGAGGGACCAACAGGC
ApoE OT1-3	AGCCCTGTGGCCCCTGCTGTGGG	GGACCTTATAGCTGCCCTGTC	GAAACCATGGGACAATGGTATAGG
ApoE OT1-4	GGCCCTGAAGGCCCTGCTGTGAG	GAGGAGTTGTGTTTGGGGTCT	AACTGAACCGGCCTCTTACC
ApoE OT1-5	TGCTCTGTGTTCCCTGCTGTCAG	GGGTTCTATCCACAGTCCCAC	CAAGGCGCCCACGTGCGTAC
ApoE OT1-6	GGGTCAGTGAGCCCTGCTGTTGG	TCTCTGTCTTCCAGGCAAGC	ACAGGATTTGGGTCGTGCTT
ApoE OT1-7	GACCGTGGGGGCCCTGCTGTTAG	ACCTGATTTCAGGAAGGCTTGA	TTGTGTGGCCCGATGTTCAG
ApoE OT7-1	GTGGTGGTCATTGATTCTCCAGG	GGGTCGGTGACATCTTCCATT	TAAGACATTCGAGCCTGGAGC
ApoE OT7-2	GTGTTGATCATTGATTCTCAAGG	GTCAAAGCACATCCTTCCCC	GGGTTTGTCACGGCAGTCTT
ApoE OT7-3	CAAATGAGCATTGATTCTCCAGG	TCTGGAACCTTCCTTAGGGGT	ACGTGCCTTCTTAGTTTGTGC
ApoE OT7-4	TGCTTGTTCATTGATTCTCCCAG	ACACATGGGGCTTAGCTCTTT	CCAAGGAGTGAACAGTGAGCA
ApoE OT7-5	CGAATCAGCATTGATTCTCCAGG	GTCGGACATCTGACACGAGTT	CCCCTGCTGGAGTTCCTAGT
ApoE OT7-6	TGGCTGATGTTTGATTCTCCAGG	AGGGTGAGACAGCTTACAGTTT	ATCAAGTCAGTGAAGTTGCTTTT
ApoE OT7-7	CGGAGGAACATTGATTATCCAAG	ATGTGACTGCGTCTCGTACC	CAATTGGAGTCCAGCCACCT
ApoE OT7-8	GGGATGAAGATTCATTCTCCTAG	GGGCCCAGGAATGTTCTTTTG	CTCTCAGTAGCAGACAGATGCT
mRosa26 (ZFNs)	TGCAACTCCAGTCTTTCTAGAAGATGGGCGGGAGTC	AAGGGAGCTGCAGTGGAGTA	CAAAACCGAAAATCTGTGGG
rRosa26 (ZFNs)	TCCCGCCTATCTTCCAGAAAGACTCCAGTTGCAGATCACGAG	GGCGGATCACAAGCAATAAT	CAGTGGAGTAGGCGGAGAAA

For each sgRNA target site, PAM sequence is in red. For the ZFN target sites, the cleavage sites are in red.

Table S2 sgRNAs purified by ethanol precipitation are overestimated by Nanodrop measurement.

sgRNA prep	Concentration by Nanodrop (ng/μl)		Concentration by Qubit (ng/μl)	Fold of overestimation by Nanodrop
	Original[a]	Repeat[b]		
1	1186	1181	114	10.36
2	941	1009	132	7.64
3	1293	1292	277	4.66
4	3000	4433	1240	3.58
5	2017	2116	314	6.74
6	479	1916	250	7.66
7	1250	1362	560	2.43
8	1109	1345	457	2.94
9	1695	1333	227	5.87
10	1560	1639	1000	1.64
11	2450	2412	1030	2.34
12	1247	1149	186	6.18
13	2120	2327	540	4.31
14	4360	4047	1560	2.59
15	4000	3794	1790	2.12
16	2300	2150	1040	2.07

Sixteen sgRNAs for various targets prepared at different times were initially measured on a Nanodrop. All of these samples were measured side by side using a Nanodrop and a Qubit.
[a]Concentrations measured using a Nanodrop at the time of each sgRNA preparation
[b]Concentrations measured using a Nanodrop at a later time, in parallel with Qubit measurement

Table S3 Column purification of sgRNAs reduced concentration overestimation by a Nanodrop.

sgRNA prep after column purification	Concentration by Nanodrop (μg/μl)	Concentration by Qubit (μg/μl)	Fold of overestimation by Nanodrop
1	3.49	1.91	1.83
2	2.54	1.43	1.77
3	1.69	1.37	1.24
4	3.04	2.32	1.31
5	3.23	2.66	1.21
6	2.57	2.06	1.25
7	2.28	1.05	2.17
8	2.39	0.85	2.81
9	2.46	1.43	1.72
10	2.03	1.40	1.45
11	1.94	1.36	1.43
12	2.27	1.49	1.52

Twelve sgRNAs were purified using Megaclear columns after in vitro transcription and measured side-by-side using a Nanodrop and a Qubit.

Table S4 mRNA preparations with comparable measurements by using a Nanodrop and Qubit.

Cas9 mRNA Prep	Concentration by Nanodrop (ng/μl)		Concentration by Qubit (ng/μl)	Fold of overestimation by Nanodrop
	Original[a]	Repeat[b]		
1	1330	1488	1320	1.13
2	1890	2215	1990	1.11
3	1800	1901	1650	1.15
4	1700	2540	2100	1.21

Various preparations of Cas9 mRNA, purified by using ammonium sulfate precipitation, were initially measured on a Nanodrop. All of these samples were measured side-by-side using a Nanodrop and a Qubit.
[a]Concentrations measured using a Nanodrop at the time of each mRNA preparation
[b]Concentrations measured using a Nanodrop at a later time, in parallel with Qubit measurement

Table S5 Double stranded DNA with comparable measurements by using a Nanodrop and Qubit.

