Electronic Supplementary Material (ESI) for Nanoscale.

Electronic Supplementary Information

Non-viral delivery of CRISPR/Cas9 complex using CRISPR-GPS nanocomplexes

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Supplementary Figures

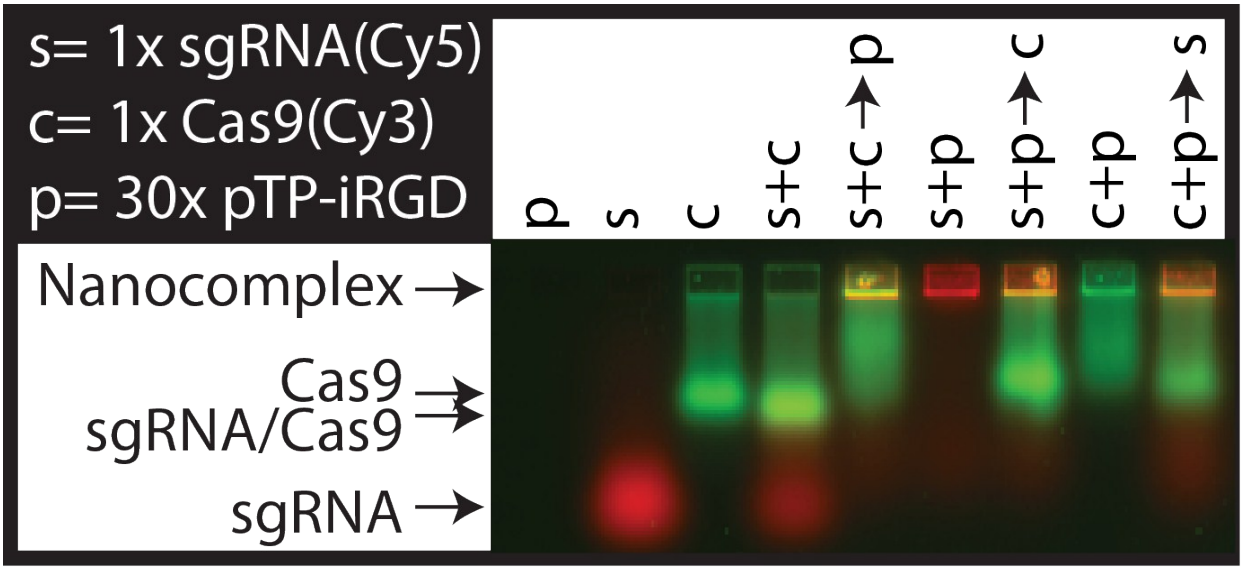


Figure S1: Effect of the order of mixing different components on the complexation. 1x of 5'-Cy5-labeled sgRNA, Cy3-labeled Cas9, and 30x of unlabeled peptides were mixed in different orders as indicated in the figure and then analyzed by an agarose-based gel-retardation assay.

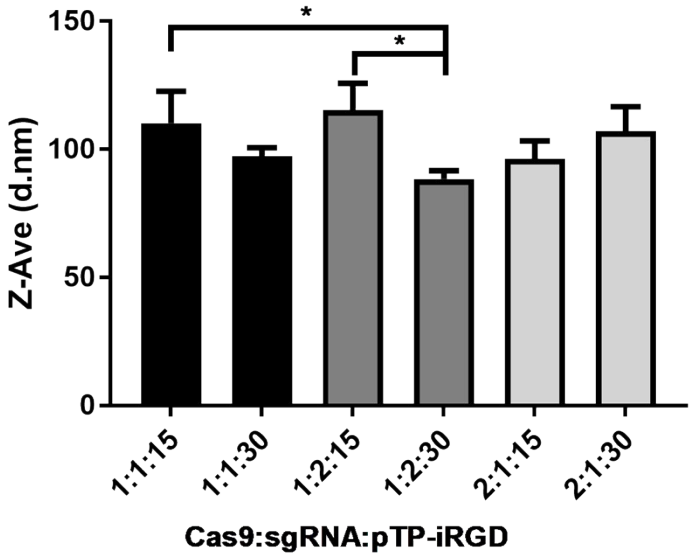


Figure S2: Effect of the ratio of Cas9, sgRNA and peptide on size of the nanocomplexes. Cas9 (25-50 nM), sgRNA (25 nM) and pTP-iRGD (750-1500 nM) were mixed in different ratios as indicated and then after 15-20 minutes, size was analyzed by dynamic light scattering (DLS). Experiments were carried out in triplicate, error bars represent standard deviation (n=3), and the asterisks indicate *P<0.05 as analyzed by a two-way ANOVA test.

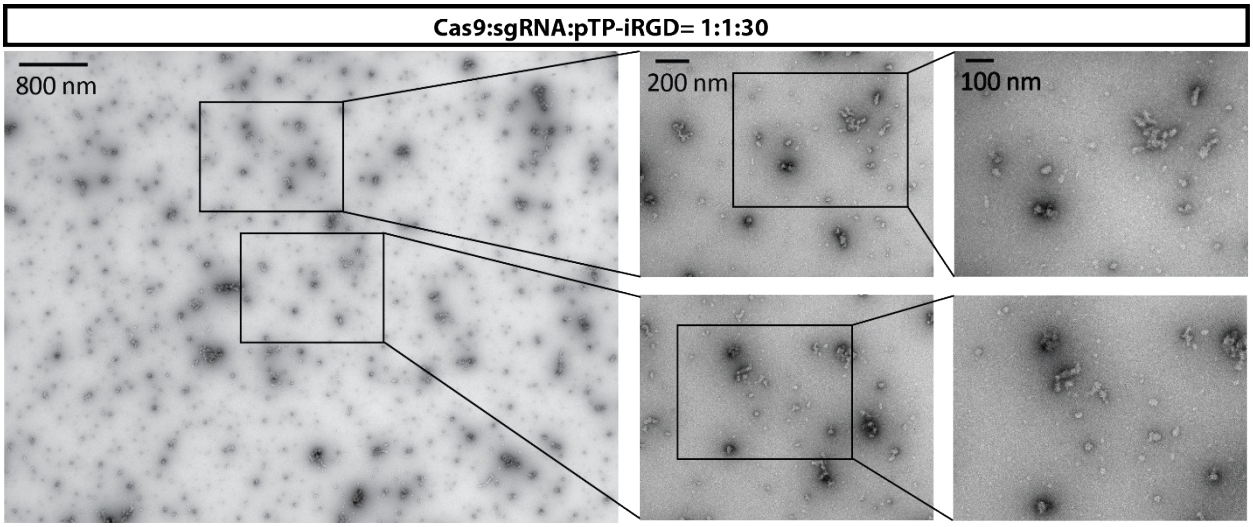


Figure S3: TEM analysis of nanocomplexes. TEM analysis of particles formed in water using 50 nM sgRNA, 50 nM Cas9, and 1500 nM pTP-iRGD were imaged immediately using 2% uranyl acetate as a counter stain at different magnifications. The top two images on the right are the same set of images used in the figure 2d. Scale bars of each image are indicated individually.

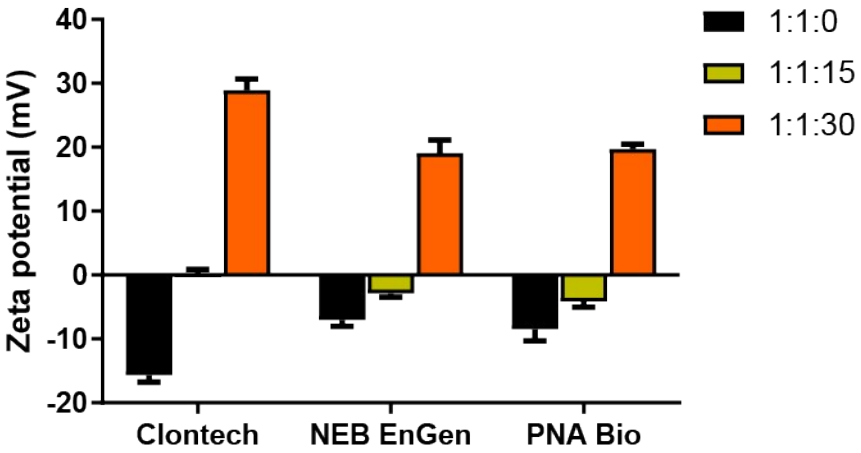


Figure S4: Effect of the source of Cas9 and ratio of peptide on zeta potential of the nanocomplexes. Cas9 (25 nM), sgRNA (25 nM) and pTP-iRGD (750-1500 nM) were mixed in different ratios as indicated and then after 15-20 minutes, zeta potential was analyzed by further diluting the mixture by 25x with RNase free water using DLS. Experiments were carried out in triplicate, error bars represent standard deviation (n=3).

