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Supplementary Information

G-quadruplex motif at the 3' end of sgRNAs improves CRISPR-Cas9 based genome editing efficiency

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Material and Methods

Expression and purification of *S. pyogenes* Cas9:

pET-28b-Cas9-His was a gift from Alex Schier (Addgene plasmid # 47327).[1] *E. coli* BL21 DE3 strain was transformed with pET-28b-Cas9-His plasmid containing *S. pyogenes* Cas9 fused with 6xHis tag. The strain was inoculated in 10 ml Luria Bertani (LB) broth containing 100ug/ml of Ampicillin and 30ug/ml of Chloramphenicol at 37°C for overnight. The cells were inoculated in 1 litre of LB broth and grown at 37°C to OD_{600} = 0.4-0.6. Subsequently, 0.1mM of isopropyl β-D-1- thiogalactopyranoside (IPTG) was added to the culture for induction and incubated at 18°C for overnight. Next day, cells collected by centrifugation at 6000Xg for 5minutes at 4°C and resuspended in lysis buffer (20 mM Tris pH-8, 500mM NaCl, 5% glycerol, 1X protease inhibitor cocktail, 0.1 mg/ml lysozyme and 200 mM PMSF). The cells were lysed and sonicated (5 sec pulse ON, 30 sec pulse OFF, Amplitude 35% for 5 min total), and the cell debris was pelleted by centrifugation at 20000Xg for 1 hr.

The lysate supernatant was added with the cOmplete His-tag purification (Nickel-NTA) resin (Roche) at 4°C for 1 hr with gentle rotation. The resins were transferred to a 30ml Column and washed with 10 column volumes of the wash buffer (20mM Tris pH-8, 500mM NaCl, 5mM Imidazole). The resin along with Cas9 was incubated with 30ml of elution buffer (20 mM Tris pH-8, 500mM NaCl, 500mM Imidazole) at 4°C for 1 hr with gentle rotation. The Cas9 was eluted and the concentrated by Amicon ultracentrifugal filter (Millipore, 100-Kda MW cut-off). The eluted Cas9 further purified with superdex 200 column (GE Healthcare) in final buffer (20mM Tris pH-8, 500mM KCl, 10mM $MgCl_2$, 10% glycerol, and 1mM TCEP). The eluted fractions were stored in aliquots at -80°C.

In Vitro transcription

DNA template for sgRNA IVT were prepared by using 52-nt long forward primer containing T7 Promoter, 20-nt guide sequence and 15-nt overlapping sequence to the reverse primer which has whole tracrRNA sequence (variable length) given in supplementary table no. 1. In a 50 ul reaction, forward and reverse primer were mixed in equal amounts along with 10X PCR buffer, dNTPs (2.5mM) and *Taq* polymerase. The template was made by annealing and

extension PCR reaction as: 95°C- 3 min; 30 cycles of 95°C- 10sec, 45°C-30 sec, 72°C- 30 sec; final extension 72°C- 7 min. RNA was transcribed using T7 MegaScript Kit (Ambion) with modification of using 5ul of PCR product of DNA template and 5% of Dimethylsulfoxide (DMSO) in per reaction as per the manufacturer's instruction. *In vitro* transcribed were treated with Turbo DNase (Ambion) and purified with NucAway column as per the manufacturer's instruction.

Electromobility Shift Assay

pET-dCas9-VP64-6xHis was a gift from David Liu (Addgene plasmid # 62935).[2] dCas9-VP64 protein was purified using same protocol as described above for SpCas9 protein. For electro mobility shift assay, dCas9-VP64 (50-200nM): sgRNA (100-400nM) complex was made in the ratio of 1:2 at 25°C for 10 min. Then 100ng of the dsDNA substrate was added to the complex and incubated at 37°C for 15 min in 15ul reaction volume. All the samples were run on the 8% Native PAGE for 2-3 hrs at 4°C. The gel was stained with SYBR Gold dye and visualised in Typhoon.

***In Vitro* Cleavage**

In vitro cleavage of plasmid DNA was performed as; Cas9 protein (50nM) and sgRNA (150nM) was incubated at 25°C for 10 min to make Cas9-sgRNA complex. Then 200ng of 700bp long PCR product of eGFP with target sequence was added and incubated at 37°C for 1 hr in 15ul of reaction buffer containing 20mM Tris pH-8, 200mM KCl, 10mM $MgCl_2$ and 1mM TCEP. PCR for 700bp product was done using *Taq* DNA polymerase (GeneAid) with PCR gene specific primers using conditions: 95°C for 5 min; 35 cycles (95°C for 30 s, 58°C for 30 s, 72°C for 30 s) and 72 °C for 7 min. Reaction products were resolved on an ethidium-bromide-stained 1% agarose gel and then visualized using SynGene G: BOX.

Serum Stability

IVT purified sgRNA, 3µM each of unmodified RNA, 3′G2U1 RNA, 3′G3U3 RNA were incubated with 10% FBS at 37°C. An aliquot (10µl) was removed from the incubation at the each of the eight-time points i.e. 0, 15, 30, 60, 90, 120, 150, 180 min. Every reaction was stopped by snap freezing the aliquot in liquid nitrogen and their degradation over the time was analyzed on 1.5% agarose gel. The RNA intensity that remained at each time point was measured using open source ImageJ software. The plot represents three replicates wherein each time point has been normalized with the RNA remaining at 0min time point and the gel

image depicts one of the replicate. The RNA used were already prepared for formation of G quadruplex structure using G-quadruplex forming buffer (10mM sodium cacodylate and 100 mM potassium chloride) and denaturation followed by slow cooling. The unmodified RNA was also provided the same G-quadruplex buffer conditions to keep the buffer same for all the RNA samples.