DNA	Concentration by Nanodrop (ng/μl)	Concentration by Qubit (ng/μl)
PCR 1	50.1	53.1
PCR 2	59.8	60.9
PCR 3	123	136.3
Plasmid 1	40	32.4
Plasmid 2	17.1	19.9

Figure S1

ApoE 5'

sgRNA #3: 3 0 1.5 2.2 2.7 3 0 1.5 2.2 2.7
sgRNA #7: 0 3 1.5 0.8 0.3 0 3 1.5 0.8 0.3
NLS: - - - - - + + + + + G

500
350
300
250
200
150

ApoE 3'

3 0 1.5 2.2 2.7 3 0 1.5 2.2 2.7
0 3 1.5 0.8 0.3 0 3 1.5 0.8 0.3
- - - - - + + + + + G

Figure S1. An NLS on Cas9 protein is not required for cleavage activity of nucleofected RNPs. The rat C6 cells were nucleofected with RNP complexes formed with either 3 µg of Cas9 protein without an NLS (-) or with NLS (+) and various amounts of ApoE sgRNAs #3 and #7 (3 µg combined in each sample). Cells were collected 48 hrs post-transfection for Cel-I analysis at both the 5' and 3' target sites. G, GFP transfection control. Weak activity of ApoE #3 in two lanes marked with arrows indicates slight contamination of sgRNA #3 in the sgRNA #7 stock.

Figure S2

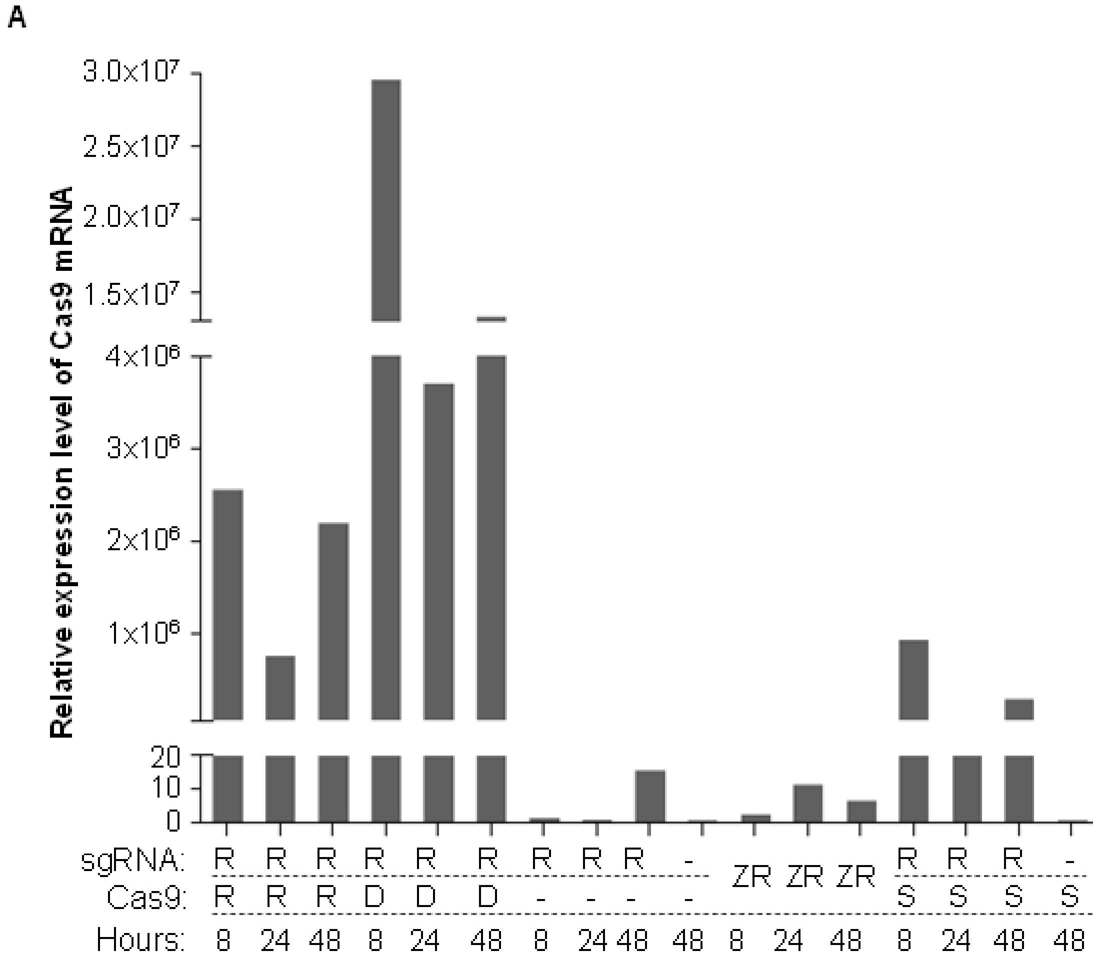


Figure S2 A Relative Cas9 mRNA levels detected by quantitative RT-PCR in samples of the right panel of Fig 1A. The rat C6 cells were transfected with sgRNA rRosa26 with Cas9 mRNA (R), Cas9 expression plasmid (D) or by itself (-). Samples from the C6 cell line stably expressing Cas9 are labeled as "S" for Cas9. Samples transfected with ZFNs against the Rosa26 locus are labeled as "ZR". Cells were collected at three time points, 8, 24 and 48 hours post transfection, for qRT-PCR.