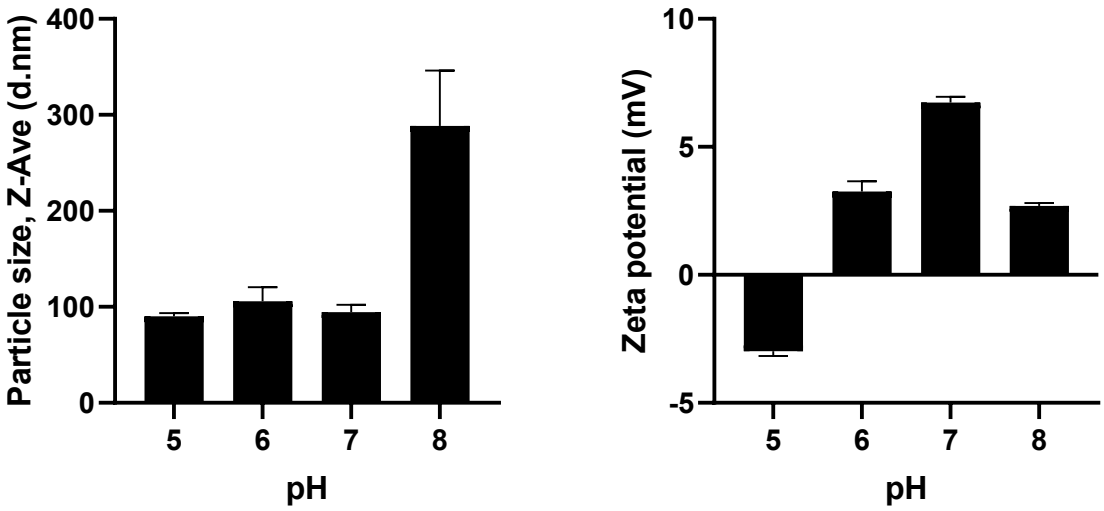


Figure S5: Effect of pH on the size and zeta potential of the Cas9, sgRNA, and peptide nanocomplexes. To measure the size of these nanocomplexes, Cas9 (200 nM), sgRNA (200 nM), and pTP-iRGD (6 μM) were mixed in buffers of varying pH, left to incubate for 15 minutes at room temperature, and then analyzed by DLS. The zeta potential was found by diluting the same sample twentyfold in buffer in a disposable folded capillary cell. Experiments were carried out in triplicate; error bars represent standard error from three different experiments (9 total measurements).

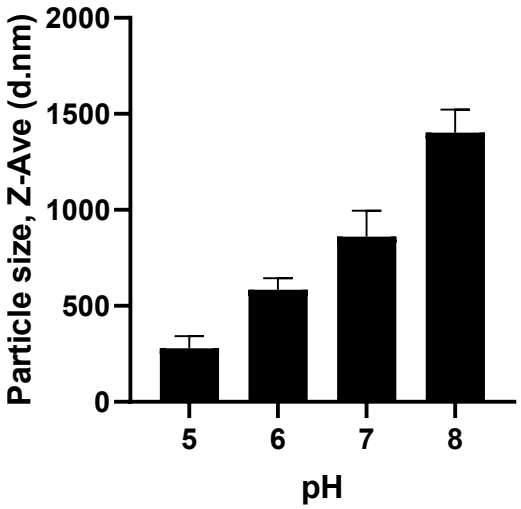


Figure S6: Effect of pH on the aggregation of Cas9. In order to measure the size of Cas9 aggregates in different environments, EnGen Cas9 (NEB) (200 nM) was used with different pH buffers. The Cas9 was then diluted in each buffer and left to incubate for 15 minutes at room temperature, to mirror the protocol we used for our nanocomplexes. We then measured the size via DLS. The Cas9 was found to gather into larger aggregates with increasing pH. The experiment was carried out in triplicate; error bars represent standard deviation (3 total measurements).

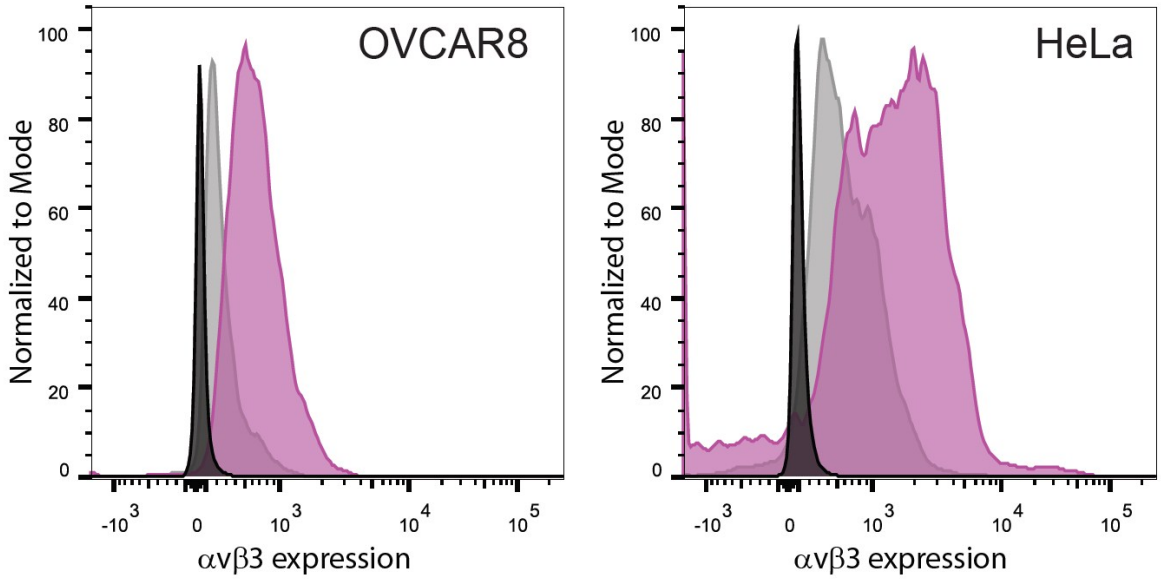


Figure S7: Expression of αvβ3 integrin receptors in OVCAR8 cells and HeLa cells by immunostaining and FACS analysis. Black histogram represents untreated cells, gray plot is phycoerythrin (PE)-labeled IgG control antibody, while the purple indicates the PE-labeled αvβ3 integrin antibody.

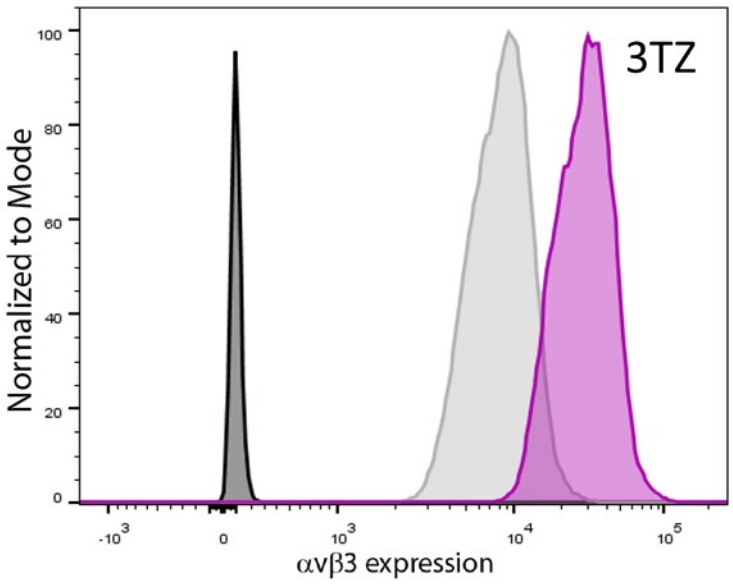


Figure S8: Expression of αvβ3 integrin receptors in 3TZ cells by immunostaining and FACS analysis. The black, gray, and purple histograms represent untreated cells, phycoerythrin (PE)-labeled IgG control antibody, and PE-labeled αvβ3 integrin antibody, respectively.

