

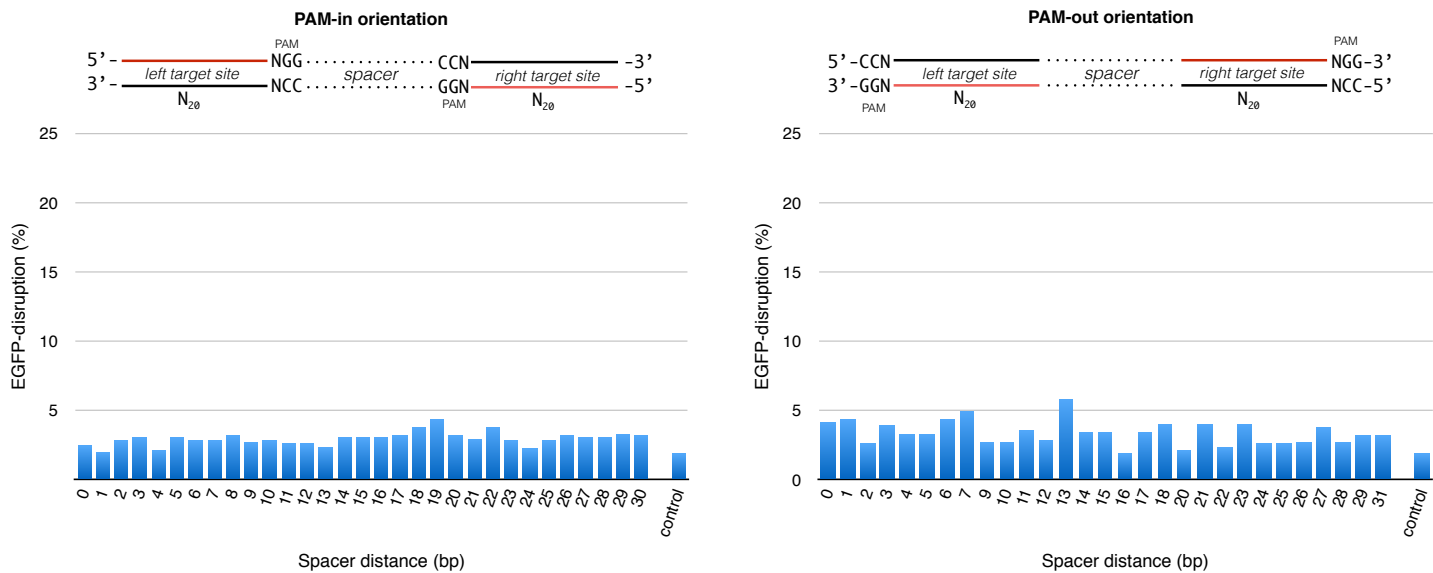
## Supplementary Discussion

### *Calculation of RFN Targeting Range in Random DNA Sequence*

We conservatively estimate that an RFN target site can be found **on average** once in every 64 bps of random DNA sequence. We arrive at this conclusion as follows: The probability of finding an RFN target with two half-sites each of the form 5'-N<sub>20</sub>-NGG-3' and a single particular spacing between them is one in every 4<sup>6</sup> or 256 bps. However, our studies show that four particular length spacer lengths between these half-sites are permitted by RFNs (13, 14, 15, or 16 bps). If one allows for all four spacer lengths, then the probability of finding a target sites rises to one site in every 4<sup>4</sup>/4 or 64 bps.

However, our work also shows that spacer lengths of 17 or 18 bps may also be permitted. If one accounts for these spacer lengths then the targeting range becomes one site in every 4<sup>4</sup>/6 or ~43 bps. Thus, the targeting range of our method is likely one site in every 43 to 64 bps in random DNA.

Supplementary Fig. 1 dCas9-FokI activities in the EGFP disruption assay



Results of a screen for dCas9-FokI activity in U2OS.EGFP cells using 60 gRNA pairs targeting sites with spacings of 0-31 bp and PAM-in or PAM-out orientations. Note the background level of EGFP disruption in these experiments shown by the control in which only a tdTomato plasmid was transfected. The control shown in both of these bar graphs and in those of Fig. 2b is the same experimental sample and is presented redundantly in each of these graphs for ease of comparison.

## Supplementary Fig. 2 Sequences of RFN-induced mutations in U2OS cells

### APC

Mutations in 9 of 23 sequences ≈ 39.1%

```
CAGGAGACGAAGAGC CCGGGCGGCGCTCGTACTTCTGG CCACTGGGCGAGCGTC TGGCAGGTGAGTGAGGCTGCAGG CATTGACGTCTCCTC WT
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGTACTTCTGGCCACTGG Cga AGCGTCTGGCAGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ0 (Δ3 +3)
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGTACTTCTGGCC ----- TCTGGCAGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ12
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGTACTTCT ----- AGCGTCTGGCAGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ12
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGTACTTC ----- GTCTGGCAGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ16
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGTACTTCTGGCCACTG ----- TGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ17
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGTACTTCTGGC ----- AGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ19
CAGGAGACGAAGAGCCCCGGGCGGCGCTCGT ----- CTGGCAGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ23
CAGGAGACGAAGAGCC ----- CGTCTGGCAGGTGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ34
ACCGGCGGCAGCAGG ----- / / ----- TGAGTGAGGCTGCAGGCATTGACGTCTCCTC Δ73
```

### BRCA1

Mutations in 7 of 86 sequences ≈ 8.1%

```
TGGGAGAGTGGAATTT CCGAAGCTGACAGATGGGTATTT TTTGAGGGGGGTAGG GCGGGAACCTGAGAGGCGTAAGG CGTTGTGAACCTGG WT
TGGGAGAGTGGAATTTCCGAAGCTGACAGATGGGTATTTCTTGA ----- GTAGGGGCGGAACCTGAGAGGCGTAAGGCGTTGTGAACCTGG Δ6
TGGGAGAGTGGAATTTCCGAAGCTGACAGATGGGTAT ----- GGGGGGTAGGGGCGGAACCTGAGAGGCGTAAGGCGTTGTGAACCTGG Δ8
TGGGAGAGTGGAATTTCCGAAGCTGACAGATGGGTATTTCTT ----- GGTAGGGGCGGAACCTGAGAGGCGTAAGGCGTTGTGAACCTGG Δ8
TGGGAGAGTGGAATTTCCGAAGCTGACAGATGGGTATTTCTTTGAGGGG ----- GAACCTGAGAGGCGTAAGGCGTTGTGAACCTGG Δ10
TGGGAGAGTGGAATTTCCGAAGCTGACAGATGGGTATTTCTT ----- GCGGGAACCTGAGAGGCGTAAGGCGTTGTGAACCTGG Δ12
TGGGAGAGTGGAATTTCCGAAGCTGACAGATGGG ----- GCGGGAACCTGAGAGGCGTAAGGCGTTGTGAACCTGG Δ22 (2x)
```

### EMX1

Mutations in 2 of 91 sequences ≈ 2.2%

```
CAAGCTGGACTCTGG CCACTCCCTGGCCAGGCTTTGGG GAGGCCTGGAGTCATG GCCCCACAGGGCTTGAAGCCCGG GGGGCCGCCATTGAC WT
CAAGCTGGACTCTGGCCACTCCCTGGCCAGGCTTT ----- ATGGCCCCACAGGGCTTGAAGCCCGGGGGGCCGCCATTGAC Δ16 (2x)
```

### FANCF site 1

Mutations in 9 of 29 sequences ≈ 31%

```
AGAGAGTCGCCGTCT CCAAGGTGAAAGCGGAAGTAGGG CCTTCGCGCACCTCAT GGAATCCCTTCTGCAGCACCTGG ATCGCTTTTCCGAGC WT
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAGTAG ----- CGCACCTCATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ8
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAGTAGGGCC ----- CTATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ9
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAGTAGG CCT ----- CATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ10
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAGTAGGGCCT ----- CATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ10
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAGTAG ----- CACCTCATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ10
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAG ----- CGCACCTCATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ11 (Δ12 +1)
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAGTAGGGCCTTCGCGCA ----- TTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ14
AGAGAGTCGCCGTCTCCAAGGTGAAAGCGGAAG ----- CCTCATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ15
AGAGAGTCGCCGTCTCCAAG ----- CATGGAATCCCTTCTGCAGCACCTGGATCGCTTTTCCGAGC Δ31
```

### FANCF site 2

Mutations in 13 of 71 sequences ≈ 18.3%

```
TTTGGTCGGCATGGC CCCATTGCGCACGGCTCTGGAGCGG GCGGCTGCACAACCAG TGGAGGCAAGAGGGCGGCTTTGG GCGGGGTCCAGTTCC WT
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCG ----- CAACAGTGGAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ3
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCT ----- AACCAGTGGAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ4
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCG ----- CAGTGGAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ8
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCTG ----- TGGAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ9
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCTG ----- GAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ11
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCGga ----- GAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ12 (Δ14 +2)
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCG ----- GAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ13
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCTG ----- GCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ14
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCG ----- GGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ15
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAG ----- TGGAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ18
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAG ----- GCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC Δ23
TTTGGTCGGCATGGCCCC ----- / / ----- GTCAGTGT Δ97
```

```
TTTGGTCGGCATGGCCCCATTTCGCACGGCTCTGGAGCGGCGGCG acactattgcaatgaaaataaatttcctttattagctagaagtgaatc +168 (Δ9 +177)
Tgaaggggttcatgatgtccccataatttttggcagagggaagaaagatctcagtggtattttgtgagccagggcatttggccacaccagccac
caccttttgataggcagcctgcacctgaggagtgcg AGTGAGGCAAGAGGGCGGCTTTGGGCGGGGTCCAGTTCC
```

### GLI1

Mutations in 4 of 66 sequences ≈ 6.1%

```
GTTCAACTCGATGAC CCCACCACCAATCAGTAGCTATG GCGAGCCCTGCTGTCT CCGGCCCTTCCCCAGTCAGGGGG CCCCCAGTGTGGGGA WT
GTTCAACTCGATGACCCACCAACCAATCAGTAGCTAT ----- CCTGCTGTCTCCGGCCCCCTCCCCAGTCAGGGGGCCCCCAGTGTGGGGA Δ7
GTTCAACTCGATGACCCACCAACCAATCAGTAGCTATG ----- TCTCGGCCCTTCCCCAGTCAGGGGGCCCCCAGTGTGGGGA Δ13
GTTCAACTCGATGACCCACCAACCAATCAGTAGCTATGGCAGCCCC ----- CCCCAGTCAGGGGGCCCCCAGTGTGGGGA Δ17
GTTCAACTCGATGACCCACCAACCA ----- CCTGCTGTCTCCGGCCCCCTCCCCAGTCAGGGGGCCCCCAGTGTGGGGA Δ18
```

## MLH1

### Mutations in 4 of 20 sequences ≈ 20%

```
TGGCTGAAGGCACCTTCCGCTTGAGCATCTAGACGTTTCCTTGGCTCTTCTGGCGCCAAAATGTCGTTTCGTGGCAGGGGTTATTTCGGCGGCTGG WT
TGGCTGAAGGCACCTTCCGCTTGAGCATCTAGACGTTTCCTTGGCTCTTCTGGCGCCAAAATGTCGTTTCGTGGCAGGGGTTATTTCGGCGGCTGG Δ9
TGGCTGAAGGCACCTTCCGCTTGAGCATCTA-----TTCTGGCGCCAAAATGTCGTTTCGTGGCAGGGGTTATTTCGGCGGCTGG Δ16
TGGCTGAAGGCACCTTCCGCTTGAGCATCTAGACGTTTCCTTGG-----ATGTCGTTTCGTGGCAGGGGTTATTTCGGCGGCTGG Δ16
TGGCTGAAGGCACCTTCCGCTTGAGCATCTAGACGTTT-----GTCGTTTCGTGGCAGGGGTTATTTCGGCGGCTGG Δ24
```

## RARA

### Mutations in 4 of 86 sequences ≈ 4.7%

```
CCCTTCTGACTGTGGCCGCTTGGCATGGCCAGCAACA-----CCTGCCCAGACCTGGGGGCGGGCACCCTCAATGGGTACCCGGTGCCTCC WT
CCCTTCTGACTGTGGCCGCTTGGCATGGCCAGCAACA-----CTGCCCAGACCTGGGGGCGGGCACCCTCAATGGGTACCCGGTGCCTCC Δ6
CCCTTCTGACTGTGGCCGCTTGGCATGGCCAGCAACA-----CTGCCCAGACCTGGGGGCGGGCACCCTCAATGGGTACCCGGTGCCTCC Δ7
CCCTTCTGACTGTGGCCGCTTGGCATGGCCAGCA-----GCCCCAGACCTGGGGGCGGGCACCCTCAATGGGTACCCGGTGCCTCC Δ12
CCCTTCTGACTGTGGCCGCTTGGCATGGCCAG-----GCCCCAGACCTGGGGGCGGGCACCCTCAATGGGTACCCGGTGCCTCC Δ14
```

