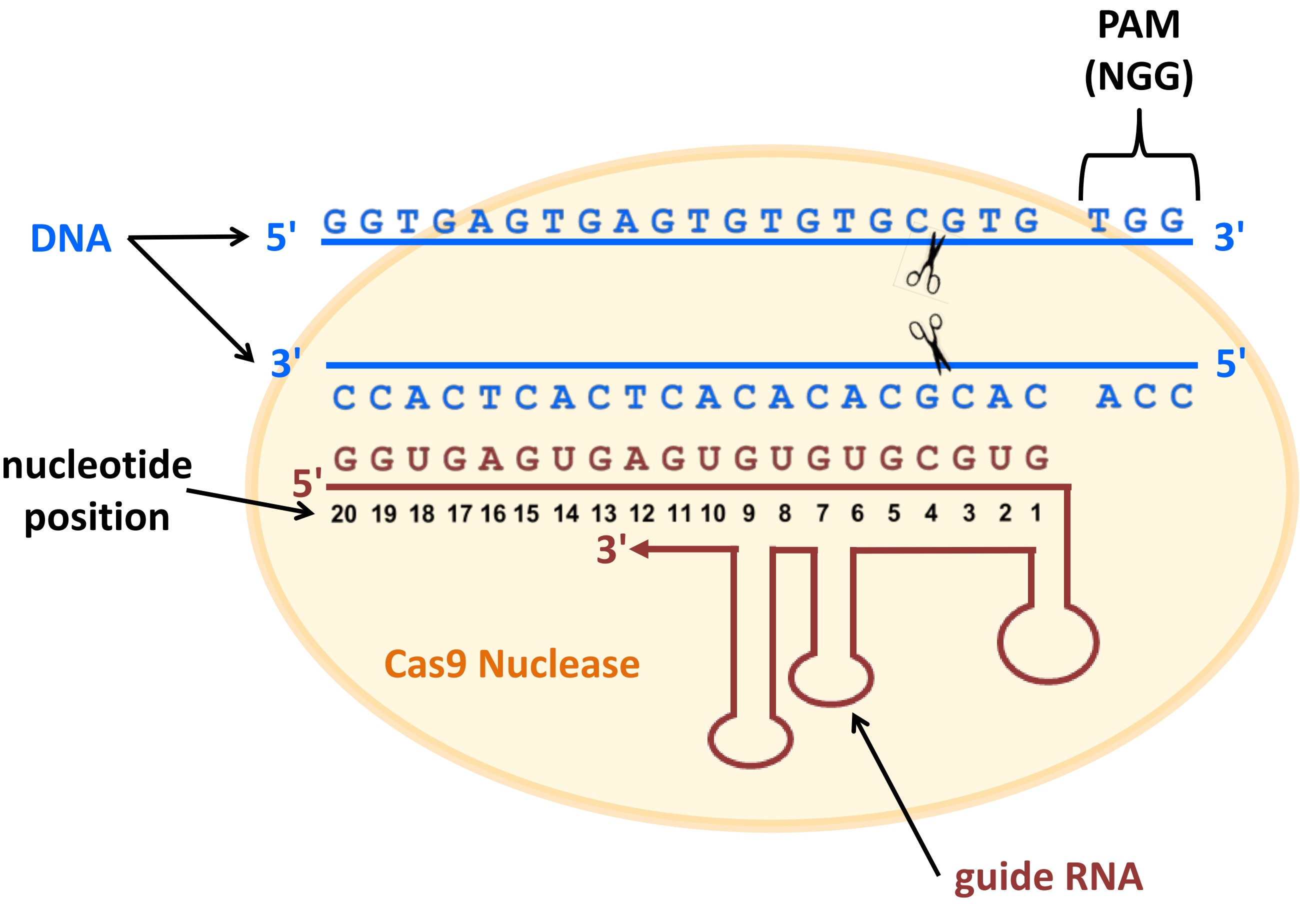
# Supplementary Results

## Titration of sgRNA- and Cas9-expressing plasmid amounts used for the EGFP disruption assay

Single guide RNAs (sgRNAs) were generated for three different sequences (**Supplementary Fig. 2a**) located upstream of *EGFP* nucleotide 502, a position at which the introduction of frameshift mutations via non-homologous end-joining can robustly disrupt expression of EGFP[1, 2]. For each of the three target sites, we initially transfected a range of sgRNA-expressing plasmid amounts (12.5 to 250 ng) together with 750 ng of a plasmid expressing a codon-optimized version of the Cas9 nuclease into our U2OS.EGFP reporter cells bearing a single copy, constitutively expressed *EGFP-PEST* reporter gene. All three RGENs efficiently disrupted EGFP expression at the highest concentration of sgRNA plasmid (250 ng) (**Supplementary Fig. 2b**). However, RGENs for target sites #1 and #3 exhibited equivalent levels of disruption when lower amounts of sgRNA-expressing plasmid were transfected whereas RGEN activity at target site #2 dropped immediately when the amount of sgRNA-expressing plasmid transfected was decreased (**Supplementary Fig. 2b**). We next titrated the amount of Cas9-encoding plasmid (range from 50 ng to 750 ng) transfected into our U2OS.EGFP reporter cells and assayed for EGFP disruption. As shown in **Supplementary Fig. 2c**, target site #1 tolerated a three-fold decrease in the amount of Cas9-encoding plasmid transfected without substantial loss of EGFP disruption activity. However, the activities of RGENs targeting target sites #2 and #3 decreased immediately with a three-fold reduction in the amount of Cas9 plasmid transfected (**Supplementary Fig. 2c**). Based on these results, we used 25ng/250ng, 250ng/750ng, and 200ng/750ng of sgRNA-/Cas9-expressing plasmids for *EGFP* target sites #1, #2, and #3, respectively, for the experiments described in the main text.

We do not understand the reasons why some sgRNA/Cas9 combinations work better than others in disrupting EGFP expression nor do we know why some of these combinations are more or less sensitive to the amount of plasmids used for transfection. Although it is possible that the range of off-target sites present in the genome for these three sgRNAs might influence each of their activities, we did not observe any differences in the numbers of genomic sites that differ by one to six bps for each of these particular target sites (**Supplementary Table 3**) that would account for the differential behavior of the three sgRNAs.