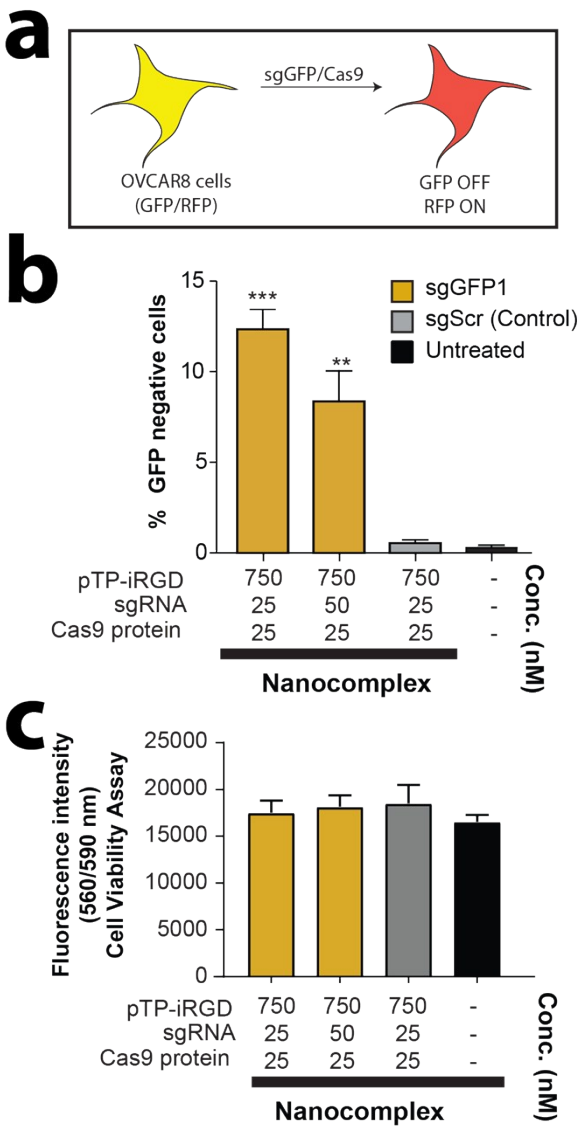


Figure S9: Tandem-lipid peptides have been shown to be effective in functional delivery of CRISPR/Cas9 components to multiple cell lines. We optimized the concentration of sgRNA, Cas9 and pTP-iRGD peptide for the delivery of Cas9/sgRNA complex in an OVCAR8/eGFP/RFP cell line. (a) Schematic representation of the cell line. (b) OVCAR8/eGFP/RFP cells were plated in a 96-well plate and then transfected with sgRNA (50 nM)/Cas9 (50 nM) RNP complex using 1500 nM of integrin targeting peptide (pTP-iRGD) in OptiMEM at the indicated ratios. After treatment for 6 h with the transfection mixture containing either a sgRNA against GFP (sgGFP1) or its scrambled sgGFP1 sequence (sgScr), cells were treated with the fresh cell culture media and GFP disruption was quantified using flow cytometry after 3 days. Untreated sample represent cells treated with OptiMEM without any transfection complex. (c) After 72 h, cytotoxicity was determined by treating cells with PrestoBlue reagent (Thermo Fisher Scientific) following manufacturer's protocol. Experiments were carried out in triplicate, error bars

represent standard deviation, and the asterisks indicate *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 analyzed by two-way ANOVA test.

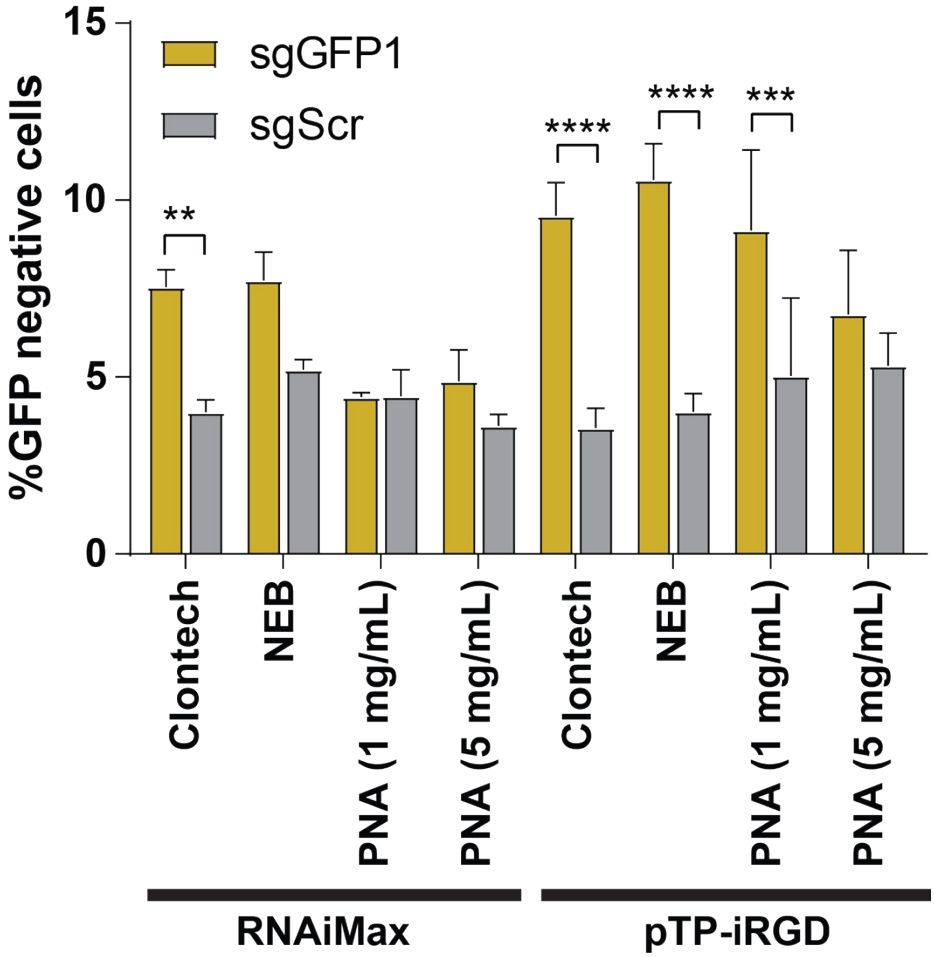


Figure S10: Tandem-lipid peptides achieve functional delivery of Cas9 protein from different vendors. We tested the Cas9 protein from different commercial sources (Clontech, NEB, PNA Bio- 1 mg/mL stock, and PNA Bio- 5 mg/mL stock) for the delivery of Cas9/sgRNA RNP complex. HeLa cells expressing destabilized GFP (d2eGFP) were plated in a 96-well plate and then transfected with sgRNA (50 nM)/Cas9 (50 nM) RNP complex using either RNAiMAX (0.3 μL/well) or 1500 nM of integrin targeting peptide (pTP-iRGD) in Opti-MEM media. After treatment for 8 h with the transfection mixture containing either a sgRNA against GFP (sgGFP1) or its scrambled sgGFP1 sequence (sgScr) cells were treated with fresh cell culture media and GFP disruption was quantified using flow cytometry after 2 days. Error bars represent standard deviation (n=3) and the asterisks indicate **P<0.01, ****P<0.0001 analyzed by two-way ANOVA test using GraphPad Prism software.