## VEGFA site 1

### Mutations in 19 of 74 sequences ≈ 25.7%

```
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCCAAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT WT
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCCAAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCA +2
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----GCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ3
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----AGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ5
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----AAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ5
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----AAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ6 (2x)
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----TTCCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ7
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----ATTCCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ8
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----AAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ9 (2x)
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----ATTCCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ14 (3x)
TCAGAAATAGGGGGTCCAGGAGCAAAC-----CAAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ17 (2x)
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----CTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ18
TCAGAAATAGGGGGTCCAGGAGCAAACCTCCCCCACCCTTTCC-----AGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ20
TCAGAAATAGGGGGTCCAGG-----CAAAGCCCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ24
TCAGAAATAGGGGGTCCAGGAGC-----TCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT Δ35
```

## VEGFA site 2

### Mutations in 26 of 80 sequences ≈ 32.5%

```
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCGGACAGTGGACGCGGCGGGAGAGCCCGCGCCCGGAG WT
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCG-----TGGACGCGCGCGGAGCCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAG Δ8 (2x)
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCG-----ACGCGCGCGGAGCCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAG Δ13
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCG-----TGGACGCGCGCGGAGCCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAG Δ13
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCGGACAG-----CCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAG Δ16
CCCCAGCCCCAGCTACCACTCTCTCCCGG-----ACGCGCGCGGAGCCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAG Δ17
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCG-----AGCCCGCGCCGAG Δ43
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCGGACAGTGG-----/ /-----GGGGGTCGGGGCTCG Δ54 (3x)
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCG-----/ /-----AG Δ56 (2x)
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCG-----/ /-----GGTGGAGGGGGTCCG Δ56 (2x)
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCGGACACa-----/ /-----CGCGGCGTCGCAC Δ69 (Δ71 +2)
C-----/ /-----GGAG Δ87 (2x)
CCCCAGCCCCAGCTACCACTCTCTCCCGGCCGCGCG-----/ /-----CTGAACTTTTCGTC Δ89 (7x)
CCGGAGCGCGGCGTG-----/ /-----GAGGGGGTTCGGGGCT Δ165 (2x)
```

## VEGFA site 3

### Mutations in 31 of 77 sequences ≈ 40.3%

```
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTCACTAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC WT
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT-----TAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ3
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCA-----CTAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ4
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTCA-----GGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ5
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCA-----TAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ5
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGAC-----CTAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ8 (2x)
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAG-----GGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ9
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGAC-----TAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ9 (2x)
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----CTAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ10
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----AGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ11
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----CTAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ11
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGT-----TCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ13
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----CTAGGGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ14
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----GGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ15
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----GGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ16 (2x)
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTCACTA-----CACAGGGAAGCTGGGTGAATGGAGCGAGC Δ16
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGC-----TCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ16
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACG-----CTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ17
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAG-----GGGGCGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ19
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGC-----GCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ21
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTCAC-----AGGGAAGCTGGGTGAATGGAGCGAGC Δ21
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGC-----CACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ21 (2x)
CCATTCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTCA-----GGGAAGCTGGGTGAATGGAGCGAGC Δ23
```

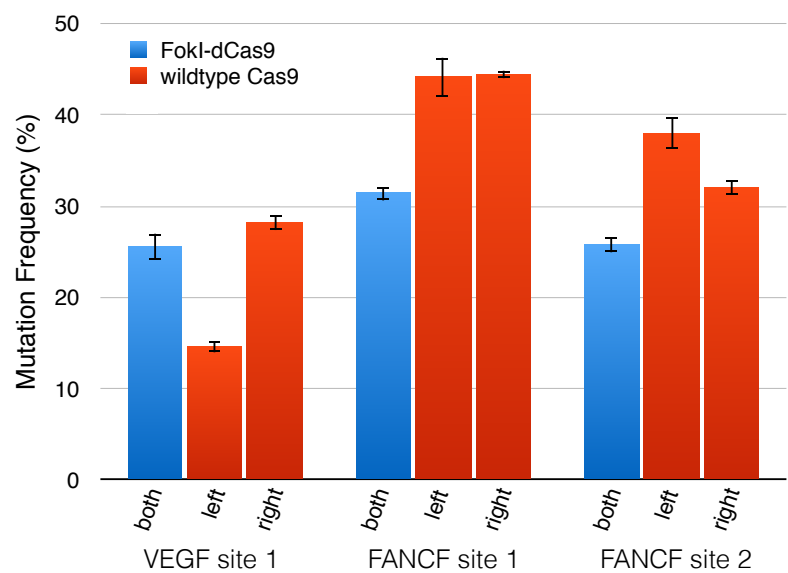
```

CCATTCCCTCTTTAGCCAGAGCCGGGGTGTGCAG-GCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ24
CCATTCCCTCTTTAGCCAGAGCCGGGGTGTGCAGAC-CACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ24
CCATTCCCTCTTTAGCCAGAGCCGGGGTGTGCAGAC-CACAGGGAAGCTGGGTGAATGGAGCGAGC Δ27
CCATTCCCTCTT-AGTGACTGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGC Δ34 (2x)

```

Sequences were determined using standard Sanger sequencing. For each target site, wild-type sequence is shown in the top line with 23-nt “half-sites” highlighted in yellow and the PAM sequence shown in boldface, underlined red text. Deletions are marked with red-dashes on a light grey background and insertions are highlighted in blue. The net number of bases inserted or deleted is shown directly to the right of each sequence. Indel mutations isolated more than once are also indicated to the right of the net base change.

**Supplementary Fig. 3** Direct activity comparisons of FokI-dCas9 with paired gRNAs and wild-type Cas9 with single gRNAs at three endogenous human gene sites



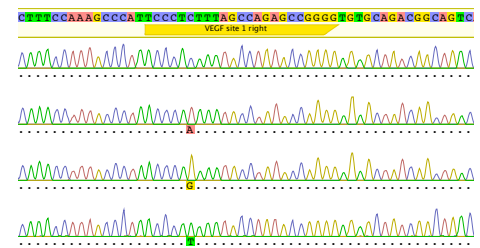
Mutation frequencies induced by dimeric RFNs directed by a pair of gRNAs (blue bars) or by wild-type Cas9 with one or the other gRNA in the same pair (red bars, labeled as “left” and “right”) for three different endogenous human gene target sites are shown. Experiments were performed in U2OS cells and T7EI assay was used to quantify the rates of mutagenesis. Error bars represent standard errors of the mean, n=4.

## Supplementary Fig. 4 Sanger sequencing confirms induction of base substitution mutations by Cas9 nickases

### VEGFA site 1, right

#### Mutations in 10 of 86 sequences

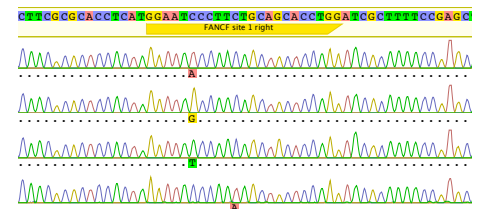
CTTTCCAAAGCCCAT**TCCCTCTTTAGCCAGAGCCGGG**TGTGCAGACGGCAGTC  
CTTTCCAAAGCCCAT**TCCCT****A**TTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTC  
CTTTCCAAAGCCCAT**TCCCT****G**TTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTC (7X)  
CTTTCCAAAGCCCAT**TCCCT****T**TTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTC (2X)



### FANCF site 1, right

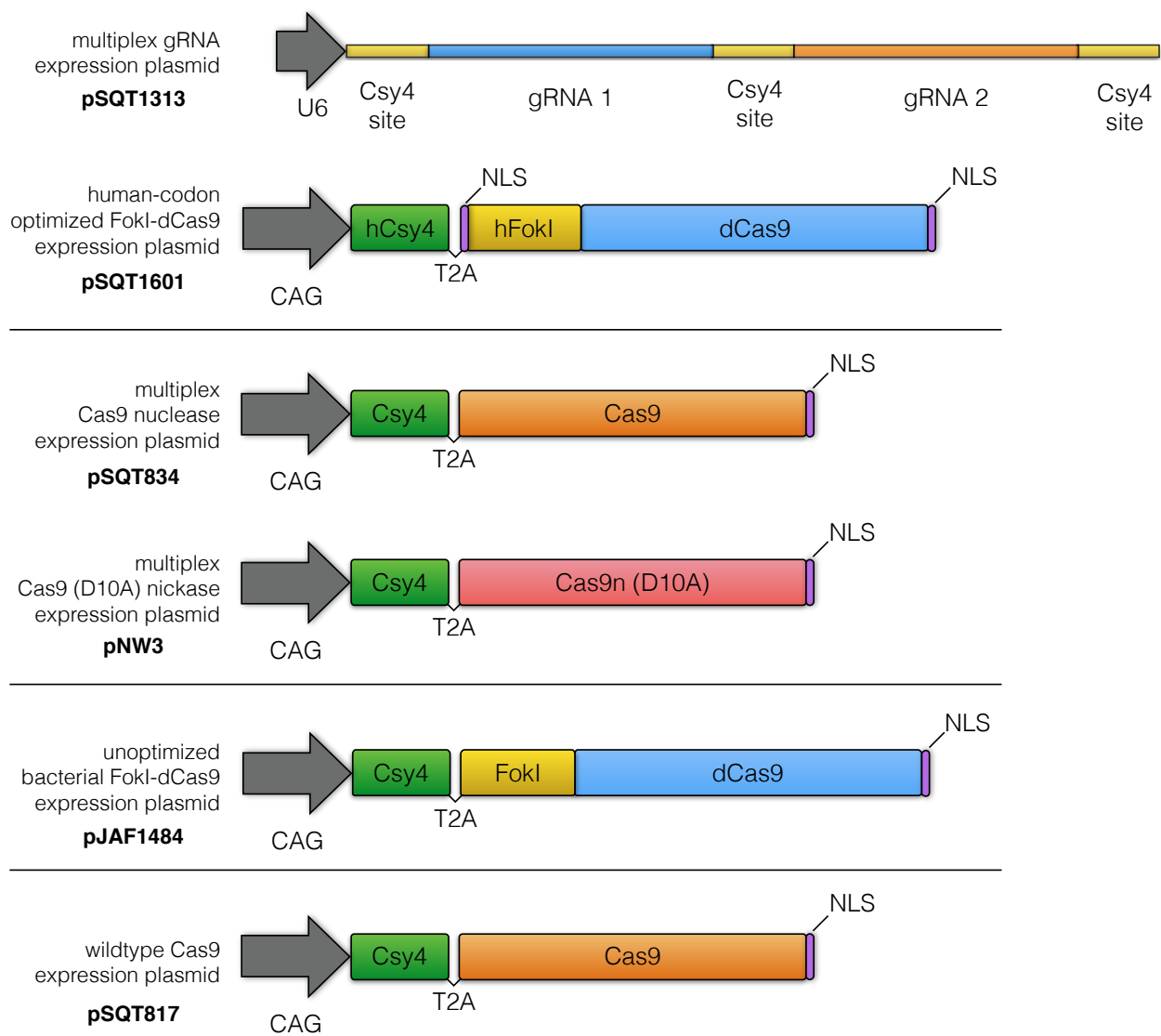
#### Mutations in 19 of 66 sequences

CTTCGCGCACCTCAT**GGAATCCCTTCTGCAGCACCTGG**ATCGCTTTTCCGAGC  
CTTCGCGCACCTCATGGAAT**A**CCTTCTGCAGCACCTGGATCGCTTTTCCGAGC (5X)  
CTTCGCGCACCTCATGGAAT**G**CCTTCTGCAGCACCTGGATCGCTTTTCCGAGC (6X)  
CTTCGCGCACCTCATGGAAT**T**CCTTCTGCAGCACCTGGATCGCTTTTCCGAGC (6X)  
CTTCGCGCACCTCATGGAATCCCT**A**TGCAGCACCTGGATCGCTTTTCCGAGC  
CTTCGCGCACCTCATGGAATCC-----ACCTGGATCGCTTTTCCGAGC



For each target half-site, the wild-type sequence is shown in the top line with the 23-nt sequence recognized by each Cas9 nickase highlighted in yellow and the PAM shown in red, underlined, bold-face text. Point mutations are shown in bold text and highlighted in blue (A), yellow (G), or magenta (T) with the number of each mutant sequence identified shown to the right of the sequence if it was isolated more than once. Representative DNA sequencing chromatograms are shown to the right of the sequence alignments. The sequences shown were obtained from the same genomic DNA used in the experiments shown in **Figs. 5a** and **5b**.