Figure S2

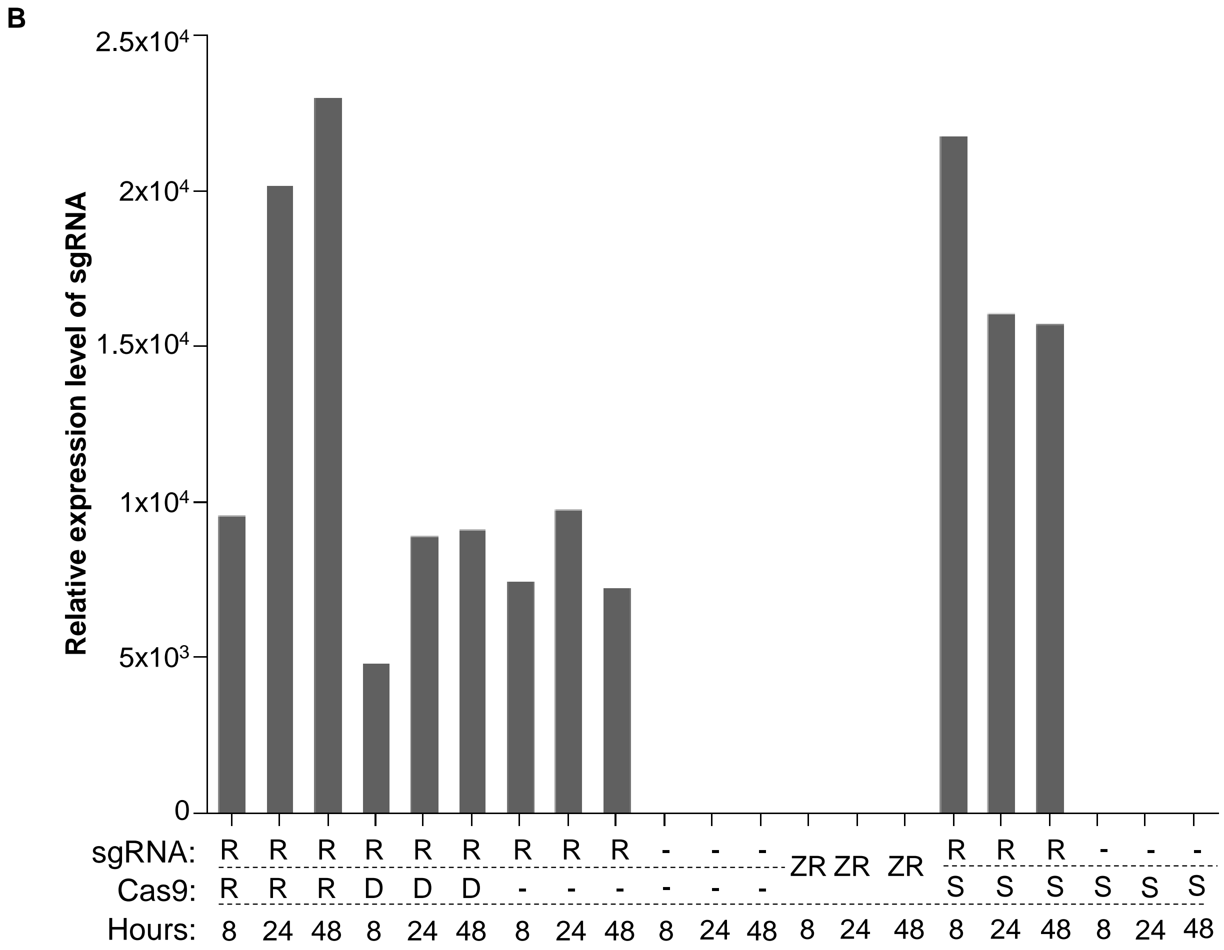


Figure S2B Relative sgRNA Rosa26 levels detected by qRT-PCR from the same samples in **A**.

Figure S2

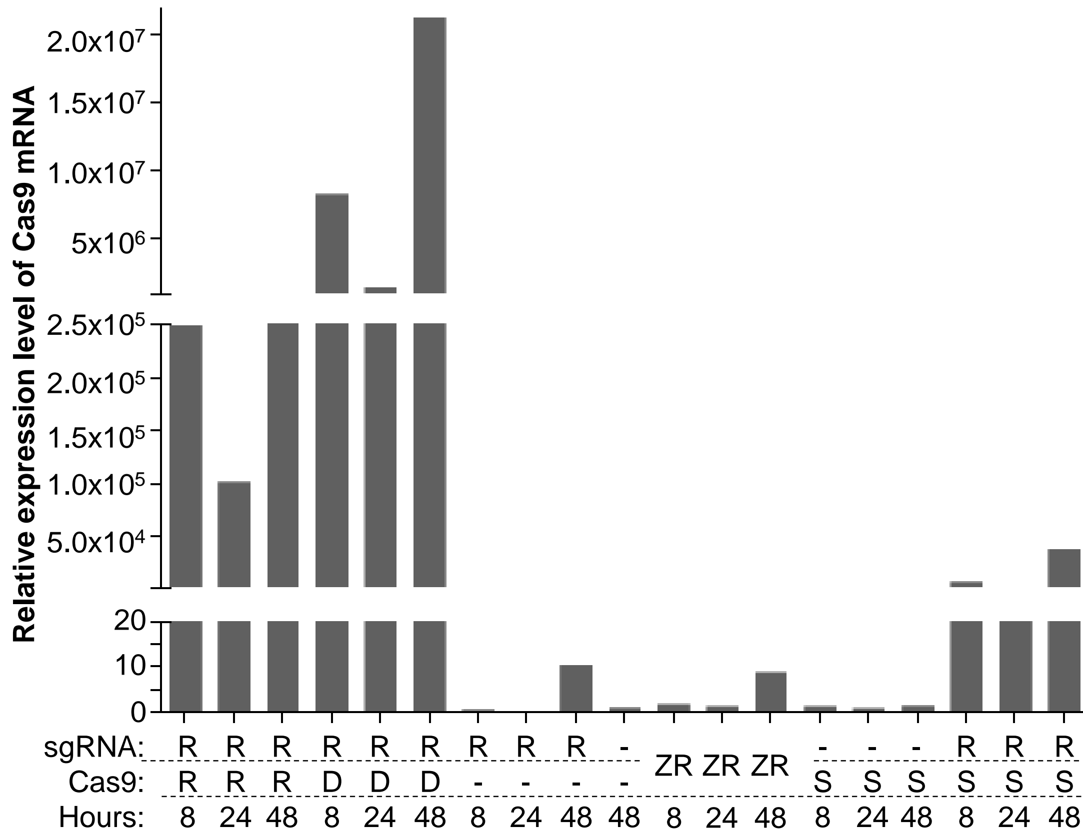


Figure S2C Relative Cas9 mRNA levels detected by quantitative RT-PCR in samples of the right panel of **Fig. 1B**. The mouse Neuro-2a cells were transfected with sgRNA mRosa26 with Cas9 mRNA (R), Cas9 expression plasmid (D) or by itself (-). Samples from the Neuro-2a cell line stably expressing Cas9 are labeled as “S” for Cas9. Samples transfected with ZFNs against the Rosa26 locus are labeled as “ZR”. Cells were collected at three time points, 8, 24 and 48 hours post transfection, for qRT-PCR.