Cell culture and transfection.

Mouse embryonic stem cells (Oct4-GFP) [3] were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 4.5 g l–1 D-glucose and pyruvate, 20% FBS (Pan Sera), 30 μM 2-mercaptoethanol, 300 units of penicillin-streptomycin (Invitrogen), 0.6X nonessential amino acids and 8 ng of leukemia inhibitory factor (LIF). Medium was changed every 24 h and ESCs were split every 2-3 days using Trypsin-EDTA treatment. The cells were seeded in gelatine coated 12 well plate at a seeding density of 5 x 10^4 cells/well. For Cas9 mRNA transfection, 800 ng of cas9 mRNA (Invitrogen) was added to 50 ul OPTIMEM media followed by addition of 400 ng of sgRNAs. Lipofectamine 3000 was added to the OPTIMEM media according to manufacturer's protocol. The 100μl mixture was incubated for 30 min prior to addition in the cells. The cells were incubated for 48 h at 37°C in incubator having 5%CO_2.

Flow Cytometry

At the designated time points after transfection, mESCs-eGFP were washed with ice cold 1X PBS. Next, the cells were trypsinized and pelleted by centrifugation at 200*g* for 5 min at 4°C, following which the supernatant was aspirated and decanted. The pellet was resuspended in ice cold 1X PBS and analysed in BD Accuri C6 flow cytometer.

Lactate Dehydrogenase (LDH) assay

The mESCs were seeded in 96 well plates at a density of 8000cells/well. The cells were transfected with various sgRNA-RNP complexes (10nM of Cas9 protein and 24nM of sgRNA) and incubated for 48 hrs in humidified incubator at 37°C. For LDH measurement, the LDH Cytotoxicity Detection Kit (Roche) was used according to the manufacturer's recommendations. The supernatant of the cells was reacted with the freshly prepared reaction mixture and incubated for 20 min protected from the light. The percentage of LDH release was calculated compared to 100% cell lysis control (cells lysed with lysis buffer provided in

the kit) for 10 min. LDH released into the supernatant from damaged cells is a biomarker for cytotoxicity.

Zebrafish husbandry and Microinjections:

Zebrafish utilized in the work were accommodated in CSIR-Institute of genomics and integrative biology and employed in the study in accordance to the guidelines specified by the Animal Ethics Committee of the institute. To obtain the embryos crosses were set among the double transgenic Tg*(fli1:EGFP, gata1a: DsRed)* zebrafish or wild-type *ASWT* zebrafish. 50pg of various sgRNAs and 500pg of Cas9 protein were injected consecutively into single cell zebrafish embryos using microinjection techniques as explained previously. Injected Tg*(fli1:EGFP, gata1a: DsRed)* zebrafish embryos were maintained in 0.003% phenyl thiourea in embryo water(5mM NaCl, 0.17mM KCl, 0.33mM CaCl, 0.33 $MgSO_4$) and ASWT embryos were maintained in 0.05% methylene blue in embryo water.

DNA isolation and T7 Endonuclease Assay

For DNA isolation, 7 embryos per tube were freezed in replicates. Genomic DNA was isolated as mentioned previously. In short, embryos were grinded in 50ul of the DNA isolation buffer (composition) and further 670ul of buffer was added. 1mg of proteinase K was added and solution was incubated at 65°C for about 3 hrs with regular vortexing. Protein were separated by adding 60ul of 8M KCl and centrifugation. Supernatant was used for precipitating the nucleic acids using isopropanol and separated using centrifugation. Finally, pellet was washed with 70% ethanol and dissolved in nuclease free water (Ambion, USA). 50ng of the DNA was used for amplification of the targeted region using the oligos (supplementary table no. 1). 1µg of the PCR amplicon was used and proceeded for the T7 endonuclease assay. For T7EI assay, PCR was done using *Taq* DNA polymerase (Thermo Scientific) with gene-specific primers using the following conditions: 95°C for 3 min; 35 cycles (95°C for 30 s, 54°C for 30 s, 72°C for 30 s) and 72 °C for 7 min. PCR products were run on 2% agarose gels showing single band, PCR cleaned, denatured, annealed and treated with T7EI (New England Biolabs) following the manufacturer's instruction After the T7 endonuclease treatment products were visualized on 2% agarose gel and imaged using SynGene G:BOX. The gel images were quantified using the Image J software and indel frequency was calculated by applying the method as recommended previously. The indel frequencies were plotted into box plot in R using ggPlot.

Identification of off- targets

In order to identify the potential off targets of sgRNA targeting eGFP, we employed the Cas-OFFinder tool.[4] We set the following parameters- PAM type: 5'-NGG-3', Target genome- *Danio rerio* (GRCz10), Mismatch number: 3, DNA bulge size:1, RNA bulge size: 1. The top four predicted regions with highest number of off- target hits were chosen for determining possible off- target effects of the sgRNA. Primers were designed around the potential off-target site with an approximate amplicon size of 200 bp.