Supplementary Fig. 5 Schematic maps of expression plasmids



## Supplementary Fig. 6 DNA and amino acid sequences of Cas9 and Csy4 proteins used in this study

### hCsy4-T2A-hFokI-dCas9 coding sequence in pSQT1601

(Csy4 colored in **green**, T2A peptide colored in **grey**, FokI colored in **red**, NLS underlined, Gly<sub>4</sub>Ser linker colored in **purple**, dCas9 colored in **blue**, mutated codons shaded in **yellow**, 3xFLAG tag in **bold**)

ATGGGTGATCATTATCTGGATATTCGGCTGAGGCTGATCCAGAGTTCCACCTGCGCAGCTGATGTCTGTCTTTTGGCAAACCTCATCAGGCCCTGGTTGCCAGG  
GCGGAGATCGGATAGGGGTAAGCTTTCCAGACCTCGACGAAAGCCGAGCCGCCTGGGAGAAGCCCTGCGGATCCACGCTTCTGCCGACGATCTGAGAGCCTTGCTGGC  
AAGGCCATGGCTTGAGGGGCTCCGGGATCACCTGCAGTTTGGCGAACCCGCCGTTGTTCCCCACCAACCCCTTATCGGCAGGTGTCTAGAGTGCAGGCCAAATCTAAT  
CCAGAACGGCTGCGACGGCGACTCATGCGGCGACATGATCTTAGCGAGGAAGAGGCCGAAAAAGAATCCCTGATACCGTGGCCCGCGCCCTTGACTTGCCCTTTGTCA  
CACTGCGGTCCCAGAGTACGGGGCAGCATTTTCAGACTTTTCATTGACACGGGCCACTGCAAGTTACCGCCGAAGAAGGAGGCTTTACTTGTTATGGACTCTCCAAGGG  
AGGTTTCGTGCCCTGGTTTGAGGGCAGAGGAAGTCTGTTAACATGCGGTGACGTGAGGAGAATCCTGGCCCAATGCCCTAAGCCTAAGAAGAAGCGGAAGGTGAGCAGCAACTT  
GTGAAGTCTGAAGTCTGAGGAGAAAAATCAGAGTTGAGACACAAGTTGAAGTACGTGCCACACGAATACATCGAGCTTATCGAGATCGCCAGAAACAGTACCCAGGATA  
GGATCCTTGAGATGAAAGTCATGGAGTTCTTTATGAAGGTCTACGGTTATAGAGGAAAGCACCTTGGCGGTAGCAGAAAGCCCGATGGCGCCATCTATACTGTGGATC  
TCCTATCGATTATGGGGTGATCGTGGATACCAAAGCTTACTCAGGCGGGTACAACCTGCCCATAGGACAAGCCGACGAGATGCAGCGGTATGTGGAAGAGAACCAGACG  
CGCAACAAGCACATCAACCCCAATGAATGGTGGAAAGTGTACCAAGTAGTGTGACTGAGTTCAAGTTCCTGTTTGTCTCCGCCCACTTAAGGGCAATTATAAAGCTC  
AGTCACTAGACTCAATCACATCACAACCTGCAACGGAGCTGTGTTGTCAAGTGGAGGAGCTCCTGATTGGAGGCGAGATGATCAAAGCCGGCACCTTACACTGGAGGA  
GGTGCGGCGGAAGTTCAACAATGGAGAGATCAACTTCGGTGGCGGTGGATCCGATAAAAAGTATTCATTGGTTTATGCCATCGGCACTAATTCGTTGGATGGGCTGTC  
ATAACCGATGAATACAAAGTACCTTCAAAGAAATTTAAGGTGTTGGGGAACACAGACCGTCATTGATTAAGAAGTCTTATCGGTGCCCTCTATTGATAGTGGCG  
AAACGGCAGAGGCGACTCGCTGAAACGAACCGCTCGGAGAAGTATACACGTGCAAGAACCGAATATGTTACTTACAAGAAATTTTAGCAATGAGATGGCCAAAGT  
TGACGATTTCTTTTCCACGTTTGAAGAGTCTTCTTGTGGAAGGACAAGAAACATGAACGGCACCCCATCTTGGAAACATAGTAGATGAGGTGGCATATCAT  
GAAAAGTACCAACGATTTATCACCTCAGAAAAAGTAGTTGACTCAACTGATAAAGCGGACCTGAGGTTAATCTACTTGGCTCTTGCCCATATGATAAAGTCCGTG  
GGCACTTTCTCATTGAGGGTGATCTAAATCCGGACAACCTCGGATGTCGACAACTGTTTCATCCAGTTAGTACAAACCTATAATCAGTTGTTTGAAGAGAACCCTATAAA  
TGCAAGTGGCGTGGATGCGAAGGCTATTCTTAGCGCCCGCTCTCTAAATCCGACGGCTAGAAAACCTGATCGCACAATTACCCGGAGAGAAGAAAAATGGGTTGTTT  
GGTAACCTTATAGCGCTCTCACTAGGCTGACACCAATTTAAGTCGAACCTCGACTTAGCTGAAGATGCCAAATGCAGCTTAGTAAGGACACGTACGATGACGATC  
TCGACAATCTACTGGCACAATTTGGAGATCAGTATGCGGACTTATTTTGGCTGCCAAAAACCTTAGCGATGCAATCTCTCTATCTGACATAGTGAAGTTAATACTGA  
GATTACCAAGGCGCGTTATCCGCTTCAATGATCAAAAGGTACGATGAACATCACAAGACTTGACACTTCTCAAGGCCCTAGTCCGTCAGCAACTGCCTGAGAAATAT  
AAGGAAATATTCTTTGATCAGTCGAAAAACGGGTACGAGGTATATTGACGGCGGAGCGAGTCAAGAGGAATTTACAAGTTTATCAAACCCATATTAGAGAAGATGG  
ATGGGACGGAAGAGTTGCTTGTAAGTCAATCGCGAAGATCTACTGCGAAAGCAGCGGACTTTCGACAACGGTAGCATTCCACATCAAATCCACTTAGCGGAATTGCA  
TGCTATACTTAGAAGGCAGGAGGATTTTATCCGTTCTCAAGACAATCGTGAAGAGATTGAGAAAAATCTAACCTTTCGCATACCTTACTATGTGGGACCCCTGGCC  
CGAGGGAACCTCGGTTTCGATGGATGACAAGAAAGTCCGAAGAAACGATTACTCCATGGAATTTTGAAGGAGTTGTCGATAAAGGTGCGTCAGCTCAATCGTTCATCG  
AGAGGATGACCAACTTTGACAAGAATTTACCGAACGAAAAAGTATTGCCTAAGCACAGTTTACTTTACGAGTATTTACAGGTGTAACAATGAACACGAAAGTTAAGTA  
TGCTACTGAGGGCATGCGTAAACCCGCTTTCTAAGCGGAGAACAGAAGAAAGCAATAGTAGATCTGTTATTCAAGACCAACCGCAAAGTGACAGTTAAGCAATTGAAA  
GAGGACTACTTTAAGAAAAATGAATGCTTCGATTCTGTGAGATCTCCGGGTAGAAGATCGATTTAATGCGTCACCTGGTACGTATCATGACCTCTAAAGATAATTA  
AAGATAAGGACTTCTGGATAACGAAGAGAATGAAGATATCTTAGAAGATAGTGTGACTCTTACCCTCTTTGAAGATCGGGAAATGATTGAGGAAAGACTAAAAAC  
ATACGCTCACCTGTTTCGACGATAAGGTTATGAAACAGTTAAAGAGGCGTCGCTATACGGCTGGGACGATTGTGCGGAAACTTATCAACGGGATAAGAGACAAGCAA  
AGTGGTAAACTATTCTCGATTTTCTAAGAGCGACGGCTTCGCCAATAGGAACCTTATGCAGCTGATCCATGATGACTCTTTAACCTTCAAAGAGGATATACAAAAGG  
CACAGGTTTCCGGACAAGGGGACTCATTGCACGAACATATTGCGAATCTTGTGGTTGCCAGCCATCAAAAAGGGCATACTCCAGACAGTCAAAGTAGTGGATGAGCT  
AGTTAAGGTGATGGGACGTCACAAACCGGAAAAATTTGTAATCGAGATGGCAGCGGAAATCAAACGACTCAGAAGGGGCAAAAAACAGTCGAGAGCGGATGAAGAGA  
ATAGAAGAGGGTATTAAAGAACTGGGACGCCAGATCTTAAAGGAGCATCTGTGGAAAAATACCAATTGCAGAACGAGAACTTTACCTCTATTACCTACAAAATGGAA  
GGGACATGTATGTTGATCAGGAACGGACATAAACCGTTTATCTGATTACGACGTCGATGCTATTGTACCCCAATCTTTTGAAGGACGATTCAATCGACAATAAAGT  
GCTTACACGCTCGGATAAGAACCGAGGAAAAAGTGACAATGTTCCAAGCGAGGAAGTCGTAAGAAAAATGAAGAATATTGGCGGCAGCTCTAAATGCGAAACTGATA  
ACGCAAGAAAGTTGATACTTAACATAAGCTGAGAGGGGTGGCTGTCTGAACCTGACAAGGCCGATTTATTAACGTCAGCTCGTGAAACCCGCCAAATCACAA  
AGCATGTTGCACAGATACTAGATTTCCGAATGAATACGAAATACGACGAGAACGATAAGCTGATTGCGGAAGTCAAAGTAATCACTTTAAAGTCAAAATTTGGTGTGCGGA  
CTTCAGAAAGGATTTTCAATTTCTATAAGTTAGGGAGATAAATAACTACCACCTGCGCAGCAGCTTATCTTAATGCCGTCGTAGGGACCGCACTCATTAAAGAAATAC  
CCGAAGCTAGAAAGTGAGTTTGTGATGGTATTACAAAGTTTATGACGTCGTAAGATGATCGCGAAAAGCGAACAGGAGATAGGCAAGGCTACAGCCAAATACCTTCT  
TTTATTCTAACATTATGAATTTCTTTAAGACGGAAATCACTCTGGCAACCGAGAGATACGCAACGACCTTAAATTGAAACCAATGGGGAGACAGGTGAAATCGTATG  
GGATAAGGGCCGGGACTTCGCGACGGTGAGAAAAATTTGTCCATGCCCAAGTCAACATAGTAAGAAAAACTGAGGTGACAGCCGAGGGTTTTCAAAGGAATCGATT  
CTTCAAAAAGGAATAGTGATAAGCTCATCGCTCGTAAAAAGGACTGGGACCCGAAAAAGTACGGTGGCTTCGATAGCCCTACAGTTGCCATTCTGTCTAGTAGTG  
CAAAAGTTGAGAAGGAAAAATCCAAGAACTGAAGTCAGTCAAAGAATTATTGGGGATAACGATTATGGAGCGCTCGTCTTTTGAAGAAACCCCATCGACTTCTTGA  
GGCGAAAGGTTACAAGGAAGTAAAAAGGATCTCATAATTAACCTACCAAGTATAGTCTGTTTGAAGTAGAAAAATGGCCGAAACCGGATGTTGGCTAGCGCCGAGAG  
CTTCAAAAAGGGAACGAACTCGCACTACCGTCTAAATACGTGAATTTCTGTATTTAGCGTCCATTACGAGAAGTTGAAAGGTTACCTGAAGATAACGAACAGAAGC  
AACTTTTTTGTGAGCAGCACAAACATTATCTCGACGAAATCATAGAGCAAAATTTCGGAATTCAGTAAGAGAGTCATCTAGCTGATGCCAATCTGGACAAAGTATTAAG  
CGCATACAACAAGCACAGGGATAAACCCATACGTGAGCAGGCGGAAAAATATTATCCATTGTTTACTCTTACCAACCTCGCGCTCCAGCGCATTCAAGTATTTTAC  
ACAACGATAGATCGCAACGATACACTTACCAAGGAGGTGTAGACGCGACACTGATTACCAATCCATCACGGGATTATATGAAACTCGGATAGATTTGTACAGC  
TTGGGGGTGACGGATCCCCAAGAAGAAGAGGAAAGTCTCGAGCGACTACAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAA  
GTGA

## hCsy4-T2A-hFokI-dCas9 amino acid sequence

(Csy4 colored in **green**, T2A peptide colored in **grey**, FokI colored in **red**, NLS underlined, Gly<sub>4</sub>Ser linker colored in **purple**, dCas9 colored in **blue**, mutated amino acids shaded in **yellow**, 3xFLAG tag in **bold**)