Figure S2

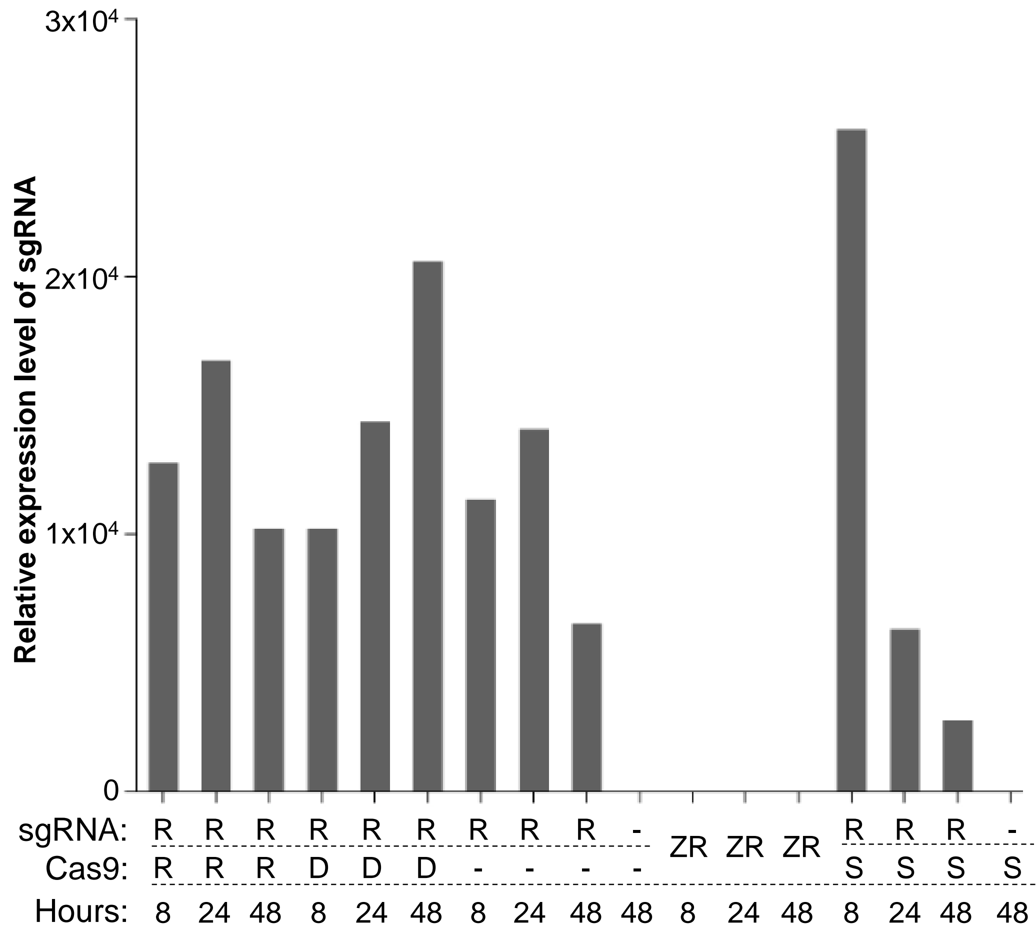
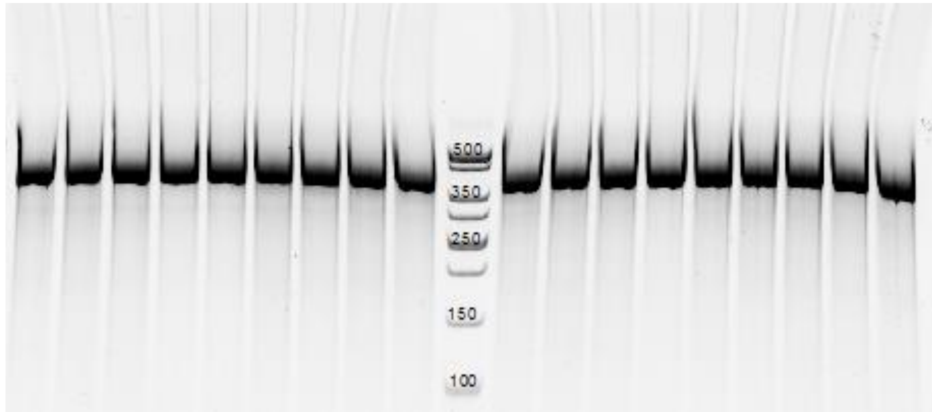


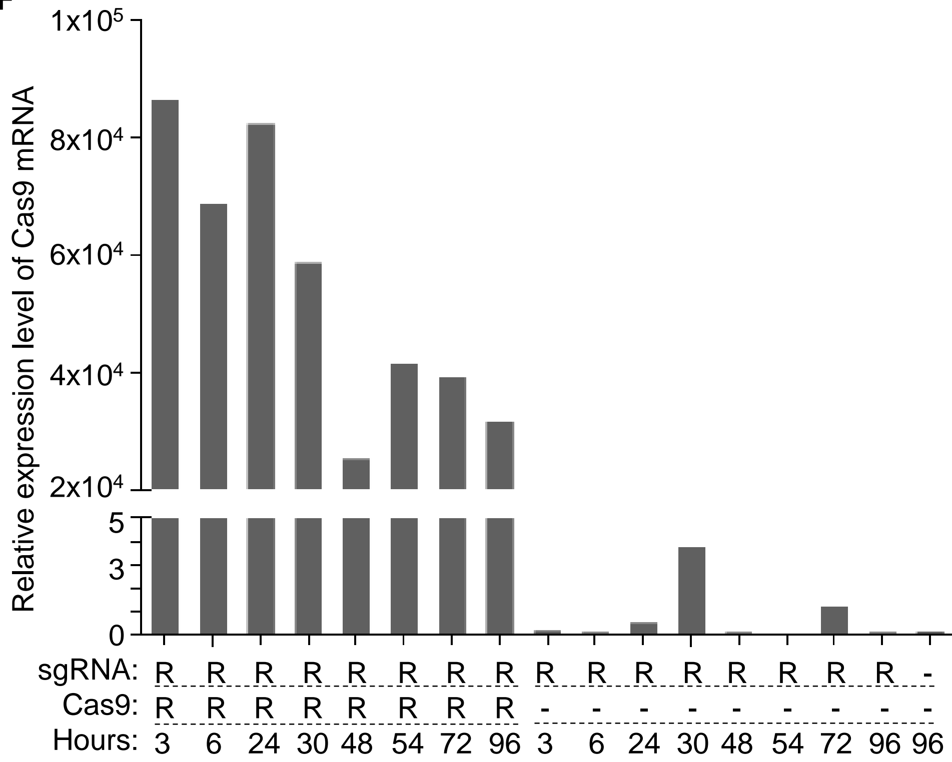
Figure S2D Relative sgRNA mRosa26 levels detected by qRT-PCR from the same samples in **Fig. S2C**.