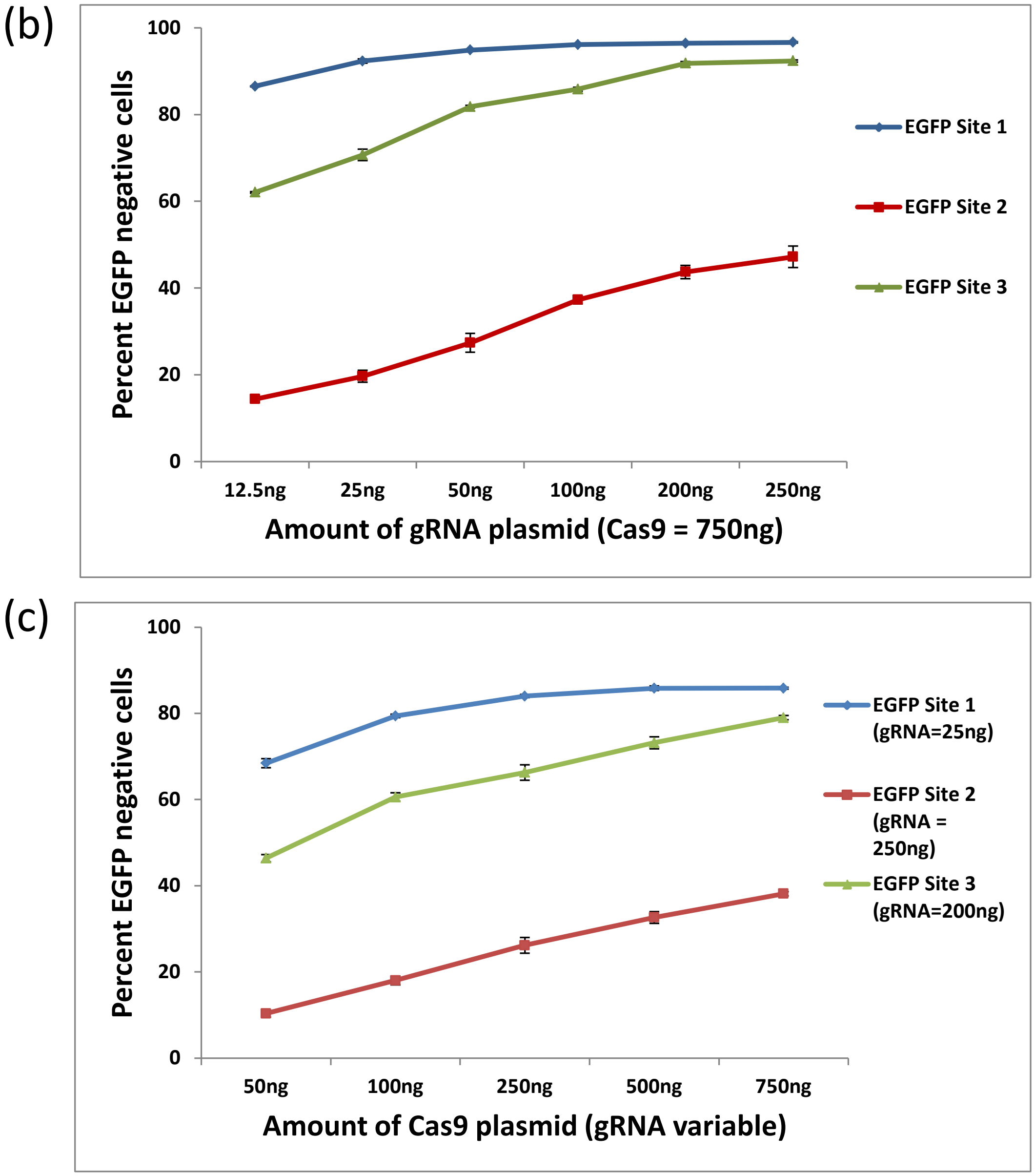


**Supplementary Fig. 1 Schematic illustrating a sgRNA/Cas9 nuclease complex bound to its target DNA site**

Scissors indicate approximate cleavage points of the Cas9 nuclease on the genomic DNA target site.

Note the numbering of nucleotides on the guide RNA proceeds in an inverse fashion from 3’ to 5’.

(a) **EGFP Site 1** GGGCACGGGCAGCTTGCCGGTGG
**EGFP Site 2** GATGCCGTTCTTCTGCTTGTCGG
**EGFP Site 3** GGTGGTGCAGATGAACTTCAGGG



**Supplementary Fig. 2 Titration of sgRNA- and Cas9-expressing plasmid amounts used for the human cell-based EGFP disruption assay**

(a) Sequences for three RGEN target sites in *EGFP* used in this study. (b) Activities of RGENs in the EGFP disruption assay performed using varying amounts of sgRNA-expressing plasmids. (c) Activities of RGENs in the EGFP disruption assay performed using varying amounts of Cas9-expression plasmid.

## Target 1 (*VEGFA* Site 1):

### OT1-3
AGACAGGACATTCTGACACCCCAGGAGCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTGCA Wild-type x18
<----------------------------AACTCCCTCCATCCCACAAATCCGTCCTTAGATGTGCA Δ53
AGACAGGACATTCTGACACC-----------------CCATCCCACAAATCCGTCCTTAGATGTGCA Δ17
AGACAGGACATTCTGACACCCCAGGA---------------CCCACAAATCCGTCCTTAGATGTGCA Δ15
AGACAGGACATTCTGACACCCCAG--GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTGCA Δ2

AGACAGGACATTCTGACACCCCAGGA*GG*GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTGCA +2