MGDHYLDIRLPDPEFPQAQLMSVLFGKLGKALVAQGGDRIGVSFPDLDESRSRLGERLRIHASADDLRALLARPWLEGLRDHLQFGEPVAVPHPTPYRQVSRVQAKSN  
PERLRRRLMRRHDLSEEEARKRIPDVARALDLPFVTLRSQSTGQHFRFLIRHGPLQVTAEEGGFTCYGLSKGGFVPWFEGRGSLLTCGDVEENPGP**MPKKKRKVS**SQL  
VKSELEEKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAITYTVGSPIDYGVIVDTKAYSGGYNLP**IGQADEMQRYVEENQT**  
RNKHINPNEWKVPSSVTEFKFLFVSGHFKGNYKAQLTRLNHNITNCNGAVLSVEELLIGGEMIKAGT**L**LEEVRRKFNNGEINFGGGSDKKYSIGL**A**IGTNSVGWAV  
ITDEYKVPSSKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLSEESFLVEEDKKHERHPIFGNIVDEVAYH  
EKYPTIYHLRKKLVDSDDKADRLIYLALAHMIKFRGHFLIEGDLNPDNSDVKLFILQVQTYNQLFEEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLF  
GNLIAALSGLTPNFKSNFDLAEDAKLQLSKDYYDDDLNLLAQIGDQYADFLAAKNLSDAIILSDILRVNTEITKAPLSASMIKRYDEHHQDLTLKALVRQQLPEKY  
KEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYFPLKDNREKIEKILTFRIPYYVGPLA  
RGNSRFAWMTRKSEETITPWNFEVVDKGASAQSFIERMTNFDKNLPNEKVLPHKSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKAIVDILLFKNRKYVTKQLK  
EDYFKKIECFDSVEISGVEDRFNASLGYHDLKKIKDKDFLDNEENEDILEDIVLTLTFEDREMIERLKYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQ  
SGKTIIDFLKSDGFANRNFMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAICKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKR  
IEEGIKELGSQILKEHPVENTQLQNEKLYLYLQNGRDMYVDQELDINRLSDYVD**A**IVPQSFLKDDSIDNKVLRSDKNRGKSDNPSEEVKKMKNYWRQLNNAKLI  
TQRKFDNLTKAERGGSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYKREINNYHHAHDAYLNAVVGTA**IKKY**  
PKLESEFVYGDYKVYDVRKMIKSEQEI**GKATAKYFFYSNIMNFFKTEIT**LANGEIRKRL**LIET**NGETGEIVWDKGRDFATVRKVL**SMPQVNIVKKTEVQTGGFSKESI**  
LPKRNSDKLIARKKDWDPKYYGGFDSPTVAYSVLVAKVEKGSKKLKSVKELLGITIMERSSEFKNPIDFLEAKGYKEVKKDLIKLPKYSLELENGRKRMLASAGE  
LQKGNELALPSKYVNFYLYLASHYEKLKGGPEDNEQQLFVEQHKHYLDEIEQISEFSKRIVILADANLDKVL**SAYNKH**RDKPIREQAENIHLFTLNLGAPAAFKYFD  
TTIDRKRYTSTKEVLDA**LIHQ**SITGLYETRIDLSQLGGDGS**PKKKRKVS**SDYKDHDGDYKDHDIDYKDDDDK

## Csy4-T2A-Cas9 coding sequence in pSQ834

(Csy4 colored in **green**, T2A peptide colored in **grey**, NLS underlined, Cas9 colored in **blue**, 3xFLAG tag in **bold**)

ATGGACCACTACCTCGACATTCGCTTGCAGCCGACCCGGAATTTCCCCGGCGCAACTCATGAGCGTGCTCTTCGGCAAGCTCCACCAAGGCCCTGGTGGCACAGGGCG  
GGGACAGGATCGCGTGAGCTTCCCCGACCTCGACGAAAGCCGCTCCCGGCTGGGCGAGCGCTGCGCATTATGCCTCGGCGGACGACCTTCGTGCCCTGCTCGCCCG  
GCCCTGGCTGGAAGGGTTGCGGGACCATCTGCAATTCGGAGAACCGGACGTCGTGCCTCACCCACACCGTACCGTCAGGTAGTCGGGTTACAGCGCAAAAGCAATCCG  
GAACGCCCTGCGGGCGCGGCTCATGCGCCGGCACGATCTGAGTGAGGAGGAGGCTCGGAAACGCAATCCCGATACGGTCGCGAGAGCCTTGGACCTGCCCTTCGTACGC  
TACGCAGCCAGAGACCCGGACAGCACTTCGTCTCTTCATCCGCCACGGGCCGTTGCAGGTGACGGCAGAGGAAGGAGGATTACCTGTTACGGGTTAGACAAAGGAGG  
TTTCGTTCCCTGGTTGAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAATCCTGGCCCAATGGATAAAAAAGTATTCTATTGGTTTAGACATCGGCACT  
AATTCCGTTGGATGGGCTGTCTATAACCGATGAATACAAAGTACCTTCAAAGAAATTTAAGGTGTTGGGGAACACAGACCGTCATTGATTAAAAAGAATCTTATCGGTG  
CCCTCCTATTGATAGTGGCGAAACGGCAGAGGCGACTCGCCTGAAACGAACCGCTCGGAGAAGGTATACAGTCGCAAGAACCGAATATGTTACTTACAAGAAATTTT  
TAGCAATGAGATGGCCAAAGTTGACGATCTTTCTTTTACCCTGTTGGAAGAGTCTTCTTGTCGAAGAGGACAAGAAACATGAACGGCACCCCATCTTTGGAAACATA  
GTAGATGAGGTGGCATATCATGAAAGTACCAACGATTTATCACCTCAGAAAAAGCTAGTTGACTCAACTGATAAAGCGGACCTGAGGTTAATCTACTTGGCTCTTG  
CCCATATGATAAAGTTCCGTGGGCACCTTCTCATTGAGGGTGATCTAAATCCGACAACCTCGGATGTCGACAAACTGTTTCATCCAGTTAGTACAAACCTATAATCAGTT  
GTTTGAAGAGAACCTATAAATGCAAGTGGCGTGATGCGAAGGCTATTCTTAGCGCCCGCTCTCTAAATCCGACGGCTAGAAAACCTGATCGCACAATTACCCGGA  
GAGAAGAAAAATGGGTTGTTGCGTAACCTTATAGCGCTCTCACTAGGCTGACACCAAAATTTAAGTCGAACCTCGACTTAGCTGAAGATGCCAAATTCAGCTTAGTA  
AGGACACGTACGATGACGATCTCGACAATCTACTGGCACAATTTGGAGATCAGTATGCGGACTTATTTTGGCTGCCAAAAACCTTAGCGATGCAATCCTCTATCTGA  
CATACTGAGAGTTAATACTGAGATTACCAAGCGCCGTTATCCGCTTCAATGATCAAAGGTACGATGAACATCACAAGACTTGACACTTCTCAAGGCCCTAGTCCGT  
CAGCAACTGCCTGAGAAATATAAGGAAATATTCTTTGATCAGTCGAAAAACGGGTACGCAGGTTATATTGACGGCGGAGCGAGTCAAGAGGAATCTACAAGTTTATCA  
AACCATATTAGAGAAGATGGATGGGACGGAAGAGTTGCTTGTAAAACCAATCGCAAGATCTACTGCGAAAGCAGCGGACTTCGACAACGGTAGCATTCCACATCA  
AATCCACTTAGGCGAATTGCATGCTATACTTAGAAGGCAGGAGGATTTTATCCGTTCTCAAAGACAATCGTGAAGAGATTGAGAAAAATCTAACCTTTGCATACCT  
TACTATGTGGGACCCCTGGCCCGAGGGAACCTCGGTTGCGATGGATGACAAGAAAGTCCGAAGAAACGATTACTCCATGGAATTTTGAGGAAGTTGCGATAAAGGTG  
CGTCAGCTCAATCGTTCATCGAGAGGATGACCAACTTTGACAAGAATTTACCGAACGAAAAAGTATTGCCTAAGCACAGTTTACTTTACGAGTATTTACAGTGATACAA  
TGAACCTCACGAAAGTTAAGTATGTCAGTGAAGGATGCGTAACCCGCCTTTCTAAGCGGAGAACAGAAGAAAGCAATAGTAGATCTGTTATTCAAGACCAACCGCAAA  
GTGACAGTTAAGCAATTGAAAGAGGACTACTTTAAGAAAATTTGAATGCTTCGATTCTGTGAGATCTCCGGGGTAGAAGATCGATTTAATGCGTCACTTGGTACGTATC  
ATGACCTCTAAAGATAATTAAAGATAAGGACTTCTGGATAACGAAGAGAATGAAGATATCTTAGAAGATATAGTGTGACTCTTACCCTCTTTGAAGATCGGGAAAT  
GATTGAGGAAAGACTAAAAACATACGCTCACCTGTTGACGATAAGGTTATGAAACAGTTAAAGAGGCGTCGCTATACGGGCTGGGGACGATTGTCGCGGAAACTTATC  
AACGGGATAAGAGACAAGCAAAGTGGTAAACTATTCTGATTTTCTAAAGAGCGACGGCTTCGCCAATAGGAACCTTATGACAGCTGATCCATGATGACTCTTTAACCT  
TCAAAGAGGATATACAAAAGGCACAGGTTTCCGACAAGGGGACTCATTGCACGAACATATTGCGAATCTTGCTGGTTCGCCAGCCATCAAAAAGGGCATACTCCAGAC  
AGTCAAAGTAGTGGATGAGCTAGTTAAGGTGATGGGACGTCACAAACCGGAAAAACATTGTAATCGAGATGGCACGCGAAAAACAAACGACTCAGAAGGGGCAAAAAAC  
AGTCGAGAGCGGATGAAGAGAATAGAAGAGGGTATTAAGAAGTGGGACGACGATCTTAAGAGGAGCATCTGTGGAAAAATACCAATTGCAGAACGAGAACTTTACC  
TCTATTACCTACAAAATGGAAGGGACATGTATGTTGATCAGGAACGGACATAAACCGTTTATCTGATTACGACGTCGATCACATTGTACCCCAATCTTTTGAAGGA  
CGATTCAATCGACAATAAAGTGCTTACACGCTCGGATAAGAACCAGGGGAAAGTGACAATGTTCCAAGCGAGGAAGTCGTAAGAAAAATGAAGAATATTGGCGGCAG  
CTCCTAAATGCGAACTGATAACGCAAGAAAGTTGATAACTTAACTAAGCTGAGAGGGGTGGCTGTCTGAACCTTGACAAGGCCGGATTATTAAACGTCAGCTCG  
TGGAAACCCGCCAATCACAAGCATGTTGCACAGATACTAGATTCCCGAATGAATACGAAATACGACGAGAACGATAAGCTGATTGGGAAGTCAAAGTAATCACTTT

AAAGTCAAATTTGGTGTGCGACTTCAGAAAGGATTTTCAATTCTATAAAGTTAGGGAGATAAATAACTACCACCATGCGCACGACGCTTATCTTAATGCCGTCGTAGGG  
ACCGCACTCATTAAGAAATACCCGAAGCTAGAAAGTGAGTTTGTGTATGGTGATTACAAAGTTTATGACGTCCGTAAGATGATCGCGAAAAGCGAACAGGAGATAGGCA  
AGGCTACAGCCAAATACTTCTTTTATTCTAACATTATGAATTTCTTTAAGACGGAAATCACTCTGGCAAACGGAGAGATACGCAAACGACCTTTAATTGAAACCAATGG  
GGAGACAGGTGAAATCGTATGGGATAAGGGCCGGGACTTCGCGACGGTGAGAAAAGTTTGTCCATGCCCAAGTCAACATAGTAAAGAAAAGTACGGTGCAGACCGGA  
GGGTTTTCAAAGGAATCGATTCTTCCAAAAAGGAATAGTGATAAGCTCATCGCTCGTAAAAAGGACTGGGACCCGAAAAAGTACGGTGGCTTCGATAGCCCTACAGTTG  
CCTATTCTGTCTAGTAGTGGCAAAAGTTGAGAAGGGAAAAATCCAAGAACTGAAGTCAGTCAAAGAATTATTGGGGATAACGATTATGGAGCGCTCGTCTTTTAAAA  
GAACCCCATCGACTTCTTTGAGGCGAAAGGTTACAAGGAAGTAAAAAGGATCTCATAATTAACTACCAAAGTATAGTCTGTTTGGTTAGAAAATGGCCGAAAACGG  
ATGTTGGCTAGCGCCGGAGAGCTTCAAAGGGGAACGAACCTCGCACTACCGTCTAAATACGTGAATTTCTGTATTTAGCGTCCCATTACGAGAAGTTGAAAGGTTAC  
CTGAAGATAACGAACAGAAGCAACTTTTTGTTGAGCAGCACAAACATTATCTCGACGAAATCATAGAGCAAATTCGGAATTCAGTAAGAGAGTCATCTAGCTGATGC  
CAATCTGGACAAAGTATTAAGCGCATACAACAAGCACAGGGATAAACCCATACGTGAGCAGGCGGAAAAATATTATCCATTTGTTTACTCTTACCAACCTCGGCGCTCCA  
GCCGCAATCAAGTATTTTGACACAACGATAGATCGCAAACGATACACTTCTACCAAGGAGGTGCTAGACGCGACACTGATTACCAATCCATCACGGGATTATATGAAA  
CTCGGATAGATTTGTACAGCTTGGGGGTGACGGATCCCCCAAGAAGAAGAGGAAAGTCTCGAGCGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGA  
TTACAAGGATGACGATGACAAGTGA