Amplicon sequencing

For amplicon sequencing 50ng of the genomic DNA was used for amplification of the targeted region and regions arround the potential off- target sites using the oligos as mentioned in supplementary table no. 2 and table no.3 respectively. The PCR amplicons were purified and preceded further for amplicon sequencing.

Amplicon sequencing done using ion torrent platform-

For the potential off- target sites, we designed fusion primers using manual ion amplicon library preparation (Fusion Method) (Publication Number 4468326, Revision C). The amplicons were design in such a way such that the read length is 200 bp for all amplicons. Most of the amplicons were fell into the size range of ~190-220 pb including adapter indexes. For on- target amplicon sequencing library prepration were done using manual ion xpres Plus gDNA and amplicon library preparation (Publication Part Number 4471989 Rev. B).

After PCR amplification equal amount of amplicon ware pooled and Template preparation (emulsion PCR) was done using manual Ion PG Hi‑Q OT2 Kit User guide (Catalogue Number A27739 Publication Number MAN0010902, Revision B.0). The sequencing was done in 318-Chip using manual Ion PGM Hi‑Q Sequencing Kit user guide (Publication Number MAN0009816 Revision E.0) in ion PGM instrument.

Data Analysis-

The QC of output fastq file ware done using Fastqc tools and low-quality data were removed using Trimomatic (v0.43) tool. To calculate the % indel in on and off target site we developed the in house screpts in python. The script is written in such a way that it searches matching sequence up and down stream of potential target design and count the total no of variant sequence in between flanking reson from the reference sequence, which will be the

potential indel frequency. The result is further confirmed by an independently developed tool, Cas analyser [5] (http://www.rgenome.net/cas-analyzer).

Supplementary Table no. 1- Primer detail of different sgRNAs used in the study

Primer	Sequence
eGFP_sgRNA_F.P	TAATACGACTCACTATAGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
Tyr_sgRNA_F.P	TAATACGACTCACTATATGTCCAGTCTGGCCCGGCGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
Tert_sgRNA_FP	TAATACGACTCACTATAGGCGGAGCTGGAAGGTGAAGGTTTTAGAGCTAGAA
cmyc_sgRNA_FP	TAATACGACTCACTATAGGGCAGAGGGAGCGAGCGGGGTTTTAGAGCTAGAA
3′ G2T1_R.P	CCACCACCACCAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCT
3′ G3T3_R.P	CCCAAACCCAAACCCAAACCCAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCT
3′ H.P_R.P	AAAAAGCGCGCGCGCAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
5′ G2T1_F.P_eGFP	TAATACGACTCACTATAGGTGGTGGTGGTTTTGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
5′ G3T3_F.P_eGFP	TAATACGACTCACTATAGGGTTTGGGTTTGGGTTTGGGTTTTGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
5′ H.P_FP_eGFP	TAATACGACTCACTATAGCGCGCGCGCTTTTTGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
5′ G2T1_F.P_Tert	TAATACGACTCACTATAGGTGGTGGTGGTTTTGGCGGAGCTGGAAGGTGAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
5′ G3T3_F.P_Tert	TAATACGACTCACTATAGGGTTTGGGTTTGGGTTTTGGCGGAGCTGGAAGGTGAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA

5′ H.P_FP_Tert	TAATACGACTCACTATAGCGCGCGCGCTTTTTGGCGGAGC TGGAAGGTGAAGGTTTTAGAGCTAGAAATAGCAAGTTAA AATAA
5′ G2T1_F.P_cmyc	TAATACGACTCACTATAGGTGGTGGTGGTTTTGGGCAGAG GGAGCGAGCGGGGTTTTAGAGCTAGAAATAGCAAGTTAA AATAA
5′ G3T3_F.P_cmyc	TAATACGACTCACTATAGGGTTTGGGTTTGGGTTTTGGGC AGAGGGAGCGAGCGGGGTTTTAGAGCTAGAAATAGCAAG TTAAAATAA
5′ H.P_FP_cmyc	TAATACGACTCACTATAGCGCGCGCGCTTTTTGGGCAGAG GGAGCGAGCGGGGTTTTAGAGCTAGAAATAGCAAGTTAA AATAA
Universal_R. P	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Supplementary Table no. 2- Primer detail of different primers used for on-target amplification in the study

Primer	Sequence
eGFP_PCR_F.P	CAAGGGCGAGGAGCTGTTCAC
eGFP_PCR_R.P	CTCGTCCATGCCGAGAGT
Tyr_T7EI_F.P	CGGTGTGTGTGAAGCATCTCG
Tyr_T7EI_R.P	GCAGGTTTGGTTGTAAAACAC

Supplementary Table no. 3- Primer detail of different primers used for off-target amplification in the study