E

sgRNA: R R R R R R R R - R R R R R R R R -
Cas9: R R R R R R R R - - - - - - - - - -
Hours: 3 6 24 30 48 54 72 96 96 3 6 24 30 48 54 72 96 96



F



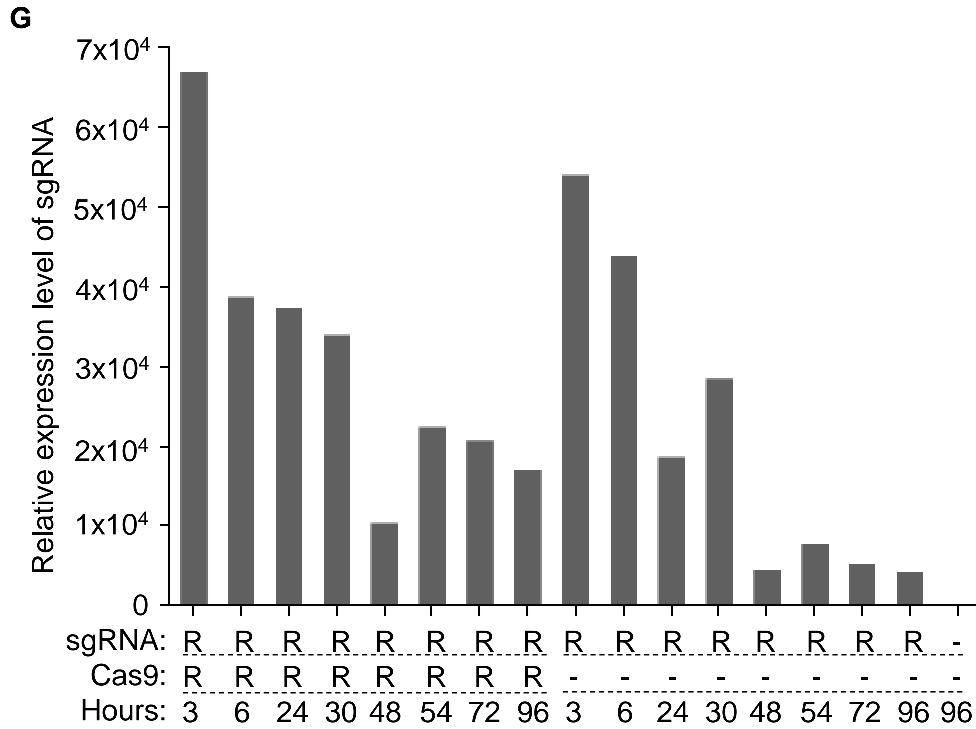
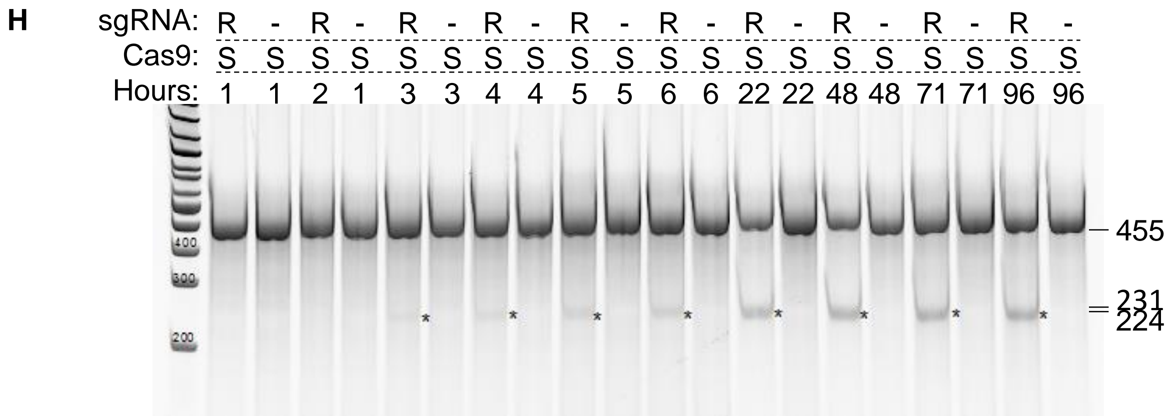


Figure S2 E-G A more detailed time course of the rat C6 cells transfected with the ApoE sgRNA #1 with and without Cas9 mRNA. No NHEJ signal was detectable in any of the samples (**E**). Relative Cas9 mRNA (**F**) and sgRNA (**G**) Relative Cas9 mRNA (**F**) and sgRNA (**G**) levels were detected by quantitative RT-PCR in each sample in **E**.