#### Csy4-T2A-Cas9 amino acid sequence

(Csy4 colored in green, T2A peptide colored in grey, NLS underlined, Cas9 colored in blue, 3xFLAG tag in bold)

MGDHYLDIRLRPDPEFPPAQLMSVLFGKLHQALVAQGGDRIGVSFPDLDESRSRLGERLRIHASADDLRALARPWLEGLRDHLQFGEPAVVPHTPYRQVSRVQAKSN  
PERLRRRLMRRHDLSEEEARKRIPDTVARALDLPFVTLRSQSTGQHFRLFIRHGPLQVTAEEGGFTCYGLSKGGFVPWFEGRGSLLTCGDVEENPGPMDDKYSIGLDIG  
TNSVGWAVITDEYKVPSSKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLSEESFLVEEDKKHERHPIFGN  
IVDEVAYHEKYPTIYHLRKKLVDSDDKADRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDFKFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLLENLIAQLP  
GEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLNLLAQIGDQYADFLAAKNLSDAILSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALV  
RQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYFPLKDNREKIEKILTFRI  
PYVVGPLARGNSRFAMWTRKSEETITPWNFEVVDKGASQSFIERMTNFDKNLPNEKVLPHKSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNR  
KVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLKIKDKDFLDNEENEDILEDIVLTLTFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKL  
INGIRDQKSGKTILDFLKSDGFANRNFMLIHDDSLTFKEDIQAQVSGQGDSLHEHIANLAGSPAICKGILQTVKVVDELVKVMGRHKPENIVEMARENQTTQKGQK  
NSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVHVDPQSLFKDDSIDNKVLTRSDKNRGKSDNPVSEEVVKMKMYWR  
QLLNAKLITQRKFDNLTKAERGGSELDAKGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKIREVKVITLKSLLVSDFRKDFQFYKVIENNYHHAHDAYLNAV  
GTALIKKYPKLESEFVYGDYKYVDVRKMIKSEQEIGKATAKYFFYSNMNFKTEITLANGEIRKRLIETNGETGEIVWDKGRDFATVRKVLSPQVNVKKEVQT  
GGFSKESILPKRNSDKLIARKDWDPKKYGGFDSPTVAYSVLVAKVEKGSKKLKSVKELLGITIMERSSEFKNPIDFLEAKGYKEVKDLIKLPKYSLELENGRK  
RMLASAGELQKGNELALPSKYVNFLLYASHYEKLGKSPEDNEQQLFVEQHKKHYLDEIIIEQISEFSKRVLADANLDKVL SAYNKHDPKPIREQAENIIHLFTLNLGA  
PAAFKYFDTTIDRKRYTSTKEVLDTLIHQSIITGLYETRIDLSQLGGDGSPPKKRKYSSDYKDHDGDKYKDHDIDYKDDDDK

#### Csy4-T2A-Cas9n coding sequence in pNW3

(Csy4 colored in green, T2A peptide colored in grey, Cas9 nickase colored in blue, D10A mutation **yellow**-shaded, NLS underlined, 3xFLAG tag in bold)

ATGGACCACTACCTCGACATTGCTTGGCGACCGGACCCGGAATTTCCCCGGCGCAACTCATGAGCGTGCTCTTCGGCAAGCTCCACCAGGCCCTGGTGGCACAGGGCG  
GGGACAGGATCGGCGTGAGCTTCCCCGACCTCGACGAAAGCCGCTCCCGGCTGGGCGAGCGCTGCGCATTATGCGCTCGGCGGACGACCTTCGTGCCCTGCTCGCCCG  
GCCCTGGCTGGAAGGGTTGCGGGACCATCTGCAATTCGGAGAACCAGGAGTCTGCTCACCACACACCGTACCGTCAGGTGAGTCGGGTTGAGCGGAAAAGCAATCCG  
GAACGCTCGCGGCGGCGCTCATGCGCCGGCACGATCTGAGTGAGGAGGAGGCTCGGAAACGCAATCCCGATACGGTCGCGAGAGCCTTGGACCTGCCCTTCGTACGCG  
TACGACAGCCAGAGCACCGGACAGCACTTCCGTCTCTTCATCCGCCACGGGCGGTTGAGGTGACGGCAGAGGAAGGAGGATTACCTGTTACGGGTTGAGCAAAGGAGG  
TTTCGTTCCCTGGTTCGAGGGCAGAGGAAGTCTGTTAACATGCGGTGACGTGAGGAGAATCCTGGCCCAATGGATAAAAAAGTATTCTATTGGTTTAGCCATCGGCACT  
AATTCCGTTGGATGGGCTGTGATAACCGATGAATACAAAGTACCTTCAAAGAAATTTAAGGTGTTGGGGAACACAGACCGTCATTGATTAAGAAAGTATTATCGGTG  
CCCTCCTATTGATAGTGGCGAAACGGCAGAGGCGACTCGCTGAAACGAACCGCTCGGAGAAGGTATACAGTCGCAAGAACC GAATATGTTACTTACAAGAAATTTT  
TAGCAATGAGATGGCCAAAGTTGACGATCTTTCTTTTACCCTGTTGGAAGAGTCTTCTTGTGCGAAGAGGACAAGAAACATGAACGGCACCCCATCTTTGGAAACATA  
GTAGATGAGGTGGCATATCATGAAAAGTACCAACGATTTATCACCTCAGAAAAAGCTAGTTGACTCAACTGATAAAGCGGACCTGAGGTTAATCTACTTGGCTCTTG  
CCCATATGATAAAGTTCCGTGGGCACCTTTCTCATTGAGGGTGATCTAAATCCGGAACCTCGGATGTCGACAAACTGTTTATCCAGTTAGTACAAACCTATAATCAGTT  
GTTTGAAGAGAACCTTATAATGCAAGTGGCGTGATGCGAAGGCTATTCTTAGCGCCCGCTCTCTAAATCCCGACGGCTAGAAAACCTGATCGCACAATTACCCGGA  
GAGAAGAAAAATGGGTTGTTGCGTAACCTTATAGCGCTCTCACTAGGCTGACACCAAATTTTAAGTGAAGTCTGACTTACTGAGATGCCAAATTCAGCTTAGTA  
AGGACACGTACGATGACGATCTCGACAATCTACTGGCACAATTTGGAGATCAGTATGCGGACTTATTTTGGCTGCCAAAAACCTTAGCGATGCAATCCTCTATCTGA  
CATACTGAGAGTTAATACTGAGATTACCAAGCGCCGTTATCCGCTTCAATGATCAAAAGGTACGATGAACATACCAAGACTTGACACTTCTCAAGGCCCTAGTCCGT  
CAGCAACTGCTTGAGAAATATAAGGAAATATTCTTTGATCAGTCGAAAAACGGGTACGAGGTTATATTGACGGCGGAGCGAGTCAAGAGGAATTCTACAAGTTTATCA  
AATCCACTTAGGCGAATTGCATGCTATACTTAGAAGCGAGGAGATTTTATCCGTTCTCAAAGACAATCGTGAAGAGATTGAGAAAATCTAACCTTTGCGATACCT  
TACTATGTGGGACCCCTGGCCCGAGGGAACCTCGGTTGCGATGGATGACAAGAAAGTCCGAAGAAACGATTACTCCATGGAATTTTGGAGGAGTTGTCGATAAAGGTG  
CGTCAGCTCAATCGTTCATCGAGAGGATGACCAACTTGGACAAGAAATTTACCGAACGAAAAAGTATTGCCTAAGCACAGTTTACTTTACGAGTATTTACAGTGTACAA

TGAACTCACGAAAGTTAAGTATGTCTACTGAGGGCATGCGTAAACCCGCCTTTCTAAGCGGAGAACAGAAGAAAGCAATAGTAGATCTGTTATTCAAGACCAACCGCAAA  
GTGACAGTTAAGCAATTGAAAGAGGACTACTTTAAGAAAATTGAATGCTTCGATTCTGTGAGATCTCCGGGGTAGAAGATCGATTTAATGCGTCACTTGGTACGTATC  
ATGACCTCCTAAAGATAATTAAAGATAAGGACTTCTGGATAACGAAGAGAATGAAGATATCTTAGAAGATATAGTGTGACTCTTACCCTCTTTGAAGATCGGGAAAT  
GATTGAGGAAAGACTAAAAACATACGCTCACCTGTTTCGACGATAAGGTTATGAAACAGTTAAAGAGGCGTCGCTATACGGGCTGGGGACGATTGTCGCGGAAACTTATC  
AACGGGATAAGAGACAAGCAAAGTGGTAAACTATTCTCGATTTTCTAAAGAGCGACGGCTTCGCCAATAGGAACTTTATGCAGCTGATCCATGATGACTCTTTAACCT  
TCAAAGAGGATATACAAAAGGCACAGGTTTCCGGACAAGGGGACTCATTGCACGAACATATTGCGAATCTTGCTGGTTCCGCCAGCCATCAAAAAGGGCATACTCCAGAC  
AGTCAAAGTAGTGGATGAGCTAGTTAAGGTCATGGGACGTCACAAACCGGAAAAACATTGTAATCGAGATGGCACGCGAAAAATCAAACGACTCAGAAGGGGCAAAAAAAC  
AGTCGAGAGCGGATGAAGAGAATAGAAGAGGGTATTAAGAAGTGGGACGCCAGATCTTAAAGGAGCATCTGTGGAAAAATACCAATTGCAGAACGAGAAACTTTACC  
TCTATTACCTACAAAATGGAAGGGACATGTATGTTGATCAGGAACCTGGACATAAACCGTTTATCTGATTACGACGTCGATCACATTGTACCCCAATCCTTTTTGAAGGA  
CGATTCAATCGACAATAAAGTGCTTACACGCTCGGATAAGAACCGAGGGAAAAAGTGACAATGTTCCAAGCGAGGAAGTCGTAAAGAAAAATGAAGAATATTGGCGGCAG  
CTCCTAAATGCGAACTGATAACGCAAAGAAAGTTCGATAACTTAACTAAAGCTGAGAGGGGTGGCTTGCTGAACTTGACAAGGCCGGATTATTAAACGTGAGCTCG  
TGGAAACCCGCCAAATCACAAAGCATGTTGCACAGATACTAGATTCCCGAATGAATACGAAATACGACGAGAACGATAAGCTGATTGGGAAGTCAAAGTAATCACTTT  
AAAGTCAAATTTGGTGTGCGACTTCAGAAAGGATTTTCAATTCTATAAAGTTAGGGAGATAAATACTACCACCATGCGCACGACGCTTATCTTAATGCCGTCGTAGGG  
ACCGCACTCATTAAAGAAATACCCGAAGCTAGAAAGTGAGTTTGTGTATGGTGATTACAAAGTTTATGACGTCGTAAGATGATCGCGAAAAGCGAACAGGAGATAGGCA  
AGGCTACAGCCAAATACTTCTTTTATTCTAACATTATGAATTTCTTTAAGACGGAAATCACTCTGGCAAACGGAGAGATACGCAAACGACCTTTAATTGAAACCAATGG  
GGAGACAGGTGAAATCGTATGGGATAAGGGCCGGGACTTCGCGACGGTGAGAAAAAGTTTGTCCATGCCCAAGTCAACATAGTAAAGAAAACTGAGGTGCAGACCGGA  
GGGTTTTCAAAGGAATCGATTCTTCAAAAAGGAATAGTGATAAGCTCATCGCTCGTAAAAAGGACTGGGACCCGAAAAAGTACGGTGGCTTCGATAGCCCTACAGTTG  
CCTATTCTGTCTAGTAGTGGCAAAAGTTGAGAAGGGAAAAATCCAAGAACTGAAGTCAGTCAAAGAATTATTGGGGATAACGATTATGGAGCGCTCGTCTTTTGAAAA  
GAACCCCATCGACTTCCTTGAGGCGAAAGGTTACAAGGAAGTAAAAAAGGATCTCATAATTAACTACCAAAGTATAGTCTGTTTGAGTTAGAAAAATGGCCGAAACGG  
ATGTTGGCTAGCGCCGAGAGCTTCAAAGGGGAACGAACCTCGCACTACCGTCTAAATACGTGAATTTCTGTATTAGCGTCCCATTACGAGAAGTTGAAAGGTTTAC  
CTGAAGATAACGAACAGAAGCAACTTTTTGTTGAGCAGCACAAACATTATCTCGACGAAATCATAGAGCAAATTTCCGAATTCAGTAAGAGAGTCATCTAGCTGATGC  
CAATCTGGACAAAGTATTAAGCGCATACAACAAGCACAGGGATAAACCCATACGTGAGCAGGCGGAAAAATATTATCCATTTGTTTACTCTTACCAACCTCGGCGCTCCA  
GCCGATTCAAGTATTTTGCACAAACGATAGATCGCAAACGATACACTTCTACCAAGGAGGTGCTAGACGCGACACTGATTACCAATCCATCACGGGATTATATGAAA  
CTCGGATAGATTTGTACAGCTTGGGGGTGACGGATCCCAAGAAGAAGAGGAAAGTCTCGAGCGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGA  
TTACAAGGATGACGATGACAAGTGA