S.NO	PRIMER NAME	SEQUENCE
1	GFP_OFF_1_ID_1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGG TAACGATGTCTCAGTGTGGACCTAAAG
2	GFP_OFF_1_ID_2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGA GAACGATGTCTCAGTGTGGACCTAAAG
3	GFP_OFF_1_ID_3	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGG ATTCGATGTCTCAGTGTGGACCTAAAG
4	GFP_OFF_1_ID_4	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAA GATCGATGTCTCAGTGTGGACCTAAAG
5	GFP_OFF_1_ID_5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAG GAACGATGTCTCAGTGTGGACCTAAAG
6	GFP_OFF_1_ID_6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAA GTTCGATGTCTCAGTGTGGACCTAAAG
7	GFP_OFF_1_ID_7	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGA TTCGATGTCTCAGTGTGGACCTAAAG
8	GFP_OFF_1_ID_8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGAT AACGATGTCTCAGTGTGGACCTAAAG
9	GFP_OFF_1_ID_9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCG GAACGATGTCTCAGTGTGGACCTAAAG
10	GFP_OFF_1_ID_10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACC GAACGATGTCTCAGTGTGGACCTAAAG
11	GFP_OFF_1_ID_11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGA ATCGATGTCTCAGTGTGGACCTAAAG
12	GFP_OFF_1_ID_12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTG GTTCGATGTCTCAGTGTGGACCTAAAG
13	GFP_OFF_2_ID_1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGG TAACGATGGTGAAGGCTCTTGTGTTGA
14	GFP_OFF_2_ID_2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGA GAACGATGGTGAAGGCTCTTGTGTTGA

15	GFP_OFF_2_ID_3	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCGATGGTGAAGGCTCTTGTGTTGA
16	GFP_OFF_2_ID_4	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGATGGTGAAGGCTCTTGTGTTGA
17	GFP_OFF_2_ID_5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGATGGTGAAGGCTCTTGTGTTGA
18	GFP_OFF_2_ID_6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCGATGGTGAAGGCTCTTGTGTTGA
19	GFP_OFF_2_ID_7	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCGATGGTGAAGGCTCTTGTGTTGA
20	GFP_OFF_2_ID_8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACGATGGTGAAGGCTCTTGTGTTGA
21	GFP_OFF_2_ID_9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACGATGGTGAAGGCTCTTGTGTTGA
22	GFP_OFF_2_ID_10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACGATGGTGAAGGCTCTTGTGTTGA
23	GFP_OFF_2_ID_11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCGATGGTGAAGGCTCTTGTGTTGA
24	GFP_OFF_2_ID_12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCGATGGTGAAGGCTCTTGTGTTGA