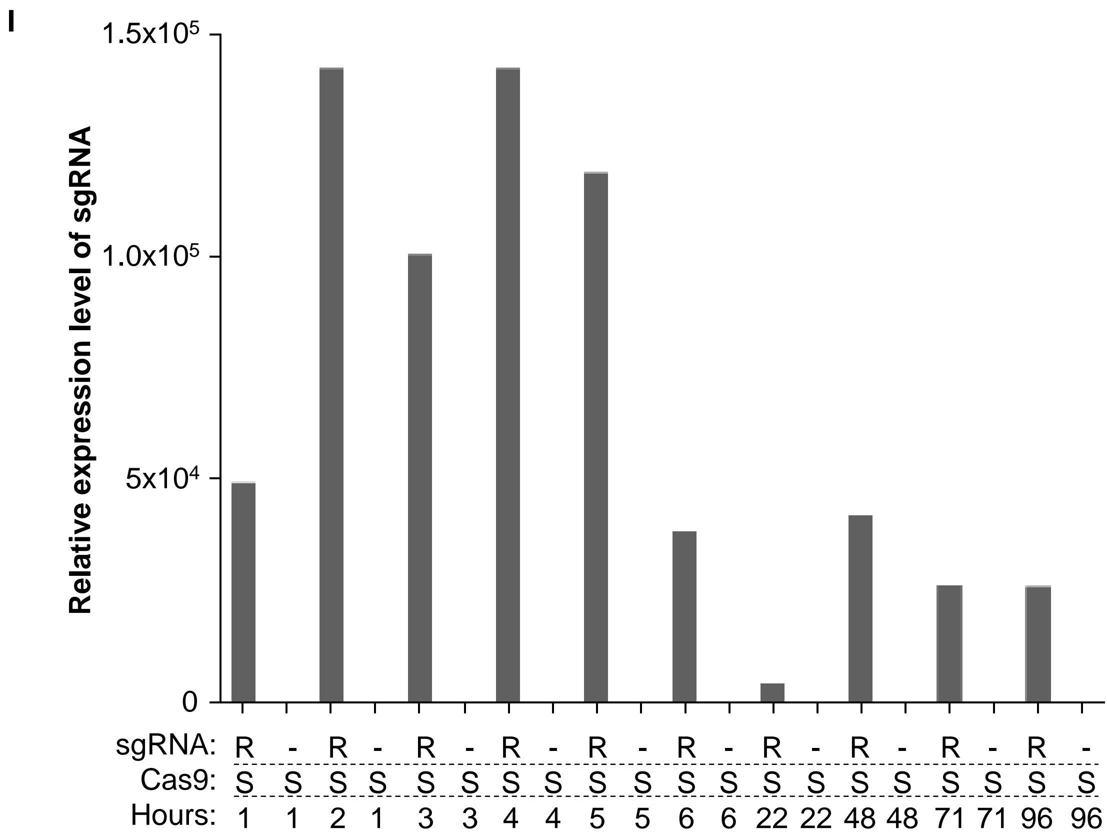


Figure S2H-I A more detailed time course of the rat C6 cells transfected with (R) and without (-) the ApoE sgRNA #1. Cleaved bands at 231 bp and 224 bp are indicative of NHEJ events in the transfected cells. Relative sgRNA levels (**I**) were detected by quantitative RT-PCR in each sample in **H**.

Figure S2 Unbound sgRNAs were relatively stable after transfection. **A** Relative Cas9 mRNA levels detected by quantitative RT-PCR in samples of the right panel of Fig 1A. The rat C6 cells were transfected with sgRNA rRosa26 with Cas9 mRNA (R), Cas9 expression plasmid (D) or by itself (-). Samples from the C6 cell line stably expressing Cas9 are labeled as "S" for Cas9. Samples transfected with ZFNs against the Rosa26 locus are labeled as "ZR". Cells were collected at three time points, 8, 24 and 48 hours post transfection, for qRT-PCR. **B** Relative sgRNA Rosa26 levels detected by qRT-PCR from the same samples in **A**. **C** Relative Cas9 mRNA levels detected by quantitative RT-PCR in samples of the right panel of **Fig. 1B**. The mouse Neuro-2a cells were transfected with sgRNA mRosa26 with Cas9 mRNA (R), Cas9 expression plasmid (D) or by itself (-). Samples from the Neuro-2a cell line stably expressing Cas9 are labeled as "S" for Cas9. Samples transfected with ZFNs against the Rosa26 locus are labeled as "ZR". Cells were collected at three time points, 8, 24 and 48 hours post transfection, for qRT-PCR. **D** Relative sgRNA mRosa26 levels detected by qRT-PCR from the same samples in **C**. **E** A more detailed time course of the rat C6 cells transfected with the ApoE sgRNA #1 with and without Cas9 mRNA. No NHEJ signal was detectable in any of the samples. Relative Cas9 mRNA (**F**) and sgRNA (**G**) levels were detected by quantitative RT-PCR in each sample in **E**. **H** A more detailed time course of the rat C6 cells transfected with (R) and without (-) the ApoE sgRNA #1. Cleaved bands at 231 bp and 224 bp are indicative of NHEJ events in the transfected cells. Relative sgRNA levels (**I**) were detected by quantitative RT-PCR in each sample in **H**.

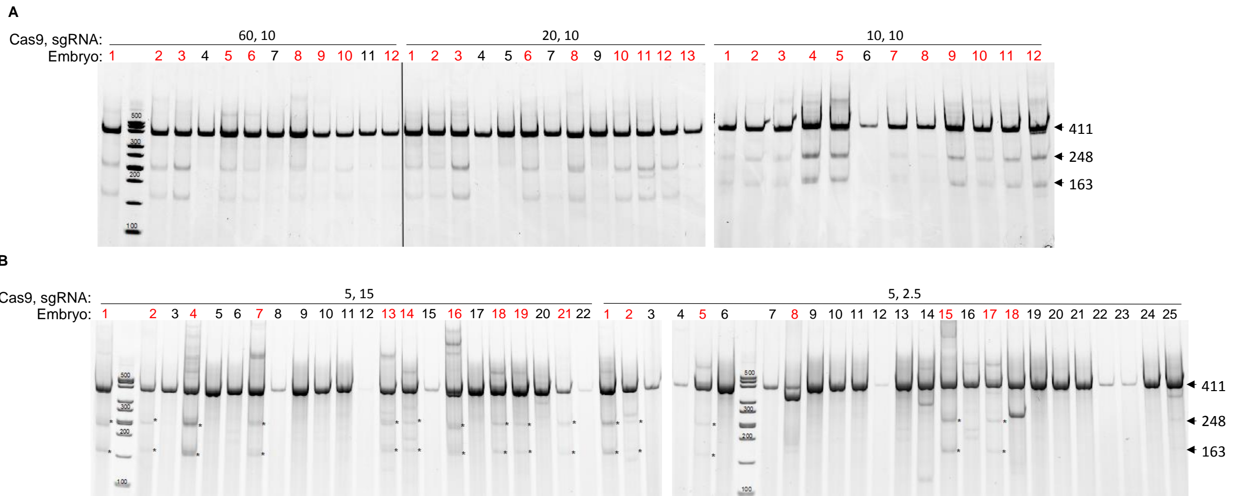


Figure S3 Titration of Cas9 mRNA and sgRNA concentrations for microinjection into single-cell mouse embryos: Different combinations of Cas9 mRNA and sgRNA concentrations were injected, and injected embryos were incubated in vitro to blastocysts and analyzed for NHEJ events using Cel-I assay. Concentrations are labeled above each group. **A** "60, 10", "20, 10", and "10, 10" refer to 60 ng/µl, 20 ng/µl and 10 ng/µl of Cas9 mRNA and 10 ng/µl sgRNA, respectively. **B** "5, 15" and "5, 2.5" refer to 5 ng/µl Cas9 mRNA coinjected with 15 ng/µl or 2.5 ng/µl sgRNA. Embryos modified by CRISPR are marked in red.