#### Csy4-T2A-Cas9n amino acid sequence

(Csy4 colored in green, T2A peptide colored in grey, Cas9 nickase colored in blue, D10A mutation shaded in yellow, NLS underlined, 3xFLAG tag in bold)

MDHYLDIRLPDPEFPQAQLMSVLFGLKHQALVAQGGDRIGVSFPDLDESRSRLGERLRIHASADDLRALLARPWLEGLRDHLQFGEPAVVPHTPYRQVSRVQAQSNP  
ERLRRRLMRHDLSEEEARKRIPDVARALDLPFVTLRSQSTGQHFRFLIRHGPLQVTAEEGGFTCYGLSKGGFVPWFEGRGSLLTCGDVEENPGPMDDKYSIGL**IGT**  
NSVGWAVITDEYKVPSSKKFKVLGNDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLVESFLVEEDKKHERHPFIGNI  
VDEVAYHEKYPTIYHLRKKLVSDTKADLRLIYALAHMIKFRGHFLIEGDLNPDNSDVKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPG  
EKKNGLFGLNIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLNLLAQIGDQYADFLAAKNLSDAILSDILRVNTEITKAPLSASMIKRYDEHHQDLTLKALVR  
QQLPKEYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYFPLKDNREKIEKILTFRIP  
YYVGPLARGNSRFAWMTRKSEETITPWNFEFVVDKGASAQSFIERMTNFDKNLPNEKVLPHKSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRK  
VTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGYHDLLKIKDKDFLDNEENEDILEDIVLTLTFEDREMIERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKL  
NGIRDQKSGKITLDFLKSDGFANRNFQILHDDSLTFKEDIQKAQVSGQGSLSHEHIANLAGSPAIAKKGILQTVKVVDLVLKVMGRHKPENIVEMARENQTTQKGQKN  
SRERMKRIIEGKELGSQILKEHPVENTQLQNEKLYLYLQNGRDMYVDQELDNRLSDYVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKMKMNYWRQ  
LLNAKLITQRKFDNLTKAERGGSELKDAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKIREVKVITLKSCLVSDFRKDFQFYKREINNYHHAHDAYLNAVVG  
TALIKKYPKLESEFVYGDYKVDVRKMIKSEQEI GKATAKYFFYSNIMNFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSPQVNIKKTEVQTG  
GFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGSKKLKSVKELLGITIMERSSEFKNPIDFLEAKGYKEVKDLIIKLPKYSLELENGRKR  
MLASAGELQKGNELALPSKYVNFYLASHYEKLGKSPEDNEQQLFVEQHKKHYLDEIIIEQISEFSKRVILADANLDKVL SAYNKHDKPIREQAENIIHLFTLNLGAP  
AAFKYFDTTIDRKRYTSTKEVLDTLIHQSIITGLYETRIDLSQLGGDGSPKKKKRVSSDYKDHDGDYKDHDIDYKDDDDK

#### Cas9 coding sequence in pSQT817

(Cas9 coding sequence colored in blue, NLS underlined, 3xFLAG tag in bold)

ATGGATAAAAAGTATTCTATTGGTTTTAGACATCGGCACTAATTCGGTTGGATGGGCTGTCATAACCGATGAATACAAAGTACCTTCAAAGAAATTTAAGGTGTTGGGGA  
ACACAGACCGTCATTTCGATTAAGAAAGAAATCTTATCGGTGCCCTCCTATTTCGATAGTGGCGAAACGGCAGAGCGCACTCGCCTGAAACGAACCGCTCGGAGAAGGTATAC  
ACGTCGCAAGAACCGAATATGTTACTTACAAGAAATTTTATGCAATGAGATGGCCTAAGTTGACGATTCTTTCTTTACCCTTTGGAAGAGTCTTCTTGTGCAAGAG  
GACAAGAAACATGAACGGCACCCCATCTTTGAAACATAGTAGATGAGGTGGCATATCATGAAAAGTACCAACGATTATCACCTCAGAAAAAGCTAGTTGACTCAA  
CTGATAAAGCGGACCTGAGGTAACTACTTGGCTCTTGCCCATATGATAAAGTTCCGTGGGCACTTTCTCATTGAGGGTGATCTAAATCCGGACAACCTCGGATGTCGA  
CAAACCTGTTTCATCCAGTTAGTACAAACCTATAATCAGTTGTTTGAAGAGAACCCTATAAATGCAAGTGGCGTGGATGCGAAGGCTATTCTTAGCGCCCGCTCTCTAAA  
TCCCGACGGCTAGAAAACCTGATCGCACAAATTACCCGGAGAGAAGAAAAATGGGTGTTTCGGTAACCTTATAGCGCTCTCACTAGGCTGACACCAAATTTAAGTCGA  
ACTTCGACTTAGCTGAAGATGCCAAATTGCAGCTTAGTAAGGACACGTACGATGACGATCTCGACAATCTACTGGCACAATTTGAGATCAGTATCGGCACTATTTTTT

GGCTGCCAAAAACCTTAGCGATGCAATCCTCCTATCTGACATACTGAGAGTTAATACTGAGATTACCAAGGCGCCGTTATCCGCTTCAATGATCAAAAGGTACGATGAA  
CATCACCAAGACTTGACACTTCTCAAGGCCCTAGTCCGTGAGCAACTGCCTGAGAAATATAAGGAAATATTCTTTGATCAGTCGAAAAACGGGTACGCAGGTTATATTG  
ACGGCGGAGCGAGTCAAGAGGAATTCTACAAGTTTATCAAACCCATATTAGAGAAGATGGATGGGACGGAAGAGTTGCTTGTAAGTCAATCGCGAAGATCTACTGCG  
AAAGCAGCGGACTTTGACAACGGTAGCATTCCACATCAAATCCACTTAGGCGAATTGCATGCTATACTTAGAAGGCAGGAGGATTTTATCCGTTCTCAAAGACAAT  
CGTGAAGATTGAGAAAACTTAACCTTTTCGCATACCTTACTATGTGGGACCCCTGGCCCGAGGGAACTCTCGGTTTCGATGGATGACAAGAAAGTCCGAAGAAACGA  
TTACTCCATGGAATTTTGAAGAGTTGTCGATAAAGGTGCGTCAGCTCAATCGTTCATCGAGAGGATGACCACTTTGACAAGAAATTTACCGAACGAAAAAGTATTGCC  
TAAGCACAGTTTACTTTACGAGTATTTACAGTGTACAATGAACTCACGAAAGTTAAGTATGTCTACTGAGGGCATGCGTAAACCCGCCTTTCTAAGCGGAGAACAGAAG  
AAAGCAATAGTAGATCTGTTATTCAAGACCAACCGCAAAGTGACAGTTAAGCAATTGAAAGAGGACTACTTTAAGAAAAATTGAATGCTTCGATTCTGTCGAGATCTCCG  
GGGTAGAAGATCGATTTAATGCGTCACTTGGTACGTATCATGACCTCCTAAAGATAATTAAGATAAGGACTTCTGGATAACGAAGAGAATGAAGATATCTTAGAAGA  
TATAGTGTGACTCTTACCCTCTTTGAAGATCGGGAAATGATTGAGGAAAGACTAAAAACATACGCTCACCTGTTTCGACGATAAGGTTATGAAACAGTTAAAGAGGCGT  
CGCTATACGGGCTGGGGACGATTGTCGCGGAAACTTATCAACGGGATAAGAGACAAGCAAAGTGGTAAAACTATTCTCGATTTTCTAAGAGCGACGCGCTTCGCCAATA  
GGAACCTTTATGCAGCTGATCCATGATGACTCTTTAACCTTCAAAGAGGATATACAAAAGGCACAGGTTTCCGGACAAGGGGACTCATTGCACGAACATATTGCGAATCT  
TGCTGGTTTCGCCAGCCATCAAAAAGGGCATACTCCAGACAGTCAAAGTAGTGGATGAGCTAGTTAAGGTCATGGGACGTCACAAACCGGAAAAACATTGTAATCGAGATG  
GCACGCGAAAAATCAAACGACTCAGAAGGGGCAAAAAACAGTCGAGAGCGGATGAAGAGAATAGAAGAGGGTATTAAGAAGTGGGACGCGAGATCTTAAGGAGCATC  
CTGTGGAAAAATACCAATTGCAGAACGAGAACTTTACCTCTATTACCTACAAAATGGAAGGGACATGTATGTTGATCAGGAAGTGGACATAAACCGTTTATCTGATTA  
CGACGTCGATCACATTGTACCCCAATCCTTTTTGAAGGACGATTCAATCGACAATAAAGTGCTTACACGCTCGGATAAGAACCAGGGGAAAAAGTGACAATGTTCCAAGC  
GAGGAAGTCGTAAGAAAAATGAAGAATATTGGCGGCAGCTCCTAAATGCGAAACTGATAACGCAAAGAAAGTTCGATAACTTAACATAAGCTGAGAGGGGTGGCTTGT  
CTGAACCTTGACAAGGCCGGATTATTAACGTCAGCTCGTGGAAACCCGCCAAATCACAAAGCATGTTGCACAGATACTAGATTCCCGAATGAATACGAAATACGACGA  
GAACGATAAGCTGATTCGGGAAGTCAAAGTAATCACTTTAAAGTCAAATTTGGTGTGCGACTTCAGAAAGGATTTTCAATTCTATAAAGTTAGGGAGATAAATAACTAC  
CACCATGCGCACGACGCTTATCTTAATGCCGTCGTAGGGACCGCACTCATTAAAGAAATACCCGAAGCTAGAAAGTAGGTTTGTGTATGGTGATTACAAAGTTTATGACG  
TCCGTAAGATGATCGCGAAAAAGCGAACAGGAGATAGGCAAGGCTACAGCCAAATACTTCTTTTATCTAACATTATGAATTTCTTTAAGACGGAATCACTCTGGCAAA  
CGGAGAGATACGCAAAACGACCTTTAATTGAAACCAATGGGGAGACAGGTGAAATCGTATGGGATAAGGGCCGGGACTTCGCGACGGTGAGAAAAAGTTTGTTCATGCC  
CAAGTCAACATAGTAAAGAAAACTGAGGTGCAGACCGGAGGGTTTTCAAAGGAATCGATTCTTCAAAAAAGGAATAGTGATAAGCTCATCGCTCGTAAAAAGGACTGGG  
ACCCGAAAAAGTACGGTGGCTTCGATAGCCCTACAGTTGCTTATTCTGTCTAGTAGTGCAAAAGTTGAGAAGGGAAAAATCCAAGAACTGAAGTCAGTCAAAGAATT  
ATTGGGGATAACGATTATGGAGCGCTCGTCTTTTGAAGAAACCCCATCGACTTCTTGAGGCGAAAGGTTACAAGGAAGTAAAAAGGATCTCATAATTAAGTACCA  
AAGTATAGTCTGTTTGAGTTAGAAAAATGGCGAAAAACGGATGTTGGCTAGCGCCGGAGAGCTTCAAAGGGGAACGAAGTTCGCACTACCGTCTAAATACGTGAATTTCC  
TGTATTTAGCGTCCATTACGAGAAGTTGAAAGGTTACCTGAAGATAACGAACAGAAGCAACTTTTTGTTGAGCAGCACAAACATTATCTCGACGAAATCATAGAGCA  
AATTTCGGAATTCAGTAAGAGAGTCATCTAGCTGATGCCAATCTGGACAAAGTATTAAGCGCATACAACAAGCACAGGGATAAAACCCATACGTGAGCAGGCGGAAAAAT  
ATTATCCATTGTTTACTCTTACCAACCTCGGCGCTCCAGCCGATTCAAGTATTTTGACACAACGATAGATCGCAACGATACACTTCTACCAAGGAGGTGCTAGACG  
CGACACTGATTACCAATCCATCACGGGATTATATGAAACTCGGATAGATTTGTACAGCTTGGGGGTGACGGATCCCAAGAAGAAGAGGAAAGTCTCGAGCGACTA  
CAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTGA