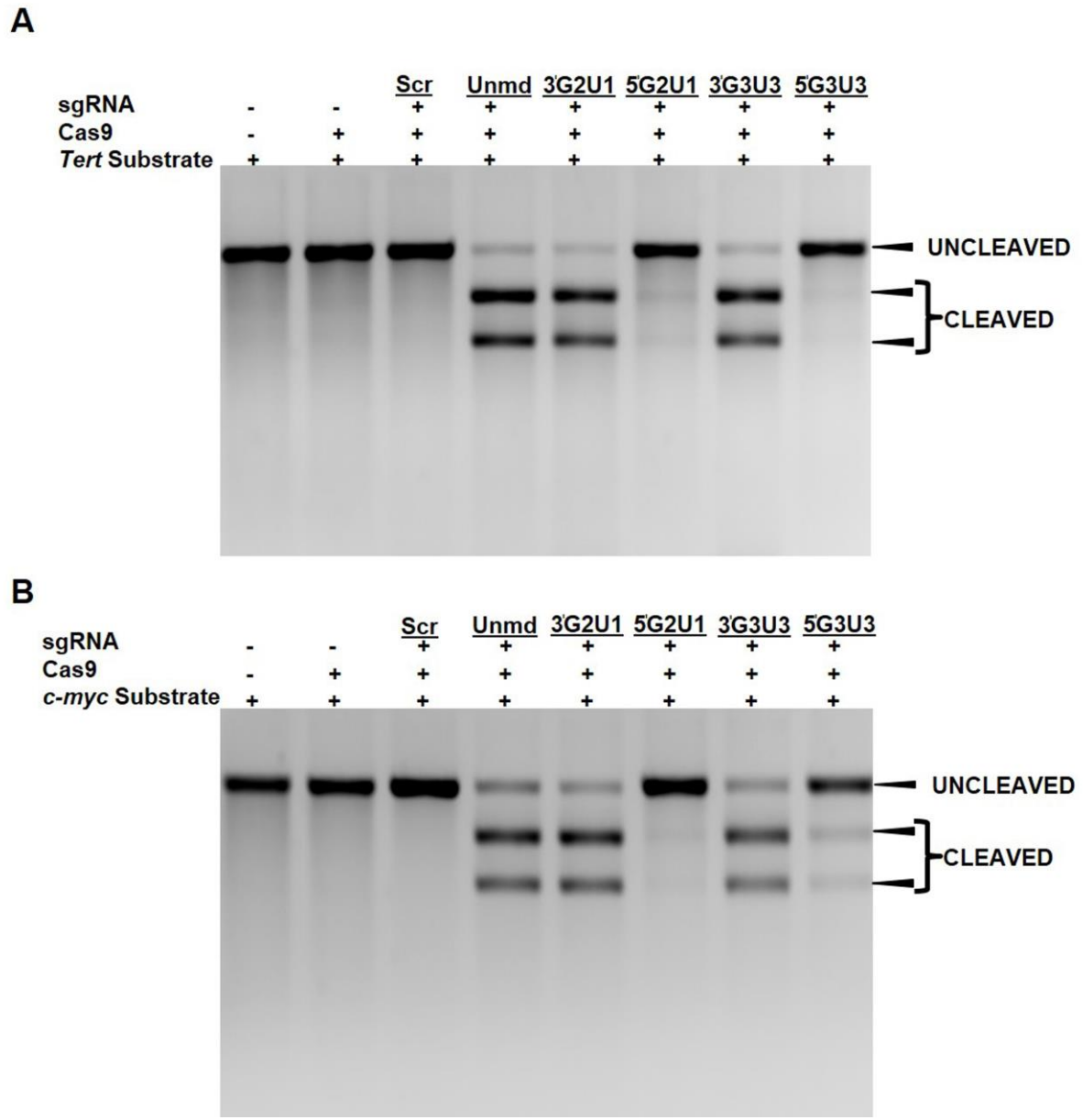

Figure S1: Evaluating the *in vitro* cleavage efficacy of various sgRNA-Cas9 complexes with endogenous DNA targets. A) *tert* and B) *c-myc* plasmids were linearized using SalI enzyme and 100 ng was incubated with various Cas9-sgRNA complexes. The cleavage products were resolved on 1% agarose gel. Scr-Scrambled, Unmd- Unmodified.

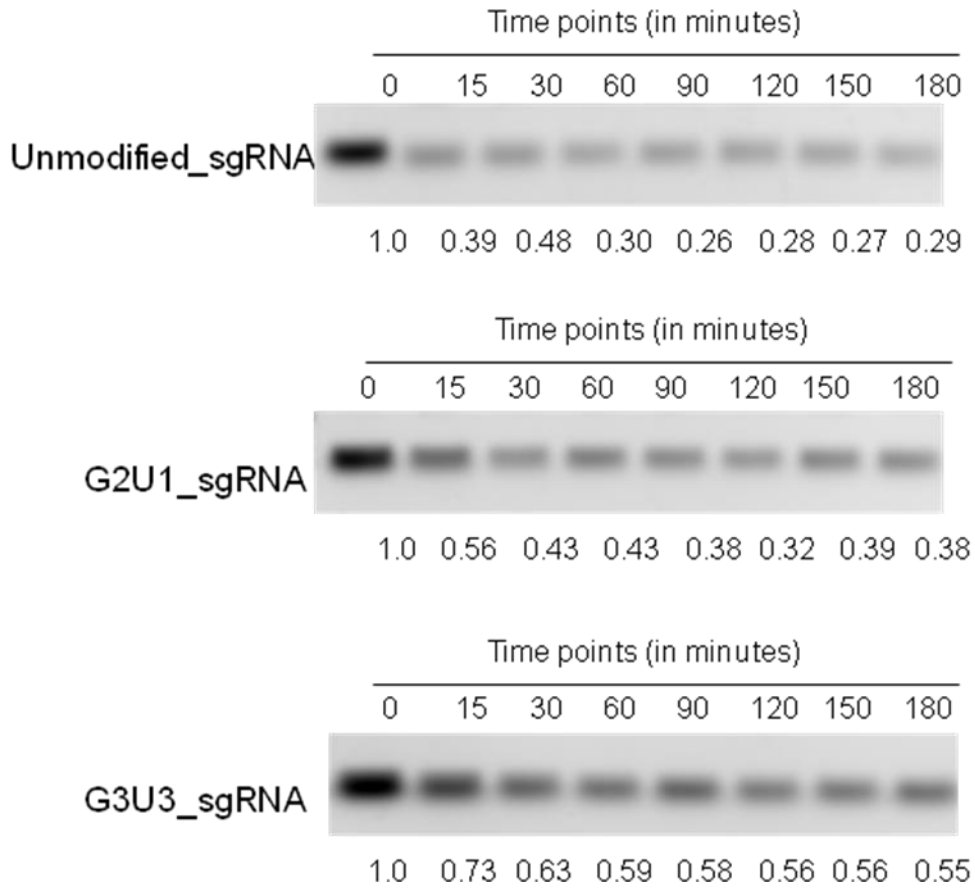

Figure S2: Stability of sgRNAs measured in 10%FBS during time course from 0 to 180 min. Representative images for each sgRNA shown with quantification indicated below the panels. The G-quadruplex motif is appended at the 3′ end for G3U3 and G2U1 sgRNAs.