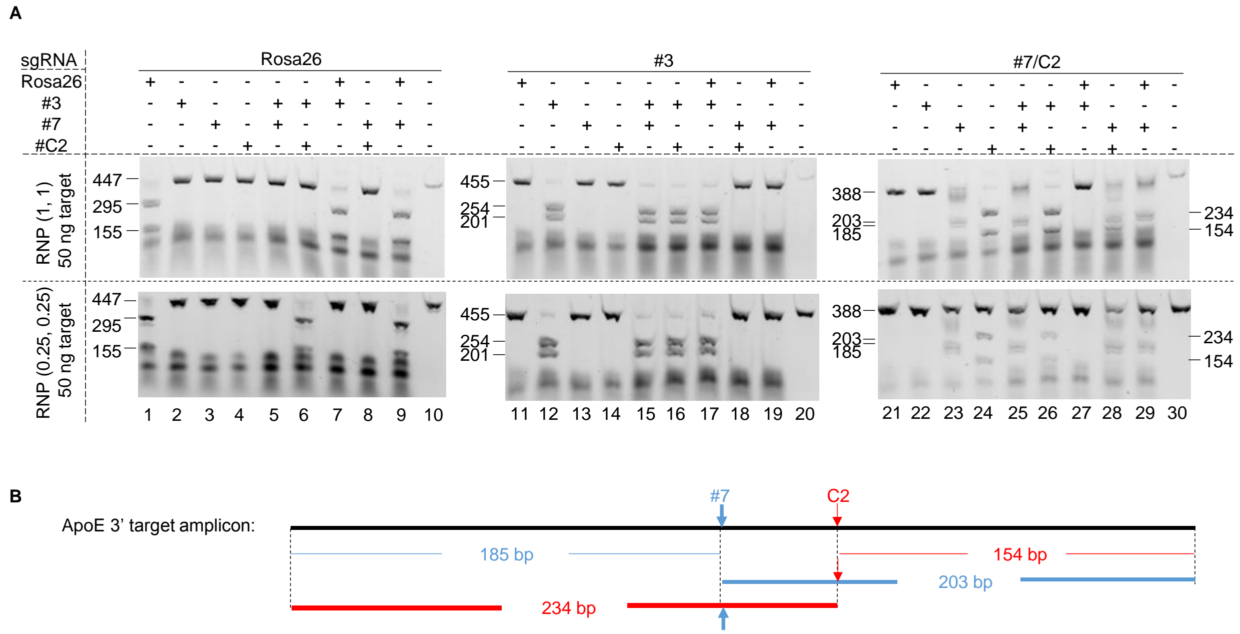


Figure S4. *In vitro* competition assay with two different amounts of RNP complex. **A** In addition to being shown in Fig. 5, where the in vitro competition assay was carried out with RNP (1, 1), referring to equivalent of 1 µg of Cas9 protein and 1 µg of each sgRNA (top panels), we also lowered the protein/sgRNA amount to RNP (0.25, 0.25), referring to 0.25 µg of Cas9 protein and 0.25 µg of each sgRNA (bottom panels). Target sites and sgRNA(s) added in each reaction are labeled on top of each lane. Lanes 6 and 7 in the bottom left panel were switched at loading. In the right hand two panels, band sizes on the left are expected for cleavage by sgRNA #7, and on the right, by C2. **B** Schematic of sgRNAs #7 and C2 cleavage sites proportional to sequences in base pairs. The exact cleavage sites are marked by arrows and resulting cleavage products from the PCR amplified target region are marked with their sizes in base pairs: #7 in blue, and C2 in red, respectively. Each of the two large cleavage products, marked in thicker lines, 234 bp from C2 and 203 bp from #7 can be cleaved by the other RNP again, demonstrated by an blue arrow on the 234 bp fragment, and red on 203 bp fragment, and result in 185 bp and 154 bp fragments, respectively.