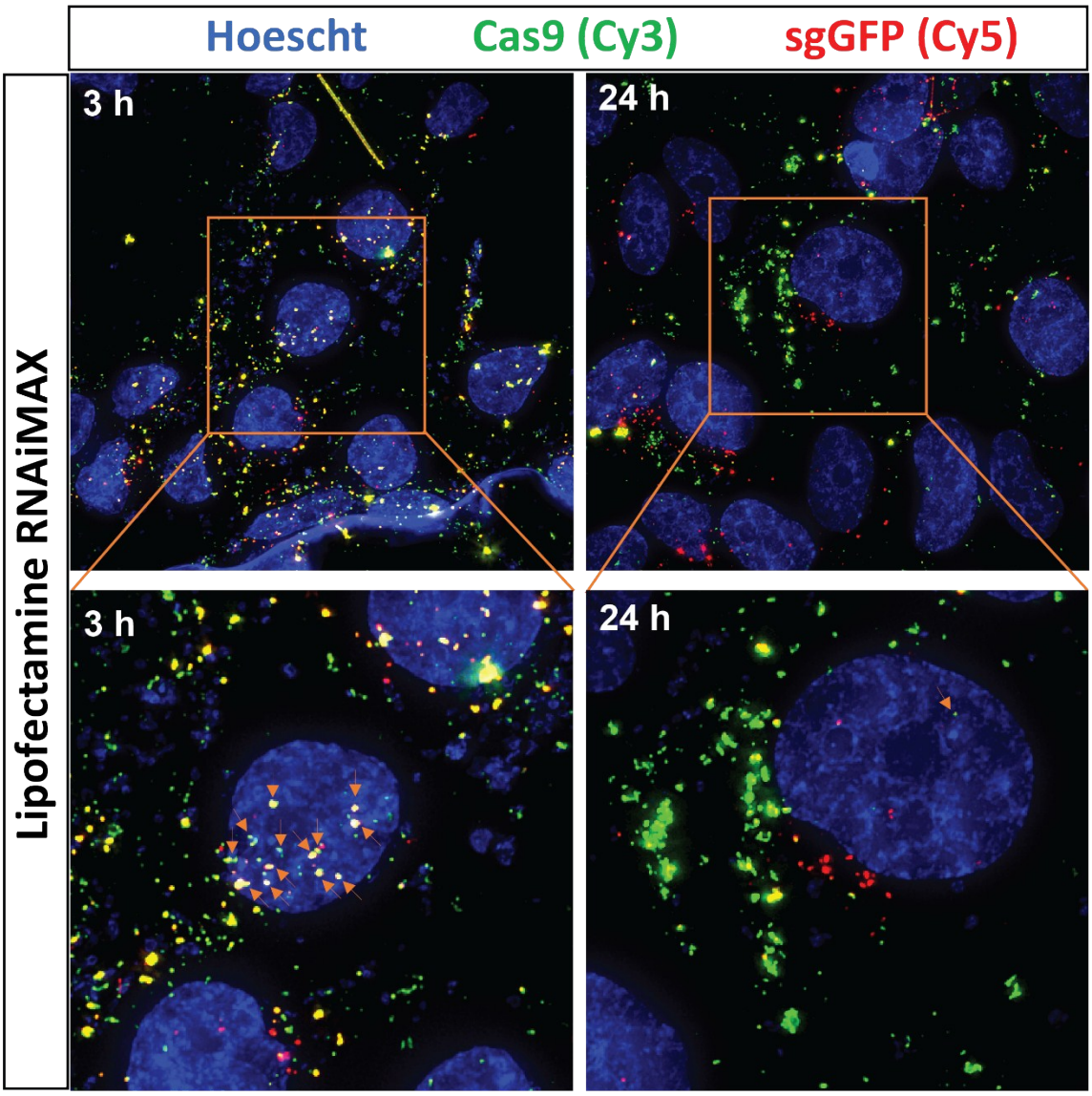


Figure S11: RNAiMAX delivers functional sgRNA/Cas9 cargo inside human tumor-derived OVCAR8 cells. Ovcar8/d2eGFP cells were transfected with lipoplexes containing 50 nM of 5'Cy5-labeled sgRNA and 50 nM of Cy3-labeled Cas9 and 1.5 µL RNAiMAX in 500 uL of OptiMEM. The cells were fixed using 4% paraformaldehyde at different time points and then imaged after 3h or 24 h using an Olympus FV1200 Laser Scanning Confocal Microscope with oil-immersed 60X/1.40, Plan Apo, IX70 objective.

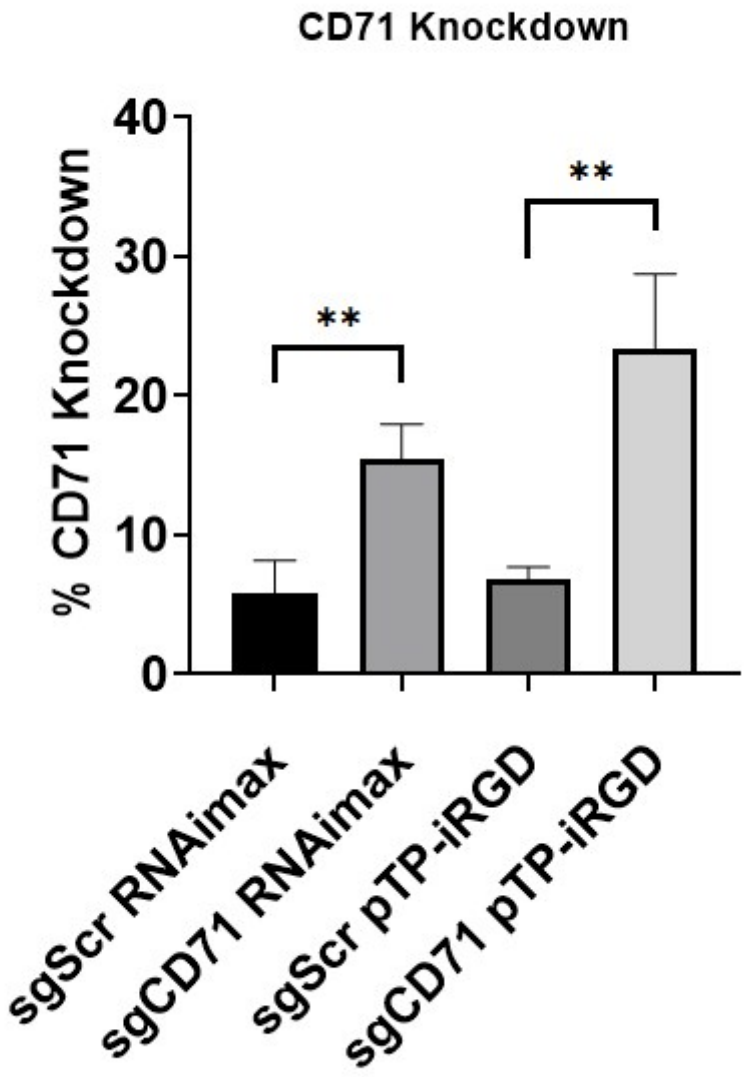


Fig.S12 Knockdown of endogenous gene CD71 using CRISPR-GPS: HEK cells were plated in a 96-well plate and transfected with sgRNA (100 nM)/Cas9 (100 nM) RNP complex using either RNAimax (0.3 µL/well) or 1500 nM of integrin targeting peptide (pTP-iRGD) in Opti-MEM media. After a 24-hour treatment with transfection mixture containing either a sgRNA against CD71 (sgCD71) or its scrambled sequence (sgScr), cells were treated with fresh culture media. After another 72-hour period, these cells were harvested, and their DNA was extracted. PCR was performed to amplify a target region flanking the cut site. CD71 disruption was quantified by Sanger sequencing the PCR products and performing TIDE Analysis for quantifying InDels. Error bars represent standard deviation (n=3) and asterisks indicate ** $p < 0.01$ analyzed by two-tailed student's t-test.