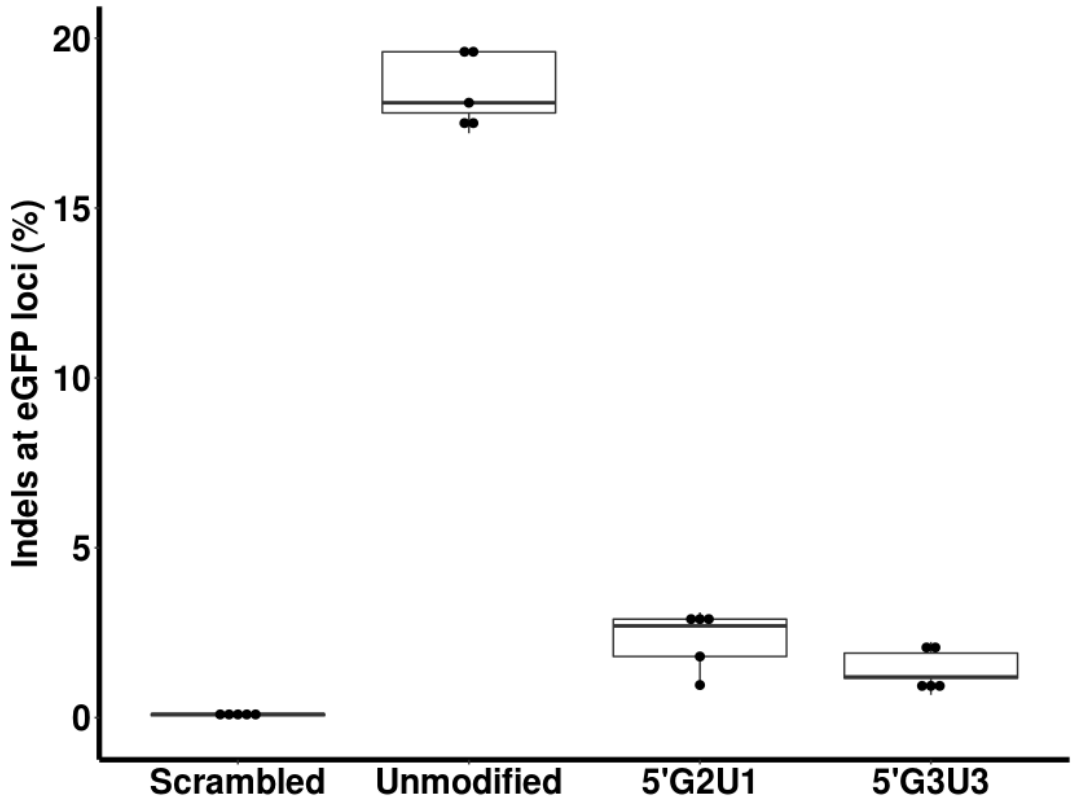

Figure S3: Indel frequencies induced by Cas9: sgRNAs assessed using the PCR-based T7EI assay. 5′G2U1 and 5′G3U3 containing sgRNAs showed minimal indels at the target eGFP loci whereas Cas9 complexed with unmodified sgRNAs produced upto ~18% indels at the same loci (n=5, 10 embryos per sample, whiskers denote positive or negative deviations from mean).