Figure S5

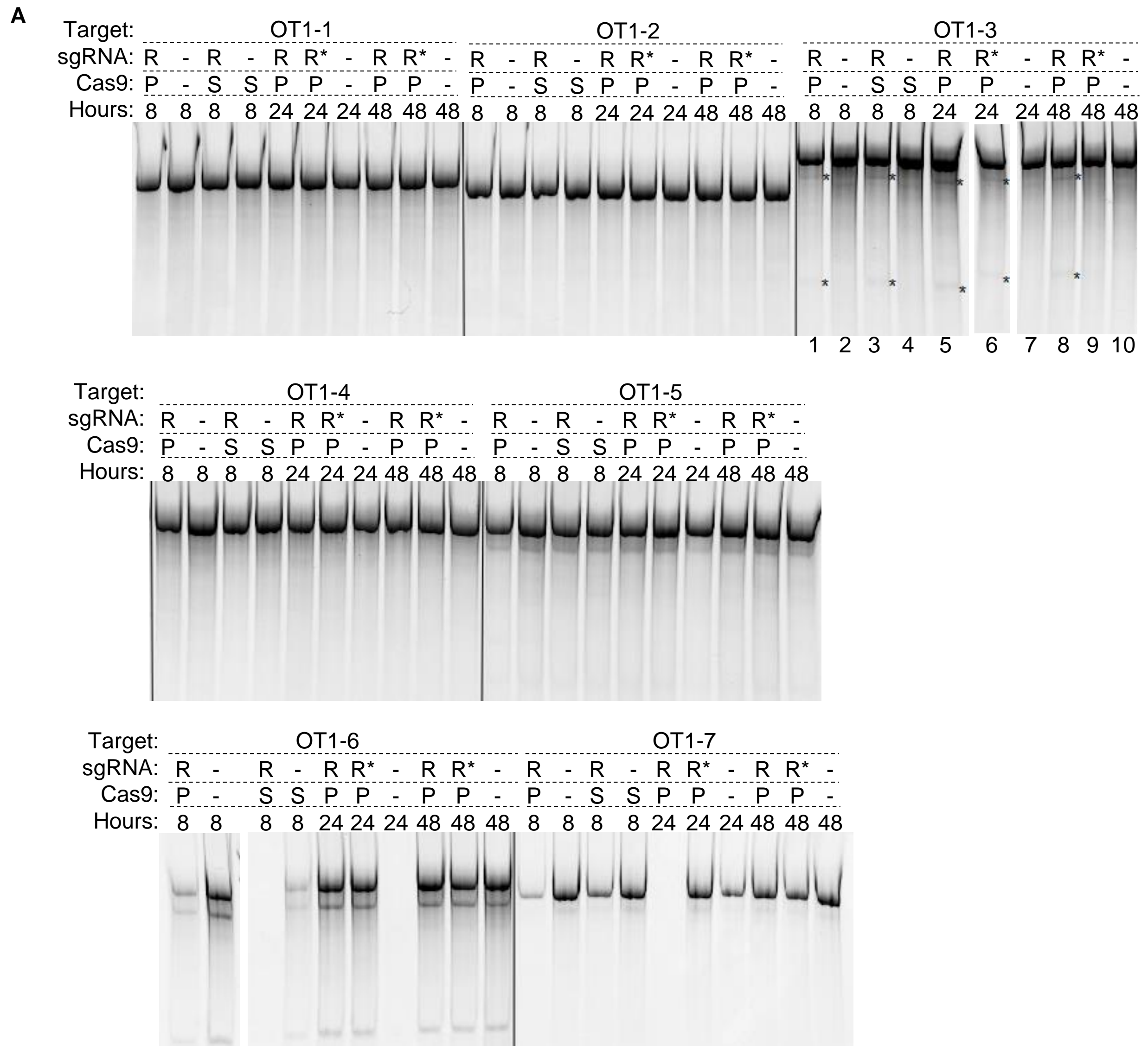


Figure S5

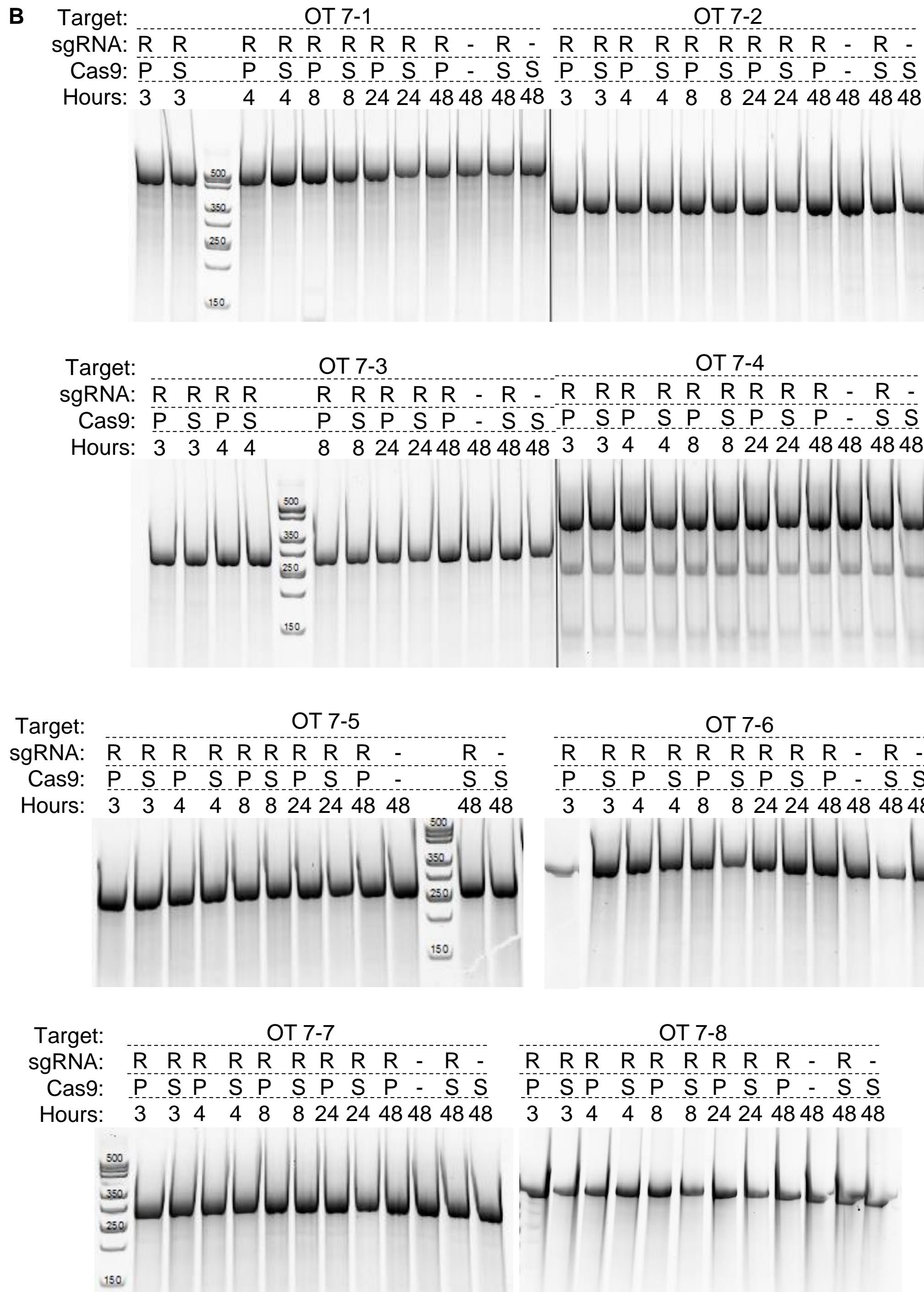


Figure S5 Off targeting detection in rat C6 cells. Samples from Fig 3 were analyzed at the top predicted off target sites for sgRNA #1 (**A**) and sgRNA #7 (**B**) using Cel-I assay. Cleaved bands indicative of positive off target events at off target site 1-3 (OT1-3) are marked with asterisks. Cas9 protein (P), Cas9 mRNA (R) or Cas9 expression plasmid (D) were cotransfected with

either 12 ug (R) or 2 ug (R*) of sgRNA into C6. Samples from Cas9 stable cell line transfections are marked as “S” for Cas9. Lanes for OT1-3 and OT1-6 were assembled from different gels due to loading. Cleaved bands in OT 1-6 and OT 7-4 resulted from SNPs.