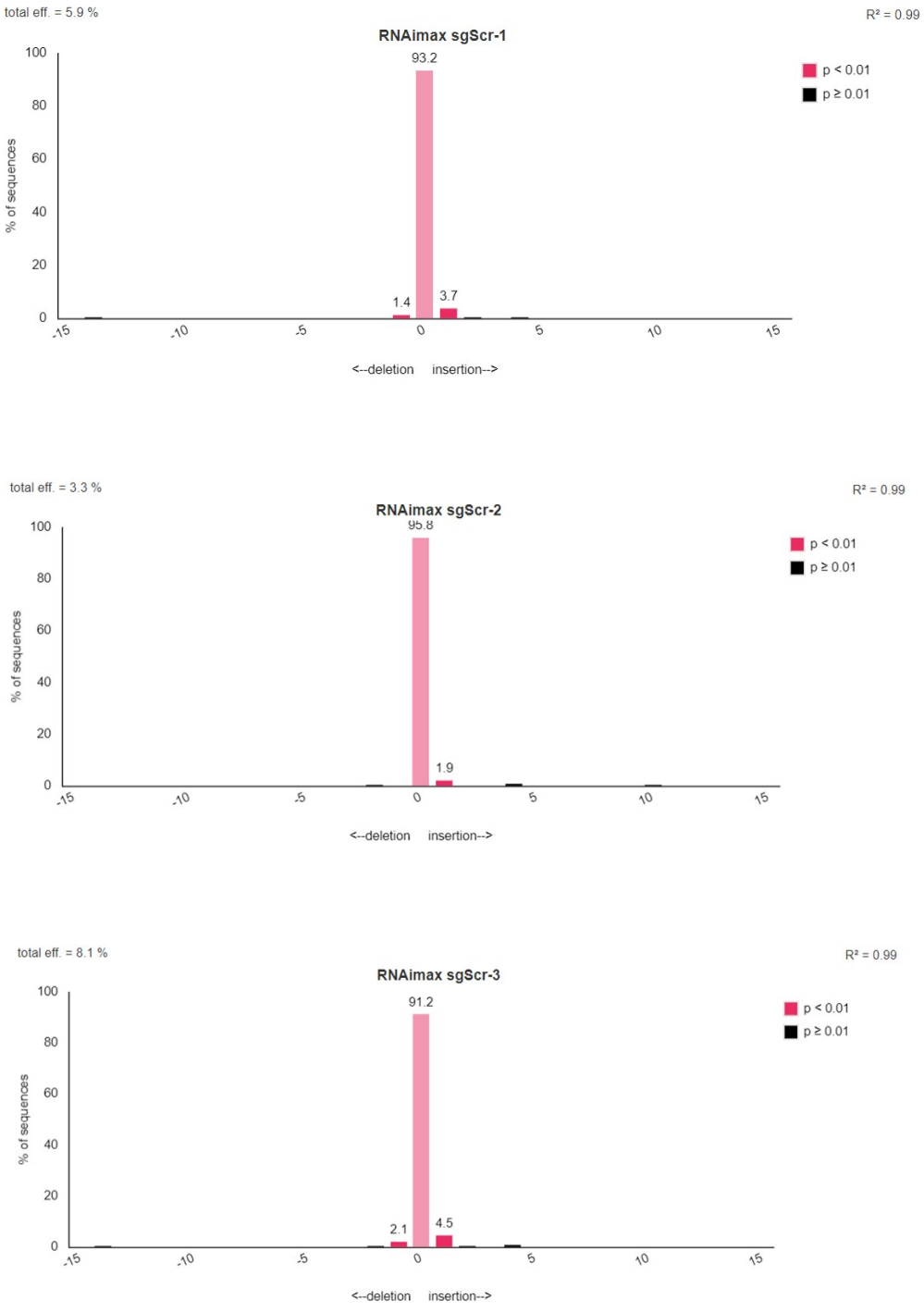


Fig. S13 (a) Quantification of InDels in RNAimax sgScr samples (negative control) using TIDE Analysis: TIDE Analysis was performed using a reference sequence from untreated samples and setting the parameters to detect a maximum InDel size of 15 nucleotides.

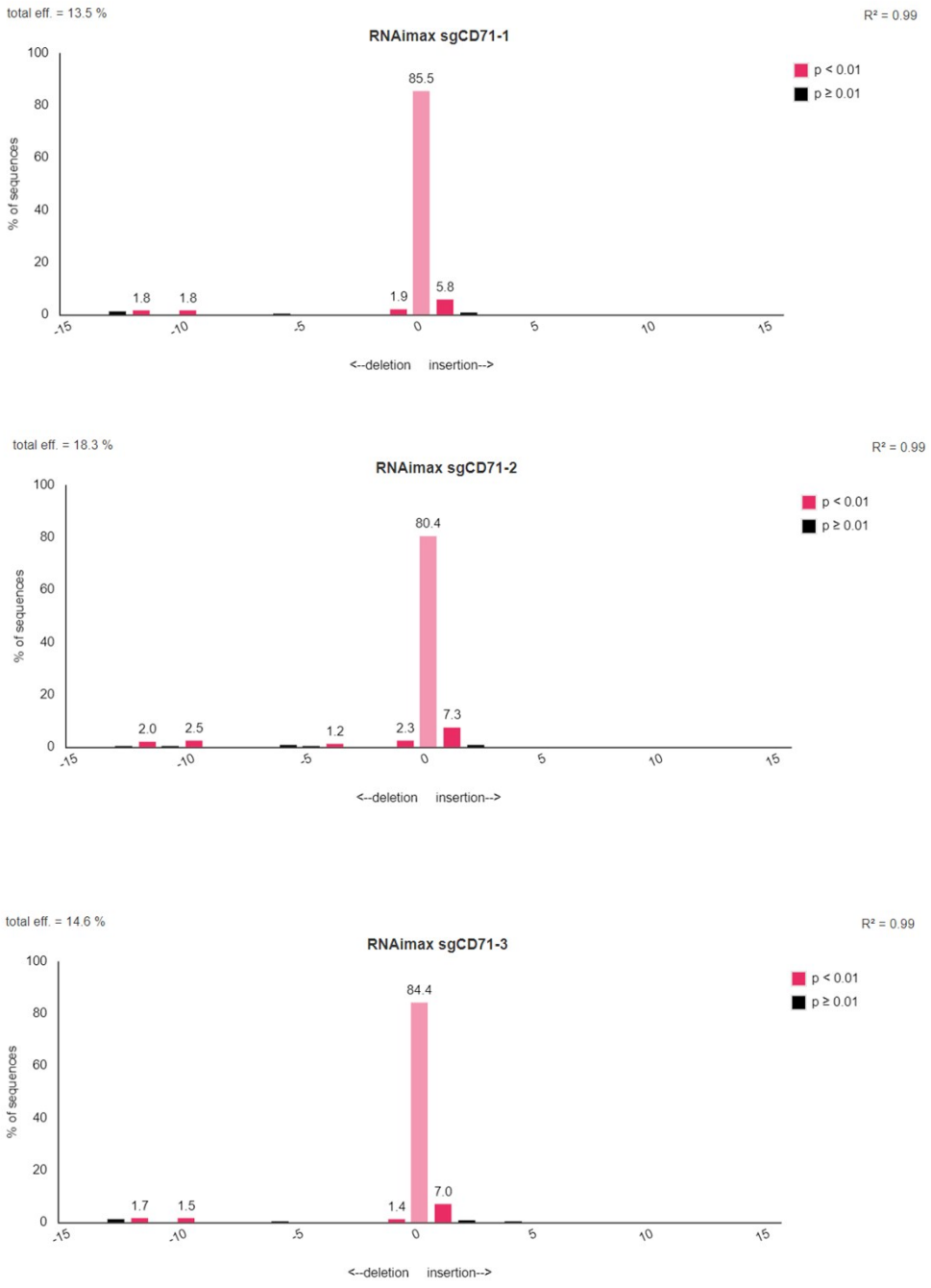


Fig. S13 (b) Quantification of InDels in RNAimax sgCD71 samples using TIDE Analysis: TIDE Analysis was performed using a reference sequence from untreated samples and setting the parameters to detect a maximum InDel size of 15 nucleotides.