### OT1-6
GAGAGAGGCTCCCATCACGGGGGAGGGAGTTTGCTCCTGGGGAACCTGTGATCCCCACAGGGAACA Wild-type x87
GAGAGAGGCTCCCATCACGGGGG-----------------------------------AGGGAACA Δ35 x3
GAGAGAGGCTCCCATCACGGGGGA--------------GGGGAACCTGTGATCCCCACAGGGAACA Δ14 x1

### OT1-11
TGGACTCTACCCACTGAATGCCAGGAGCAAACTTCCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Wild-type x27
TGGACTCTACCCACTG-------------------------------------------AATGTCTC Δ43
TGGACTCTA-------------------------CCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ25
TGGACTCTAC------------------------CCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ24
TGGACTCTACCCACTGAATG---------------CCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ15
TGGACTCTACCCACTGAATG---------------CCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ15
TGGACTCTACCCACTGAAT-------GCAAACTTCCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ7
TGGACTCTACCCACTGAATGCCAGGA------TTCCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ6
TGGACTCTACCCACTGAATGCCTGG------CATCCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ6
TGGACTCTACCCACTGAATGCCAGG---AAACTTCCCCTCCCCGAGTTGTGACAGCAAAAATGTCTC Δ3

TGGACTCTACCCACTGAATGCCAGGA*TGGAAGATAATTTTTTCCATAGACCAGGGTGGGGGAATGGTTTCGGGATGATTCAAGCACATCAC ATTTATTGTGCACTTTATTTCTATTACTATTATATTGTAATGTATACTAAAAATAATTATACAACTCACCATAATGTAGAACCAGTGGGAG CC*GCAAACTTCCCCTCCCCGAGTTGTGACAGCAAAAATGTCTCA +158

TGGACTCTACCCACTGAATGCCAGGCAAACTTCCCCTCCCCGAGTTGTGACA*GCAAAAATGTCTGGCCTAATGTCTG*GCAAAAATGTCTCA AGACATTGCCAAATGTCCCCT +23 (Δ2 +25)