## Cas9 amino acid sequence

(Cas9 amino acid sequence colored in **blue**, NLS underlined, 3xFLAG tag in **bold**)

MDKKYSIGLDIGTNSVGVAVITDEYKVPSSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRNKRICYLQEIFSNEMAKVDDSFHRLSEESFLVEE  
DKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVKLFIQLVQTYNQLFEEPNINASGVDKAILSARLSK  
SRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDLDNLQAIGDQYADLF LAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDE  
HHQDLTLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYFPLKDN  
REKIEKILTFRIPYVVGPLARGNSRFAWMTRKSEETITPWNFEVVDKGASQSFIERMTNFDKNLPNEKVLPHSLLEYEFTVYNELTKVKYVTEGMRKPAFLSGEQK  
KAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGYHDLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKYAHLFDDKVMKQLKRR  
RYTGWGRLSRKLINGIRDQSGKTILDFLKSDGFANRNFQMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIIKKGILQTVKVVDLVKVMGRHKPENIVIEI  
ARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYLQNGRDMYVDQELDNRLSDYDVHIVPQSFLKDDSIDNKVLTTRSDKNRGKSDNVPS  
EEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGSELDDKAGFIKRQLVETRQITKHVAQILD SRMNTKYDENDKLIREVKITLKSCLVSDFRKDFQFYKVINNY  
HHAHDAYLNAVVGTAIIKKYPKLESEFVYGDKVYDVRKMIKSEQEI GKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL SMP  
QVNI VKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVAKVEKGSKKLKSVKELLGITIMERSSEFKNPIDFLEAGYKEVKKDLIIKLP  
KYSLEFELNGRKRMLASAGELQKGNELALPSKYVNFYLASHYEKLGSPEDNEQKQLFVEQHKHYLDEIEQISEFSKRVLADANLDKVL SAYNKHRRDKPIREQAEN  
IIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDTLIHQSIITGLYETRIDLSQLGGDGSPPKKRKVSSDYKDHDGDYKDHDIDYKDDDDK

## Supplementary Fig. 7 DNA sequences from multiplex gRNA expression plasmids

### *BsmBI* cloning site in the multiplex gRNA expression vector pSQT1313

(U6 promoter colored in green, Csy4 recognition site colored in grey, *BsmBI* sites underlined, *BsmBI* restriction overhangs written in bold, gRNA colored in red)

```
TGTACAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCTTCATATTTGCATATAC
GATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCACT
TTTAAATTATGTTTTAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTTCACTGC
CGTATAGGCAGTGAGACGATTAATGCGTCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCG
TTCCTGCGGTATAGGCAGGTTCACTGCCGTATAGGCAG
```

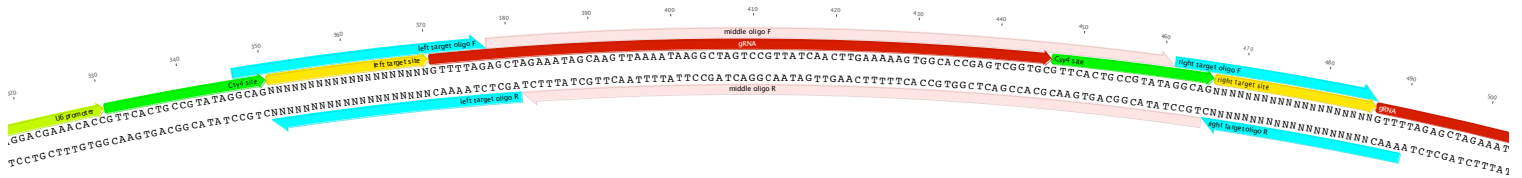
### DNA sequence of two gRNAs under the control of a single U6 promoter after cloning into pSQT1313

(U6 promoter colored in green, Csy4 recognition site colored in grey, gRNA colored in red, gRNA targeting sequences represented by (N)<sub>20</sub>)

```
TGTACAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCTTCATATTTGCATATAC
GATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCACT
TTTAAATTATGTTTTAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTTCACTGC
CGTATAGGCAGNNNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGT
TCACTGCCGTATAGGCAGNNNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTC
GGTGCGTTCACTGCCGTATAGGCAGGTTCACTGCCGTATAGGCAG
```

## Supplementary Fig. 8 Protocol for cloning gRNA sequences into the Csy4-based multiplex expression vector

### Overview



### Middle oligoduplex sequence

Synthesize oSQT875 and oSQT876 as phosphorylated desalted 'Ultrasmer' oligonucleotides. (IDT)

| Name                   | Sequence                                                                                    |
|------------------------|---------------------------------------------------------------------------------------------|
| oSQT875 middle oligo F | /5Phos/AGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCCTTCACTGCCGTATA |
| oSQT876 middle oligo R | /5Phos/TGCCTATACGGCAGTGAACGCACCGACTCGGTGCCACTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTCT    |

### Design Multiplex gRNA Target Site Oligos

1. Visit <http://zifit.partners.org> to find RNA-guided FokI nuclease target sites in your sequence of interest.
2. Order two pairs of oligos (corresponding to left and right "half-sites") for each full dimeric target site.

Target site oligos require the following overhangs for ordered ligation into SQT1313.

|                            |                                        |
|----------------------------|----------------------------------------|
| left target oligoduplex F  | 5' -GCAGNNNNNNNNNNNNNNNNNNNGTTTGTAG-3' |
| left target oligoduplex R  | 5' -AGCTCTAAAACNNNNNNNNNNNNNNNNNNN-3'  |
| right target oligoduplex F | 5' -GGCAGNNNNNNNNNNNNNNNNNNN-3'        |
| right target oligoduplex R | 5' -AAACNNNNNNNNNNNNNNNNNNNC-3'        |

### Prepare Digested Vector Backbone

1. Digest 3-5 µg of pSQT1313 (available via Addgene) with BsmBI (NEB) overnight at 55°C.
2. Heat inactivate for 20 minutes at 80°C.
3. Purify digested pSQT1313 with 1X volume of Ampure XP beads (Agencourt) according to manufacturer's instructions, or gel purify.
4. Dilute digested backbone plasmid pSQT1313 to 10 ng/µl.

### 10X Oligoduplex Annealing Buffer (STE)

| Component         | Volume (ml) | Final concentration (mM) |
|-------------------|-------------|--------------------------|
| 1 M Tris HCL      | 5           | 100                      |
| 5 M NaCl          | 5           | 500                      |
| 0.5 M EDTA        | 1           | 10                       |
| dH <sub>2</sub> O | 39          |                          |

### Anneal oligos

1. Anneal each of the oligonucleotide pairs separately at 10  $\mu$ M.

| Component             | Volume ( $\mu$ l) | Final concentration |
|-----------------------|-------------------|---------------------|
| Oligo 1 (100 $\mu$ M) | 10                | 10 $\mu$ M          |
| Oligo 2 (100 $\mu$ M) | 10                | 10 $\mu$ M          |
| 10X STE Buffer        | 10                | 1X                  |
| dH <sub>2</sub> O     | 70                |                     |

Run the following annealing protocol in a thermocycler:

1. 95°C, 5 minutes
  2. Ramp down temperature to 25°C at -1°C/30 s.
  3. 4°C,  $\infty$
2. Dilute 10  $\mu$ M annealed oligoduplexes 1:1000 to a final concentration of 0.01  $\mu$ M.

### Cloning into multiplex gRNA vectors

*left oligoduplex + middle oligoduplex + right oligoduplex + pSQT1313/BsmBI*

1. Assemble the following 10  $\mu$ l ligation reaction.

| Component                         | Volume ( $\mu$ l) |
|-----------------------------------|-------------------|
| Left oligoduplex (0.01 $\mu$ M)   | 2                 |
| Middle oligoduplex (0.01 $\mu$ M) | 2                 |
| Right oligoduplex (0.01 $\mu$ M)  | 2                 |
| pSQT1313/BsmBI (~10 ng/ $\mu$ l)  | 2                 |
| 10X T4 Ligase Buffer (NEB)        | 1                 |
| T4 Polynucleotide Kinase (NEB)    | 0.5               |
| T4 DNA Ligase (NEB)               | 0.5               |

1. Incubate sample at 16°C for 30 minutes, then at 4°C overnight.
2. The following day, transform 5  $\mu$ l of ligation into 50  $\mu$ l of chemically competent cells. (Incubate 15-30 minutes on ice, heat shock at 42°C for 45 s, incubate 2-5 minutes on ice. Recover with 150-500  $\mu$ l of SOC for 1 hour.)
3. Plate on an LB Agar plate containing 100  $\mu$ g/ml carbenicillin.
4. Pick ~3-6 colonies per target site, grow overnight cultures in LB medium supplemented with 50  $\mu$ g/ml carbenicillin, and prepare miniprep plasmid DNA.
5. Sequence verify multiplex gRNA constructs with primer oSQT379 (5'-AGGGTTATTGTCTCATGAGCGG-3')

**Supplementary Table 1 85 gRNA pairs targeted to the *EGFP* reporter gene**

*See attached Excel file.*

**Supplementary Table 2 FokI-dCas9-mediated EGFP disruption activities on target sites with half-sites in PAM-out orientation and with spacer lengths ranging from 10 to 20 bp (same data presented in Fig. 2c)**

| Target Site  | Spacer Length (bp) | Mean EGFP disruption (%)<br>[replicate 1, replicate 2] |
|--------------|--------------------|--------------------------------------------------------|
| EGFP site 41 | 10                 | 2.7 [2.9; 2.4]                                         |
| EGFP site 61 | 10                 | 2.1 [2.0; 2.1]                                         |
| EGFP site 62 | 10                 | 2.1 [2.0; 2.2]                                         |
| EGFP site 63 | 10                 | 2.3 [2.6; 2.0]                                         |
| EGFP site 64 | 10                 | 1.9 [2.0; 1.7]                                         |
| EGFP site 42 | 11                 | 2.2 [2.1; 2.2]                                         |
| EGFP site 65 | 11                 | 2.4 [2.4; 2.3]                                         |
| EGFP site 66 | 11                 | 1.9 [1.8; 2.0]                                         |
| EGFP site 67 | 11                 | 1.9 [2.0; 1.8]                                         |
| EGFP site 68 | 11                 | 1.9 [1.9; 1.9]                                         |
| EGFP site 69 | 11                 | 2.1 [2.0; 2.1]                                         |
| EGFP site 43 | 12                 | 2.4 [2.5; 2.3]                                         |
| EGFP site 70 | 12                 | 2.3 [1.9; 2.6]                                         |
| EGFP site 44 | 13                 | 12.6 [14.2; 11.0]                                      |
| EGFP site 71 | 13                 | 3.0 [2.5; 3.4]                                         |
| EGFP site 45 | 14                 | 15.3 [16.7; 13.8]                                      |
| EGFP site 72 | 14                 | 14.2 [14.1; 14.3]                                      |
| EGFP site 73 | 14                 | 17.1 [14.4; 19.7]                                      |
| EGFP site 74 | 14                 | 7.8 [6.9; 8.7]                                         |
| EGFP site 46 | 15                 | 18.9 [21.3; 16.4]                                      |
| EGFP site 75 | 15                 | 18.7 [20.7; 16.7]                                      |
| EGFP site 76 | 15                 | 11.0 [14.2; 7.8]                                       |
| EGFP site 77 | 15                 | 20.9 [26.1; 15.6]                                      |
| EGFP site 78 | 15                 | 10.1 [12.2; 8.0]                                       |
| EGFP site 79 | 15                 | 5.6 [6.8; 4.4]                                         |
| EGFP site 47 | 16                 | 21.3 [22.1; 20.4]                                      |
| EGFP site 80 | 16                 | 14.6 [17.0; 12.1]                                      |
| EGFP site 81 | 16                 | 21.9 [24.5; 19.3]                                      |
| EGFP site 82 | 16                 | 19.7 [18.2; 21.2]                                      |
| EGFP site 83 | 16                 | 18.5 [13.0; 23.9]                                      |
| EGFP site 84 | 16                 | 13.1 [9.0; 17.2]                                       |
| EGFP site 48 | 17                 | 16.8 [16.1; 17.4]                                      |
| EGFP site 49 | 18                 | 10.3 [13.2; 7.3]                                       |
| EGFP site 50 | 20                 | 2.6 [2.4; 2.8]                                         |
| EGFP site 85 | 20                 | 2.5 [2.8; 2.1]                                         |