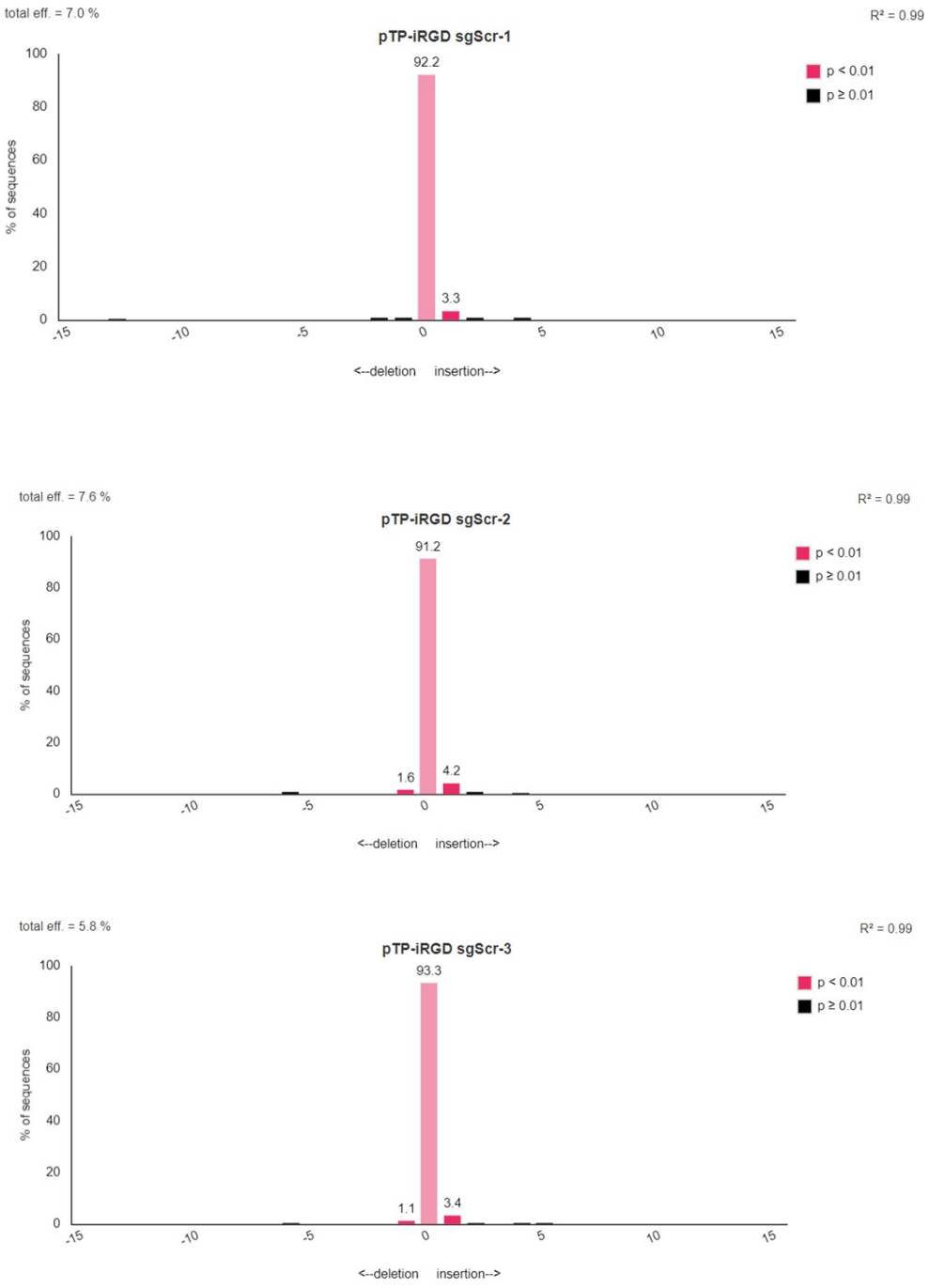


Fig. S13 (c) Quantification of InDels in pTP-iRGD sgScr (negative control) samples using TIDE Analysis: TIDE Analysis was performed using a reference sequence from untreated samples and setting the parameters to detect a maximum InDel size of 15 nucleotides.

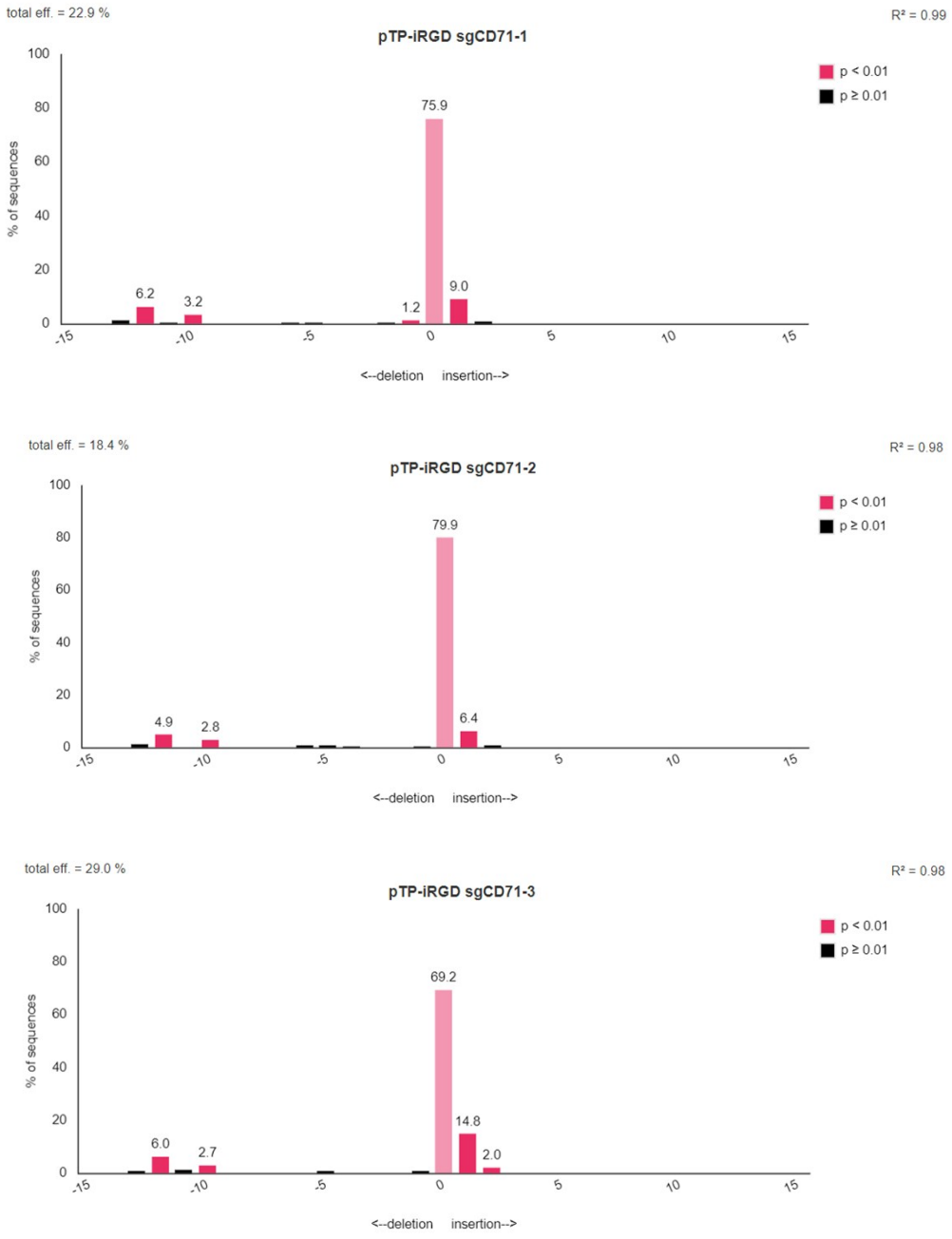


Fig. S13 (d) Quantification of InDels in pTP-iRGD sgCD71 samples using TIDE Analysis: TIDE Analysis was performed using a reference sequence from untreated samples and setting the parameters to detect a maximum InDel size of 15 nucleotides.

Methods and Materials

CRISPR-GPS components

sgGFP1 and sgScr guide RNAs were generated by using an *in vitro* transcription and screening Kit for sgRNA (Clontech) with previously described changes in the protocol (Ref). 5'-Cy5 labeled sgRNA was custom synthesized by Gene Link. A sample of sgFRT was obtained as a gift from Prof. Tyler Jack's lab at the Massachusetts Institute of Technology. Only recombinant Cas9 protein containing Nuclear localization signal (NLS) were used for developing CRISPR-GPS and were purchased from different vendors including PNA Bio (1 mg/mL and 5 mg/mL), Clontech (Guide-it™ Recombinant Cas9 with C-terminal NLS, 3 mg/mL) and NEB (EnGen® Cas9 with dual NLS, 20 μM). Cy3-labeled Cas9 (CP06, 5 mg/mL) was commercially obtained from PNA Bio. The pTP-iRGD peptide was synthesized by CPC Scientific.

Gel retardation assay with labeled Cas9 and sgRNA

The degree of complexation of nanocomplexes was measured by developing a fluorescence-based a gel retardation assay using a non-denaturing agarose gel. To develop this assay, we used a 5'-Cy5 labeled sgRNA (Gene Link) and a Cy3 labeled Cas9 (PNA Bio) along with the unlabeled pTP-iRGD peptide. For the experiment indicated in the figure S1, the three components of the nanocomplexes: the Cy5-sgRNA, the Cy3-Cas9 protein, and the pTP-iRGD peptide were combined in all three possible permutations in water. For the first set of samples, Cy5-sgRNA (2.5 μL, 1 μM) and Cy3-Cas9 (2.5 μL, 1 μM) were mixed together and incubated for 5 minutes at room temperature, followed by the addition of an equal volume of pTP-iRGD (5 μL, 15 μM). For the second set, sgRNA (2.5 μL, 1 μM) and pTP-iRGD (2.5 μL, 30 μM) were mixed together in equal volumes and then incubated for 5 minutes at room temperature, followed by the addition of Cas9 protein (5 μL, 0.5 μM). For the third set, Cas9 (2.5 μL, 1 μM) and pTP-iRGD (2.5 μL, 30 μM) were mixed together in equal volumes, followed by the addition of sgRNA (5 μL, 0.5 μM). A total of 10 μL of nanocomplex mixtures were formulated and after the addition of the last component in each set, samples were incubated for 15 minutes and then 2 μL of 40% glycerol was added before running on a 1.5% agarose gel at 170 V for 20 minutes.

For the results indicated in Figure 2a, a total of 10 μL of nanocomplex mixtures were formulated in a series of concentrations with increasing amounts of peptide. For sgRNA/Cas9/peptide samples, both Cy5-sgRNA and Cy3-Cas9 were mixed with the different concentrations of peptide (5 μL of 0.5 μM, 2.5 μM, 5 μM, 7.5 μM, 15 μM, or 30 μM) by following the protocol described in the first set above. Same protocol was used for other set of samples with following modifications. For sgRNA/peptide sample, the volumes of Cy3-Cas9 was replaced with the RNase-free water. For Cas9/peptide sample, the volumes of Cy5-sgRNA was replaced with the RNase-free water.