## Target 2 (*VEGFA* Site 2):

### OT2-2
ACCCACCTCCCTATCCTCAAAACTTGGCCAGAGGCGGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTG Wild-type   x30
ACCCAC---------------------------------------------------CGCCTTGGTG Δ51
ACCCACCTCC------------------------------------TATCCTAGGAGCGCCTTGGTG Δ36
ACCCACCTCCCTATCCTCAAAACTT---------------------------------CCCTTGGTG Δ33
ACCCACCTCCCTATCCTCAAAACTTGGCCAGAG------------------TAGGAGCGCCTTGGTG Δ18
ACCCACCTCCCTATCCTCAAAACTT----------GGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTG Δ10
ACCCACCTCCCTATCCTCAAAACTT-------GGCGGGTTGGAGGGGCCCCTAGGAGCGCCTTGGTG Δ7
ACCCACCTCCCTATCCTCAAAACTTGGCCAGA------GTGGAGGGGCCCCTAGGAGCGCCTTGGTG Δ6

ACCCACCTCCCTATCCTCAAAACT---------------------------------------
*CTGGCAGTCTGTCAGTGCGTTATCTTGTCACACTTCTACAAGGGGGCTCTCCCTGCATTCT*GA +21 (Δ40, +61)

ACCCACCTCCCTATCCTCAAAACTTGGCCAGAG*GGCGCCTCCCCAGGAAGTGCTCCGGCCAGCCCAGGGTAAACACGCTAGCCCTGCCCCT CTGGGACCATAGCCCGGGGACCCAGACTCTTGGCCACGCTCATTCCCACC*GCGGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTG +108

### OT2-15
TGACTGTCGGTGCCCCACATGTGGCAGATGCCCAGAGGCGGGGTGTGGGGGGTACTTTGTGGGCGTT Wild-type X71
TGACTGTC---------------------------------GGTGTGGGGGGTACTTTGTGGGCGTT Δ33
TGACTGTCGGTGCCCCACATGTGGCAGATGCCCAGAG-----------------------GGGCGTT Δ23
TGACTGTCGGTGCCCCACATGTGGCAGA---------------TGTGGGGGGTACTTTGTGGGCGTT Δ15
TGACTGTCGGTGCCCCACATGT--------------GGCGGGGTGTGGGGGGTACTTTGTGGGCGTT Δ14
TGACTGTCGGTGCCCCACATGTGGCAGATG----GAGGCGGGGTGTGGGGGGTACTTTGTGGGCGTT Δ4
TGACTGTCGGTGCCCCACATGTGGCAGATGCCCAGA----GGGTGTGGGGGGTACTTTGTGGGCGTT Δ4
TGACTGTCGGTGCCCCACATGTGGCAGATGCCCAGAG--GGGGTGTGGGGGGTACTTTGTGGGCGTT Δ2

```
TGACTGTCGGTGCCCCACATGTGGCAGATGCCCAGAGTTGCGGGGTGTGGGGGGTACTTTGTGGGCGTT +2
TGACTGTCGGTGCCCCACATGTGGCAGATGCCCAGAGCTGCGCGGGGTGTGGGGGGTACTTTGTGGGCGTT +2
```

**OT2-24**

```
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCCCCTCCCCACCCACCCCGCCTCAGGCTTGAAGA Wild-type x8
ACAAGA------------------------------------------------------------> Δ121
<----------------------------------------------------CTCAGGCTTGAAGA Δ82
ACAAGATG----------------------------------------------------------> Δ80
<-----------------------------------------------------------------> Δ79
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCC-------------------------------> Δ42
ACAAGATGACTATGTCCCTCTGGGC----------------------------------CTTGAAGA Δ34
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCCCCTCCC--------------CAGGCTTGAAGA Δ14
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCCCCTCC------------CCTCAGGCTTGAAGA Δ12
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCCCCTCCCCA-----------GCAGGCTTGAAGA Δ11
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCCCCTCCCCACCCAC-----------CTTGAAGA Δ11
ACAAGATGACTATGTCCCTCTGGGCCCCATCCTCCCCTCCC-----------GCTCAGGCTTGAAGA Δ11
```

```
ACAAGATGACTATGTCCCTCTGGGCCCGCCTCAAGTGATCCAGCTGCCTTGGCCTCCAAAAGTGCTAGCAGTACAGATGTGAGCCTCCATG
CCTGGCCTATTGCAACATCCCATCTCTGTGAAGCAGGGTTTTCTGCAGTGACAGCAAGAAGAGCACAGGGCCAAAAAAACTTTGTCCTTTA
GAAAGGATCTACCTTTTAGGCTGAGAATGGCA +76 (Δ43 +119)
```