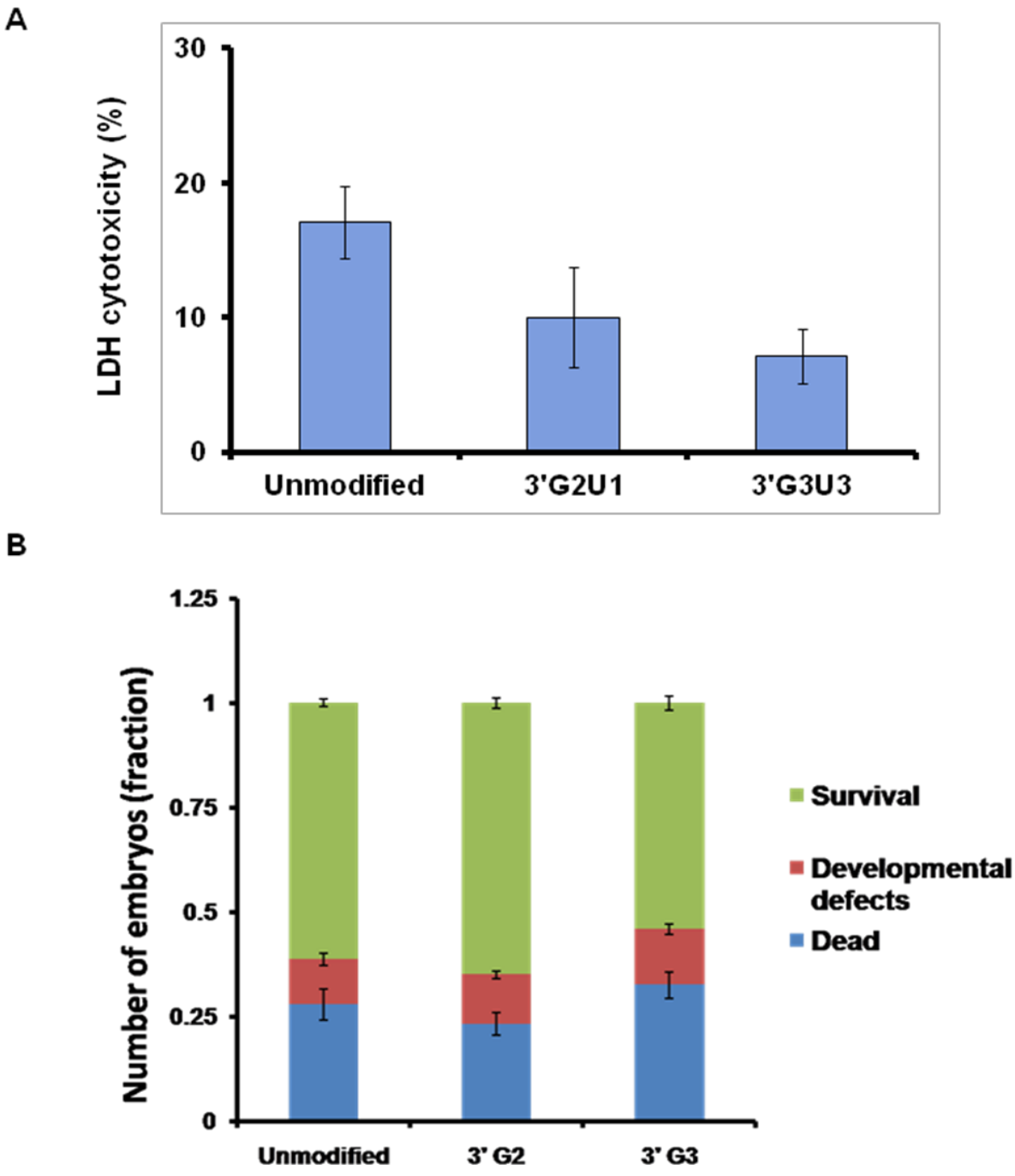

Figure S4: Measurement of Toxicity with various sgRNAs-RNP complexes. A) The LDH release was measured from the supernatant of the mouse embryonic stem cells transfected with sgRNA-RNP complexes (n=3, error bars represent standard deviation from the mean). B) The morphological analysis of the zf embryos inected with different sgRNA-RNP complexes. The 3′ containing G- quadruplex sgRNA do not show toxicity as compared to the unmodified sgRNAs (n=3, error bars represent standard deviation from the mean).

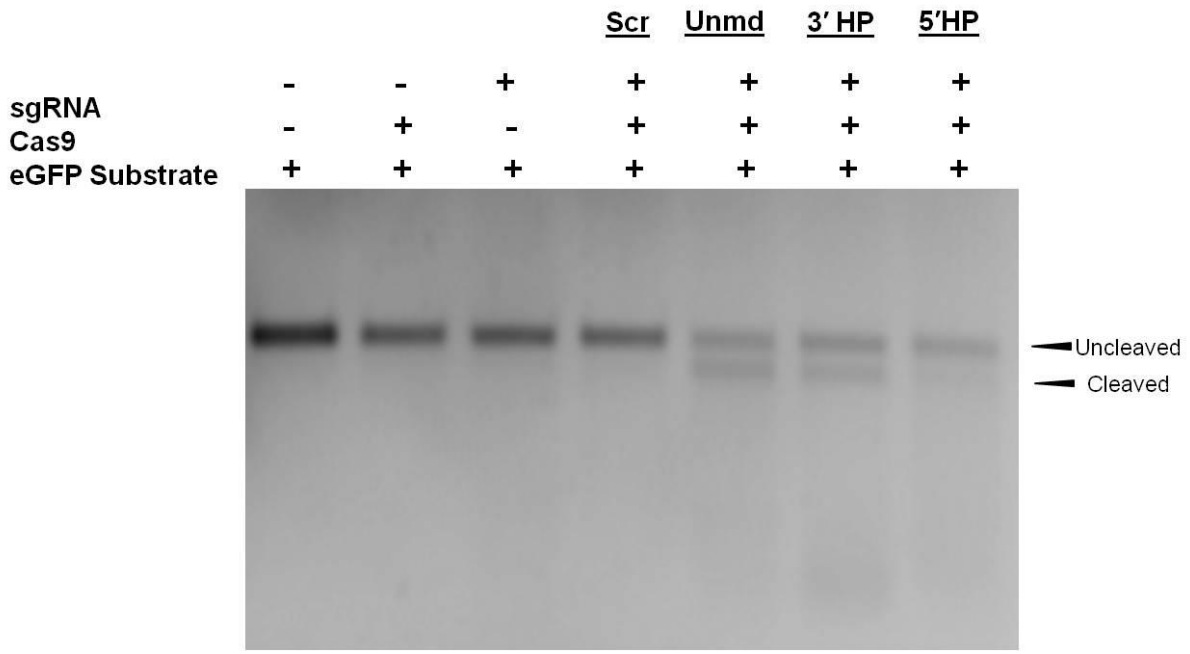

Figure S5: *In vitro* cleavage of sgRNAs containing G-rich hairpin structure at 3′ and 5′ ends. The presence of hairpin structure in 5′ end significantly reduces the cleavage efficiency of Cas9 as compared to unmodified and sgRNA with hairpin at the 3′ end. Scr denotes scrambled, Unmod- Unmodified, HP- Hairpin.