Dynamic light scattering (DLS) and zeta potential

To prepare nanocomplexes for size analysis, unlabeled sgRNA, Cas9 and peptides were used to avoid any interference with the laser of DLS instrument. To prepare, sgRNA/Cas9/peptide nanocomplexes, sgRNA (12.5 μL, 1 μM) and Cas9 (12.5 μL, 1 μM) were mixed together and incubated for 5 minutes at room temperature, followed by the addition of an equal volume of peptide pTP-iRGD (25

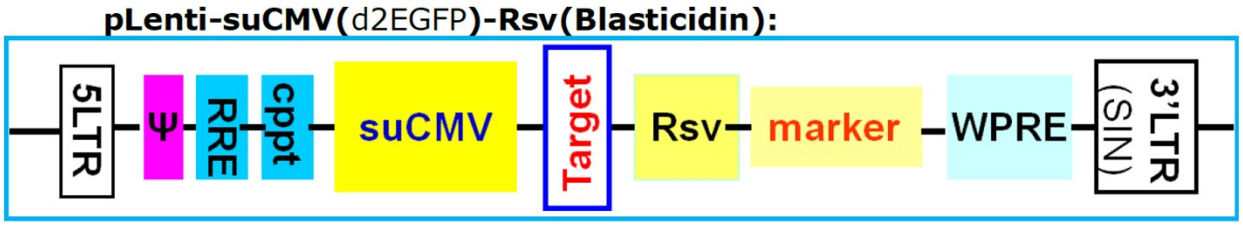
μL, 7.5 μM for 15x or 25 μL, 15 μM for 30x). Size was measured using the Zetasizer Nano ZS (Malvern) after incubating the sample for 15 minutes at room temperature to effect nanocomplexation. Nanocomplexes were further diluted to 1 mL using RNase free water and then transferred to a folded capillary zeta cell (Malvern) for Zeta potential measurement.

TEM analysis

To prepare, sgRNA/Cas9/peptide nanocomplexes for TEM analysis, sgRNA (2.5 μL, 1 μM) and Cas9 (2.5 μL, 1 μM) were mixed together and incubated for 5 minutes at room temperature. Then equal volume of peptide pTP-iRGD (25 μL, 7.5 μM for 15x or 25 μL, 15 μM for 30x) was added and incubated for 15 minutes. Samples were then placed on carbon-coated grids and then negatively stained with 2% uranyl acetate.

Generation of cell lines

HeLa cells expressing destabilized GFP (HeLa-d2eGFP) were received as a gift from Prof. Phillip A. Sharp at the Massachusetts Institute of Technology. 3TZ cells expressing FRT-STOP-FRT-GFP cassette (3TZ/ FRT-STOP-FRT-GFP) were graciously gifted by Tyler Jack's lab at the Massachusetts Institute of Technology. Both HeLa-d2eGFP and 3TZ/ FRT-STOP-FRT-GFP cell lines were cultured using Dulbecco's Modified Eagle Medium (Corning, 10-017-CV) containing 100 U/mL of Penicillin-Streptomycin (Thermo Fisher Scientific) and supplemented with 10% fetal bovine serum (Sigma) and maintained at 37 ^{o}C in the presence of 5% CO_2. OVCAR8 cells expressing destabilized GFP (OVCAR8/d2eGFP) was generated by transducing OVCAR8 cells with a custom designed lentiviral particles (Gentarget Inc.) carrying a modified d2eGFP sequence under an enhanced suCMV promoter along with blasticidin marker under a RSV promoter (Scheme 1) by following manufacturer's protocol.



Scheme 1: Schematic representation of custom lentiviral particles provided by Gentarget Inc., where 'Target' represent d2eGFP sequence and 'marker' indicates antibiotic selection marker, blasticidin.

Similarly, OVCAR8 cells co-expressing eGFP and RFP (OVCAR8/eGFP/RFP) were generated by transducing a commercially available GFP-His-RFP (Bsd) lentiviral particles (Gentarget Inc., LVP002) by following manufacturer's protocol. These particles express blasticidin selection marker under RSV promoter and they bicistronically express GFP and RFP under the suCMV promoter that are later processed by F2A peptide-mediated self-cleavage. Both OVCAR8/d2eGFP and OVCAR8/eGFP/RFP cells were cultured using RPMI-1640 Medium (ATCC) containing 100 U/mL of Penicillin-Streptomycin (Thermo Fisher Scientific) and supplemented with 10% fetal bovine serum (Sigma) and maintained at 37 ^{o}C in the presence of 5% CO_2.

Briefly, cells were plated in a 24-well plate at a confluency of 100,000 cells/well a day before the transduction. Transduction was performed by following manufacturer's protocol and then three days after transduction, cells were sorted by flow cytometry for GFP+ cells for OVCAR8/d2eGFP and GFP+/RFP+ double-positive cells for OVCAR8/eGFP/RFP. After sorting, cells were expanded for 2 days and then maintained under antibiotic selection at a final concentration of 5 μg/mL of Blasticidin for a couple of weeks.

Dynamic light scattering (DLS) and zeta potential for pH adjustment studies

To prepare nanocomplexes for size analysis, unlabeled sgRNA, Cas9, and peptides were utilized in order to avoid any interference with the laser of the DLS instrument. To adjust sample pH, buffers were prepared from RNAse free water at pH values of 5, 6, 7 and 8. The pH of the RNAse free water was adjusted with either 0.5M NaOH or 0.3% HCl to achieve desired reading. The sgRNA, Cas9, and peptides were then diluted from their stock concentration to 1 μM (15 μM for peptide) using different pH buffers. After dilution, the Cas9 (12.5 μL, 1μM) and sgRNA (12.5 μL, 1μM) were mixed together and incubated for 5 minutes at room temperature. Following the addition of an equal volume of diluted peptide pTP-iRGD (25 μL, 15 μM) for a 1:1:30 ratio of Cas9:sgRNA:pTP-iRGD, the samples were incubated for 15 minutes at room temperature, allowing for nanocomplexation. After incubation, a small quantity (3 μL) of the sample was loaded onto a low volume disposable sizing cell (Malvern) and its size was measured on a Malvern Zetasizer Ultra. The remaining sample was pipetted into a disposable folded capillary cell (Malvern DTS1070) filled with an appropriate pH buffer. Zeta potential of the sample was then measured using the same Malvern Zetasizer Ultra. For the Cas9-only measurement, the sgRNA and peptide were omitted; make-up buffer was added to dilute the 12.5 μL of 1uM Cas9 to a final volume of 50 μL.

Transfection Protocol

For transfection, cells were plated in a 96-well plate at a confluency of 8,000 cells/well in 100 uL of culture media/well and 24 hours post-plating, cells were treated with nanocomplexes or lipoplexes. Nanocomplexes were formulated by combining sgRNA (5 μL or 2.5 μL, 1 μM) and Cas9 (5 μL or 2.5 μL, 1 μM) followed by 5-minute incubation at room temperature and then addition of equal volume of pTP-iRGD (10 μL or 5 μL, 15 μM) in a total volume of 10 μL of RNase free water keeping the ratio of sgRNA: Cas9:pTP-iRGD as 1:1:30 unless otherwise indicated in the figure. Opti-MEM was then added to make up the volume of the nanocomplex transfection mix/well up to 100 uL before treating with the cells. For lipofection, sgRNA (2.5 μL, 1 μM) and Cas9 (2.5 μL, 1 μM) were incubated for 5 minutes and this RNP mix was then diluted to 50 μL with Opti-MEM. In parallel, 0.3 μL/well of RNAiMAX (Thermo Fisher Scientific) was diluted in 50 μL of Opti-MEM and then RNP mix was added to the RNAiMAX to form 100 μL of lipoplexes. Both the nanocomplex and lipoplex mixtures were added to cells approximately 24 hours after plating and cells were exposed to transfection mix for 6 hours, after which the mix was replaced with fresh media. After 2-3 days, cells were trypsinized using 150 μL of Trypsin-EDTA (0.25%) and then transferred to a U-bottom 96-well plate for FACS and analyzed by flow cytometry.

Cytotoxicity studies

Ovcar8/eGFP/RFP cells were transfected in a black clear bottom 96-well plate with CRISPR-GPS nanocomplexes, as described above in the transfection protocol. After 72 hours cells, cytotoxicity was determined by treating cells with PrestoBlue reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Fluorescence intensity was measured using a microplate reader (Tecan) with an excitation wavelength of 560 nm (bandwidth 9 nm) and emission at 590 nm (bandwidth 20 nm).