**Supplementary Table 3 Indel counts and frequencies at potential off-target sites of the RFN targeted to VEGFA site 1 as determined by deep sequencing (same data presented in Fig. 3d)**

| Chromosome | Position  | Site         | FokI-dCas9<br>Indels | FokI-dCas9<br>Total | FokI-dCas9<br>Indel<br>Frequency (%) | tdTomato<br>control<br>Indels | tdTomato<br>Total | One-<br>tailed<br>Fisher's<br>Exact<br>p-value |
|------------|-----------|--------------|----------------------|---------------------|--------------------------------------|-------------------------------|-------------------|------------------------------------------------|
| 6          | 43737290  | VEGFA site 1 | 35000                | 150158              | 23.30878                             | 10                            | 258108            | <1E-10                                         |
| 15         | 65637531  | OT1-3        | 1                    | 169681              | 0.00058                              | 1                             | 139847            | 0.7959                                         |
| 12         | 131690182 | OT1-4        | 4                    | 190111              | 0.00210                              | 5                             | 139762            | 0.8719                                         |
| 12         | 1988060   | OT1-6        | 3                    | 258976              | 0.00115                              | 2                             | 178162            | 0.6694                                         |
| 1          | 99347645  | OT1-11       | 4                    | 235853              | 0.00169                              | 4                             | 186287            | 0.7560                                         |
| 17         | 39796322  | OT1-30       | 1                    | 261605              | 0.00038                              | 1                             | 286850            | 0.7265                                         |

**Supplementary Table 4** Indel counts and frequencies induced by FokI-dCas9, Cas9 nickase (Cas9n), and tdTomato controls with single or pairs of gRNAs directed to six endogenous human gene sites as determined by deep sequencing (same data presented in Fig. 4)

| Nuclease Type or Control | Site         | guideRNA | Chromosome | Position | Indel | Totals | Percentages |
|--------------------------|--------------|----------|------------|----------|-------|--------|-------------|
| FokI-dCas9               | VEGFA site 1 | both     | 6          | 43737290 | 35000 | 150158 | 23.3088     |
| FokI-dCas9               | VEGFA site 1 | left     | 6          | 43737290 | 5     | 95476  | 0.0052      |
| FokI-dCas9               | VEGFA site 1 | right    | 6          | 43737290 | 9     | 91962  | 0.0098      |
| FokI-dCas9               | DDB2         | both     | 11         | 47236820 | 11303 | 50062  | 22.5780     |
| FokI-dCas9               | DDB2         | left     | 11         | 47236820 | 311   | 85726  | 0.3628      |
| FokI-dCas9               | DDB2         | right    | 11         | 47236820 | 153   | 95050  | 0.1610      |
| FokI-dCas9               | FANCF site 1 | both     | 11         | 22647331 | 65846 | 195311 | 33.7134     |
| FokI-dCas9               | FANCF site 1 | left     | 11         | 22647331 | 19    | 27487  | 0.0691      |
| FokI-dCas9               | FANCF site 1 | right    | 11         | 22647331 | 845   | 225154 | 0.3753      |
| FokI-dCas9               | FANCF site 2 | both     | 11         | 22647138 | 27743 | 120314 | 23.0588     |
| FokI-dCas9               | FANCF site 2 | left     | 11         | 22647138 | 989   | 205832 | 0.4805      |
| FokI-dCas9               | FANCF site 2 | right    | 11         | 22647138 | 142   | 165130 | 0.0860      |
| FokI-dCas9               | FES          | both     | 15         | 91428181 | 14260 | 125912 | 11.3254     |
| FokI-dCas9               | FES          | left     | 15         | 91428181 | 4     | 143877 | 0.0028      |
| FokI-dCas9               | FES          | right    | 15         | 91428181 | 7     | 145495 | 0.0048      |
| FokI-dCas9               | RUNX1        | both     | 21         | 36421217 | 61057 | 136164 | 44.8408     |
| FokI-dCas9               | RUNX1        | left     | 21         | 36421217 | 222   | 162636 | 0.1365      |
| FokI-dCas9               | RUNX1        | right    | 21         | 36421217 | 109   | 169122 | 0.0645      |
| Cas9n                    | VEGFA site 1 | both     | 6          | 43737290 | 14294 | 99036  | 14.4331     |
| Cas9n                    | VEGFA site 1 | left     | 6          | 43737290 | 573   | 82316  | 0.6961      |
| Cas9n                    | VEGFA site 1 | right    | 6          | 43737290 | 315   | 101957 | 0.3090      |
| Cas9n                    | DDB2         | both     | 11         | 47236820 | 6673  | 31168  | 21.4098     |
| Cas9n                    | DDB2         | left     | 11         | 47236820 | 1680  | 56019  | 2.9990      |
| Cas9n                    | DDB2         | right    | 11         | 47236820 | 172   | 42424  | 0.4054      |
| Cas9n                    | FANCF site 1 | both     | 11         | 22647331 | 66827 | 193111 | 34.6055     |
| Cas9n                    | FANCF site 1 | left     | 11         | 22647331 | 1565  | 109029 | 1.4354      |
| Cas9n                    | FANCF site 1 | right    | 11         | 22647331 | 2457  | 109289 | 2.2482      |
| Cas9n                    | FANCF site 2 | both     | 11         | 22647138 | 17007 | 111468 | 15.2573     |
| Cas9n                    | FANCF site 2 | left     | 11         | 22647138 | 120   | 100591 | 0.1193      |
| Cas9n                    | FANCF site 2 | right    | 11         | 22647138 | 1063  | 93162  | 1.1410      |
| Cas9n                    | FES          | both     | 15         | 91428181 | 16529 | 126597 | 13.0564     |
| Cas9n                    | FES          | left     | 15         | 91428181 | 6     | 125196 | 0.0048      |
| Cas9n                    | FES          | right    | 15         | 91428181 | 23    | 46102  | 0.0499      |
| Cas9n                    | RUNX1        | both     | 21         | 36421217 | 80029 | 216800 | 36.9137     |
| Cas9n                    | RUNX1        | left     | 21         | 36421217 | 1106  | 108670 | 1.0178      |
| Cas9n                    | RUNX1        | right    | 21         | 36421217 | 2169  | 121413 | 1.7865      |
| tdTomato controls (-)    | VEGF site 1  | none     | 6          | 43737290 | 29    | 313517 | 0.0092      |
| tdTomato controls (-)    | FANCF site 1 | none     | 11         | 22647331 | 18    | 578378 | 0.0031      |
| tdTomato controls (-)    | FANCF site 2 | none     | 11         | 22647138 | 81    | 393821 | 0.0206      |
| tdTomato controls (-)    | FES          | none     | 15         | 91428181 | 21    | 410620 | 0.0051      |
| tdTomato controls (-)    | DDB2         | none     | 11         | 47236820 | 14    | 165314 | 0.0085      |
| tdTomato controls (-)    | RUNX1        | none     | 21         | 36421217 | 13    | 511977 | 0.0025      |

A one-tailed Fisher's exact test was performed to determine the probability that FokI-dCas9 is associated with lower frequencies of indels than matched Cas9 nickase at 12 monomeric sites.  $P < 1E-8$  for 10 of the 12 sites tested; the remaining 2 sites that were not significantly lower are FANCF site 2 left ( $P < 1.000$ ) and FES left ( $P < 0.2951$ ).

**Supplementary Table 5** Frequencies of candidate off-target sites (bearing a defined number of mismatches) for RFNs targeted to various endogenous human gene targets

| Site         | 0 | 7 | 8 | 9 | 10 | 11 | 12 | 13  | 14   | 15   | 16    |
|--------------|---|---|---|---|----|----|----|-----|------|------|-------|
| APC          | 1 |   |   |   |    | 1  | 2  | 16  | 74   | 414  | 2254  |
| BRCA1        | 1 | 1 |   |   |    |    |    | 5   | 20   | 164  | 983   |
| DDB2         | 1 |   |   |   |    |    | 2  | 7   | 58   | 267  | 1335  |
| EMX1         | 1 |   |   | 1 |    | 2  | 8  | 40  | 175  | 828  | 3494  |
| FANCF site 1 | 1 |   |   |   |    |    | 2  | 4   | 44   | 298  | 1639  |
| FANCF site 2 | 1 |   |   |   |    |    | 2  | 12  | 79   | 358  | 1718  |
| FES          | 1 |   |   |   |    | 3  | 8  | 32  | 191  | 939  | 4505  |
| GLI1         | 1 |   |   |   |    | 2  | 1  | 7   | 69   | 343  | 1711  |
| MLH1         | 1 |   |   |   |    |    | 2  | 5   | 22   | 96   | 643   |
| RARA         | 1 |   |   |   | 1  | 2  | 8  | 39  | 187  | 698  | 2849  |
| RUNX1        | 1 |   |   |   |    |    |    | 3   | 25   | 145  | 800   |
| SS18         | 1 |   |   |   |    | 1  | 2  | 6   | 39   | 280  | 1207  |
| VEGFA site 1 | 1 |   |   |   | 1  | 2  | 3  | 22  | 103  | 543  | 2676  |
| VEGFA site 2 | 1 |   |   |   | 4  | 9  | 99 | 447 | 1675 | 5608 | 18599 |
| VEGFA site 3 | 1 |   |   |   |    |    | 3  | 20  | 120  | 623  | 2783  |

**Supplementary Table 6** Indel counts and frequencies for potential off-target sites of three dimeric RFNs as determined by deep sequencing

| Site                   | Chr | Position  | Strand | Mismatch | Off-target Sequence<br>(LEFT spacer RIGHT)                             | FokI-<br>dCas9<br>Indels | FokI-<br>dCas9<br>Total | FokI-dCas9<br>Indel<br>Frequency (%) | tdTomato<br>Indels | tdTomato<br>Total | tdTomato<br>Indel<br>Frequency (%) |
|------------------------|-----|-----------|--------|----------|------------------------------------------------------------------------|--------------------------|-------------------------|--------------------------------------|--------------------|-------------------|------------------------------------|
| APC<br>dimeric-OT1     | 22  | 24561126  | -      | 11       | GCTGGAGGCCCTGAACTCCTGG<br>cacatcgccctggcaag<br>TGGTAGGTGAGTGAGACAGCCAG | 9                        | 171867                  | 0.005                                | 9                  | 161508            | 0.006                              |
| APC<br>dimeric-OT2     | 12  | 110161444 | +      | 12       | CCTGGCTGTGCTCCCACTCCTGG<br>cgaaggctggggga<br>TGCCCGGAGAGGGAGCTGGCAGG   | 13                       | 161875                  | 0.008                                | 12                 | 153961            | 0.008                              |
| APC<br>dimeric-OT3     | 9   | 134469049 | +      | 12       | CACGCCTGCTCTTGCCTGGGGG<br>ctgcgtggggtggggc<br>TGGCAGGTGAAGGAGGCTCCGGG  | 5                        | 149755                  | 0.003                                | 5                  | 147843            | 0.003                              |
| MLH1<br>dimeric-OT1    | 5   | 110302178 | -      | 12       | GATTTGATAAACAAGAATTTTCC<br>gatgggatgaaaaaga<br>CAATATGTTGTTCTTGGCAAGGG | 8                        | 258109                  | 0.003                                | 11                 | 319697            | 0.003                              |
| MLH1<br>dimeric-OT2    | 7   | 141152982 | +      | 12       | ACAGTAGTGATCTAGTCATTTC<br>tagctttcctctgagt<br>CAAAACCTCCTTGGTGGCAGGGG  | 6                        | 124298                  | 0.005                                | 7                  | 154968            | 0.005                              |
| VEGFA-1<br>dimeric-OT1 | 7   | 71092775  | +      | 12       | CCAAGAACAATGCCCCCGCCCC<br>catagacttgatca<br>TCCCTCCTGGGCCAGAGCCAGCT    | 50                       | 317008                  | 0.016                                | 45                 | 367201            | 0.012                              |
| VEGFA-1<br>dimeric-OT2 | 11  | 102962459 | -      | 12       | CCAGGTACCATCTCCCCCACCC<br>ttcaagcttcctcc<br>TCCCTCCTTTGCTGGACAACCTGG   | 18                       | 302045                  | 0.006                                | 23                 | 356774            | 0.006                              |
| VEGFA-1<br>dimeric-OT3 | 22  | 37215219  | -      | 12       | CCTGGGGCAAACCTCCCCCACCC<br>accgcccccggtggtg<br>GGCCTCAGATTCCCCAGCCTTAG | 67                       | 314727                  | 0.021                                | 67                 | 323840            | 0.021                              |

Off-target (OT) sites examined are a subset of those shown in **Supplementary Table 5**. (Note that the sites shown for VEGFA Site 1 are the most closely mismatched in the genome because the one site with 10 mismatches and the two sites with 11 mismatches shown in **Supplementary Table 5** actually overlap with the on-target site.) Indel counts and frequencies were determined for U2OS cells transfected with RFN components or with a tdTomato-encoding plasmid as a control. Deep sequencing was performed using the same genomic DNA used in the experiments of **Fig. 3 and 4** and **Supplementary Table 4**.

**Supplementary Table 7   Primers and RFN target sites used in this study**

*See attached Excel file.*