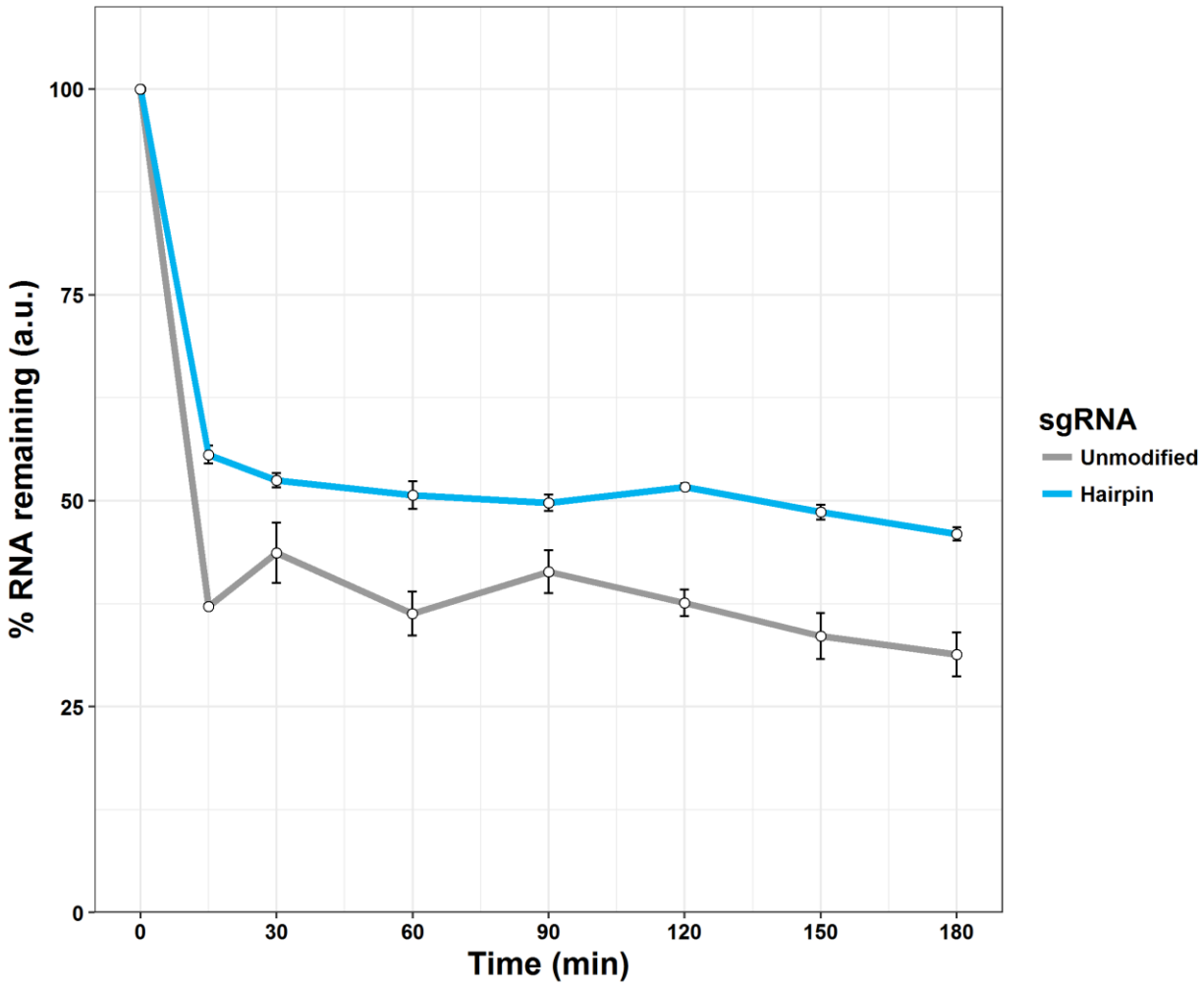

Figure S6: Serum stability of unmodified sgRNA and hairpin containing sgRNA with increasing time. The hairpin containing 3′ hairpin modified sgRNA shows ~14% higher stability that the unmodified sgRNA at 180 min (n=3, error bars represent standard deviation from the mean).

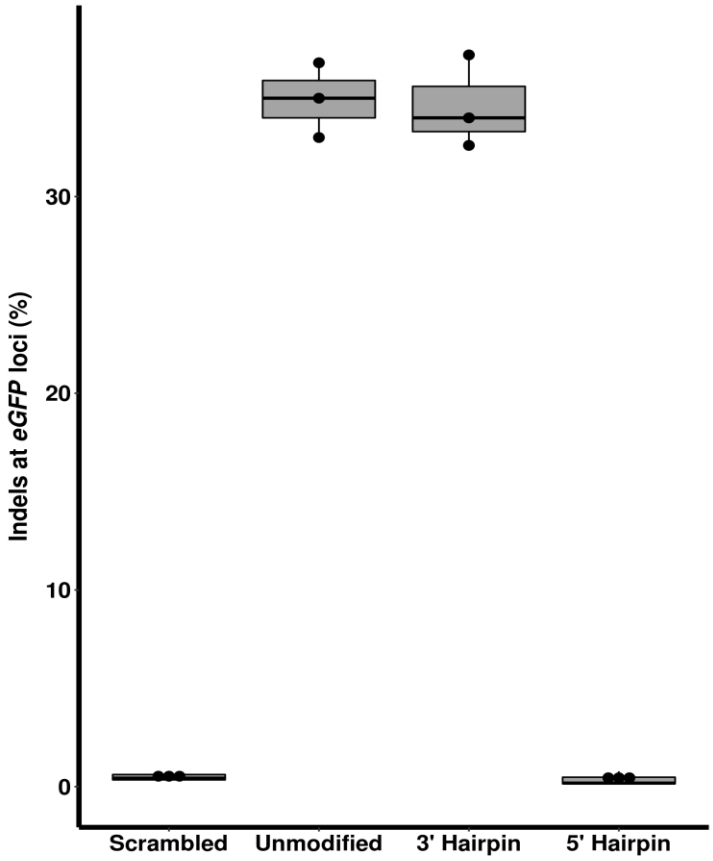

Figure S7: Percentage of Indels produced at eGFP loci upon injection of sgRNAs containing 3′HP and 5′HP and Cas9 protein in zebrafish embryos. The unmodified and 3′ HP shows ~30 % indels at the target loci; 5′ HP-sgRNA did not produce any indels, normalized with respect to scrambled sgRNA injected with Cas9 protein (n=3, 10 embryos per sample, whiskers denote positive or negative deviations from mean).

References

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