Staining of receptors and FACS analysis

To determine the level of integrin expression, HeLa/d2eGFP and OVCAR8/d2eGFP cells were stained with antibodies against αvβ3 (anti-human, PE-conjugated mouse antibody, R&D Systems) or IgG1 control and then analyzed by flow cytometry. Briefly, 500,000 cells were suspended in 1% BSA/PBS on ice and stained with 1:5 diluted antibodies in 1% BSA/PBS (200 μL/sample) for 1 hour on ice protected from light and then cells were washed once with 1% BSA/PBS (500 μL/sample) and analyzed by flow cytometry.

Method for staining of receptors and FACS analysis for 3TZ cells:

To determine the level of integrin expression, 3TZ cells were stained with antibodies against αvβ3 (anti-human, PE-conjugated mouse antibody, Santa Cruz Biotechnology sc-7312 PE) or the IgG1 control (Santa Cruz Biotechnology sc-2866) and then analyzed by flow cytometry. Briefly, 500,000 cells were suspended in 1% BSA/PBS and stained with 1:40 diluted antibodies in 1% BSA/PBS (200 μL/sample) for one hour on ice protected from light. Cells were then analyzed by flow cytometry.

Transfection of cells with labeled Cas9 and sgRNA for Wide field microscopy

Ovcar8/d2eGFP cells were plated in a 24-well plate on gelatin coated cover slips at confluency of 100,000 cells/well. After approximately 24 hours, cells were treated with nanocomplexes containing 50 nM of 5'Cy5-labeled sgRNA and 50 nM of Cy3-labeled Cas9 and 1500 nM of pTP-iRGD in 500 uL of OptiMEM. The cells were fixed using 4% paraformaldehyde at different time points as indicated in the figures and then imaged after 3h or 24 h using an Olympus FV1200 Laser Scanning Confocal Microscope with oil-immersed 60X/1.40, Plan Apo, IX70 objective.

Indel identification by colony sequencing

To confirm that CRISPR-GPS facilitated disruption of GFP is mediated formation of indels in the DNA, we used Guide-it™ Indel Identification Kit (Clontech) by following manufacturer's protocol with the following changes. After following the transfection protocol with HeLa/d2eGFP cells for 3 days in a 96-well plate, media was removed and cells were treated with 20 μL of DNA QuickExtract™ solution (Epicentre) and were scrapped out of the well using a pipette and then the sample was then heated at 65 °C for 15 minutes, 68 °C for 15 minutes and 98 °C for 10 minutes using a thermocycler for DNA extraction.

The resulting crude lysate then underwent PCR amplification for the genomic target as per manufacturer's protocol using 15 μM stocks of GFP-indel-PCR forward and reverse primers we designed

(Table 1d) to target the d2eGFP gene. The Nucleospin PCR Clean-up Kit was used purify the resulting DNA product. The cloning of PCR product was performed using manufacturer's guidelines but with half the volumes suggested. Consequently, Stellar Competent Cells (stored at -80C) were thawed in an ice bath, mixed gently, then portioned off by 50 L into Falcon tubes, to which 2.5 L of cloning product was added. Tubes were placed on ice for 30 min, followed by a heat shock for 45 seconds at 42 ^{o}C, and finally 1-2 minutes in ice. Following transformation, we added 940 μL of warmed SOC media and placed it on a shaker for 45 min in 50 ^{o}C at 160 rpm. Next, 400 μL of the mixture was spread on pre-prepared LB/Amp agar plates (with at least 20 mL of agar/plate) using cell spreaders. Plates were incubated overnight, after which colonies were picked from the plates and went through colony PCR using colony PCR primer sets (Table 1d), as per manufacturer's protocol but using half the volumes of all reagents. 5 μL of the subsequent PCR product was combined with 1 μL of loading dye and run on an agarose gel to ensure presence of desired product. The remaining DNA was purified using the Nucleospin PCR Clean-up Kit.

Measuring InDel frequencies using TIDE Analysis

For TIDE analysis, genomic DNA was extracted from cells 3 days after transfection using Lucigen QuickExtract DNA Extraction Solution (Epicentre) following the manufacturer's protocol. The pelleted cells were briefly resuspended in QuickExtract solution and incubated at 65 °C for 15 min, 68 °C for 15 min and 98 °C for 10 min. The genomic region flanking the CRISPR target site was PCR amplified (target sites and primers listed in Table 1b and 1d). The final products were purified using New England Biolab's Monarch® PCR & DNA Cleanup Kit following manufacturer's protocol and Sanger sequenced at GENEWIZ. Each sequence chromatogram was analyzed with TIDE (Tracking of InDels by decomposition)[58] software available at https://tide.deskgen.com/.

Analysis was performed using a reference sequence from the untreated samples. Parameters were set to detect a maximum indel size of 15 nucleotides. The decomposition window was adjusted to cover the largest possible range with high quality traces.

Sequence details

Table 1a: Custom d2eGFP and CD71 sequence for OVCAR8/d2eGFP cells (Bold denotes confirmed region by sequencing)

d2eGFP:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCAT**CCTGGTCGAGCTGGACGGCGACGTAAACG**
GCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGC
ACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCGCCCGCT
ACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCT
TCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCAT
CGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGC
CACAAGGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGACCCGCCACAACATCG
AGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC
GACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCT
GGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGAAGCTTAGCCATGGCTTCCCGCC

GGAGGTGGAGGAGCAGGATGATGGCACGCTGCCCATGTCTTGTGCCCAGGAGAGCGGGATGGACCGTCACCCTG
CAGCCTGTGCTTCTGCTAGGATCAATGTGTAG

CD71:

GAAGGCAGAGAGAAGGGAAGGGACGAGAGGCGAAGGGACGAGAGGCGCAGGGACGCCTCT**GCGCA**
CAGCCGCCGACCCCGTGTACCTGCAGCCGCTCGAGGCGCCGCCACAGCCCCTTCCCCACAGGCTCGCG
GCCCCGCTCCATCCCGGCCGCCCCGCCTGGGGCCGCGACCGCTTTCCCGCTCCCCGTGCGGCTCCCTATG
GCTGCTGGACAGCACCGGAGCGGCCGCTCCAGCCAGCCCCGCAGGATGAAGGGAGGACACGAGGGT
CGGTGTAGTTCTAGAAGCCCGCACTCACACTAGCGCGTCCTCCGTCCCGAGCCGCCACCCGATATCCCG
ACGCTCTGAGGGGATGGCGGCCCCTAACCGGCGGTTTATAGCCTGGCCCCGCCCCCGGCCCCCAGGGG
GGCTGTGCGTCACTTCCTGAGGCACGTACGCTCGCGAGGTGCTCTGACAGATCGCCCGGGGGGCGGG
GATGCGCGCGCAGCGGGCGGGGCCCTATGCGGAGGGCGCTGCGGGCGAGGCGAAGGCCAAGGGAA
AAGGCGCCAAGGCAGCAGGTGCCCCGAGGAGCCGCGCGATGGGCGCACTGGCCTCTGCCTCGGCGCC
TTCCTCCACCCTGGTAGCCTGGCAGCCTCCGCCTGACCTTGACCAACCTCCAGTCCCTGCAGTGCTAGGT
CCAATCTGTTGGCGTTGACGCTTAAATGTTGGATATTGCACTGCGGC

Table 1b: sgRNA targets

sgRNA	Target DNA	Length of target region
sgGFP1	**GGGCACGGGCAGCTTGCCGG**	20
sgScr	**GGGCCGATTCGCGAGCGGCG**	20
sgFRT	**TCCTATTCTCTAGAAAGTAT**	20
sgCD71	**GGGATATCGGGTGGCGGCTC**	20

Table 1c: Synthetic sgRNA with chemical modifications

sgRNA	Sequence ordered
5’ Cy5-sgGFP1	5’[Cy5]**GGGCACGGGCAGCUUGCCGG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU 3’

Table 1d: Primer sequences

Primer name	Forward or Reverse	Sequence
GFP-indel-PCR	Forward	CGGTACCCGGGGATCCTGGTCGAGCTGGACGGCGACG
	Reverse	CGACTCTAGAGGATCCACGAACTCCAGCAGGACCATG
Colony-PCR	Forward	CGTTGTAAAACGACGGCCAGTGA
	Reverse	CAATTTCACACAGGAAACAGCTATGACC
CD71-PCR	Forward	AAGGCAGAGAGAAGGGAAGG
CD71-PCR	Reverse	GCCGCAGTGCAATATCCAACA