## Target 3 (*VEGFA* Site 3):

**OT3-2**

```
GAGTGAGAGAGCGAGTGAGTGAGTGAGTGAGTGTGTGTGTGGGGGGGACTCGGCTTGTTGTTGTCGG Wild-type x14
GAGTGAGAGAGCGAGTGAGTGAGTGAGTGA----GTGTGTGGGGGGGACTCGGCTTGTTGTTGTCGG Δ4
GAGTGAGAGAGCGAGTGAGTGAGTGAGTGA------GTGTGGGGGGGACTCGGCTTGTTGTTGTCGG Δ6 x2
```

**OT3-9**

```
GTGTTGGGATGCGGGAGTGGGTGAGTGAGTGCGTGCGGGTGGCGATGCAAGCGTGTGCGAATGCGTG x173
GTGTTGGGATGCGGGA--------------------------------------------------> Δ80
GTGTTGGGATGC--------------------------------------------------GCGTG Δ50
GTGTTGGGATGCGGGAGTGGGTGAGTGA----------GTGGCGATGCAAGCGTGTGCGAATGCGTG Δ10
```

```
GTGTTGGGATGCGGGAGTGGGTGAGTGAGTGCAAGTGCGGGTGGCGATGCAAGCGTGTGCGAATGCGTG +2
```

**OT3-18**

```
TTTCAAAGACAGTAGATCTTAAATGTCCTCACGCACACACTCACCCACACATAAAAGGTGGTAACTG Wild-type x27
TTTCAAAGACAGTAGATCT--------------------------------TAAAAGGTGGTAACTG Δ32
TTTCAAAGACAGTAGATCTTAAATGT-----------------------CATAAAAGGTGGTAACTG Δ23
TTTCAAAGACAGTAGATCTTAAATGTCCT------------------CACATAAAAGGTGGTAACTG Δ18 x4
TTTCAAAGACAGTAGATCTTAAATGTCCT---------------CCACACATAAAAGGTGGTAACTG Δ15
TTTCAAAGACAGTAGATCTTAAATGTC------------CTCACCCACACATAAAAGGTGGTAACTG Δ12
TTTCAAAGACAGTAGATCTTAAATGTCCTCA--CACACACTCACCCACACATAAAAGGTGGTAACTG Δ2
```

```
TTTCAAAGACAGTAGATCTTAAATGTCCTCACAGGCTGGAGTACAGTGGCATGATATCAGCTCACTGCAATCTCGGGCTCCCGGGTTCAAG
CCATGCACACACTCACCCACACATAAAAGGTGGTAAC +63
```

## Target 4 (*EMX1*):

**OT4-1**

```
ACCTGTACATCTGCACAAGATTGCCTTTACTCCATGCCTTTCTTCTTCTGCTCTAACTCTGACAATC Wild-type x20
----------------------------------------------------------------ATC Δ64
ACCTGTACATCTGCACAAGATTGCCTTTACTCC----------------------------ACAATC Δ28
ACCTGTACATCTGCACAAGATTGCCTTTACTCCAT--------------------ACTCTGACAATC Δ20
ACCTGTACATCTGCACAAGATTGC--------------------CTTCTGCTCTAACTCTGACAATC Δ20
ACCTGTACATCTGCACAAGATTGCCTTTACTCCATGCCTTTCT-------------------CAATC Δ19
ACCTGTACATCTGCACAAGATTGCCTTTACTCCA--------------TGCTCTAACTCTGACAATC Δ14
TCCTGTACATCTGCACAAGATTGCCTTTACTCC--------CTTCTTCTGCTCTAACTCTGACAATC Δ8
```

## Supplementary Fig. 3 Sequences of off-target indel mutations induced by RGENs in human U2OS.EGFP cells

Wild-type genomic off-target sites recognized by RGENs (including the PAM sequence) are highlighted in yellow and numbered as in **Table 1** and **Supplementary Table 2**. Note that the complementary strand is shown for some sites. Deleted bases are shown as red dashes on a grey background. Inserted bases are italicized and highlighted in blue.

## Target 1 (*VEGFA* Site 1):

**OT1-3**
TCAGACAGGACATTCTGACACCCCAGGAGCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG Wild-type x41
TCAGACAGGACATT---------------CAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG Δ15
TCAGACAGGACATTCT----------GAGCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG Δ10
TCAGACAGGACATTCTGAC---------GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG Δ9
TCAGACAGGACATCCTGACAC-------GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG Δ7
TCAGACAGGACATTCTGACACCCCAG--GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG Δ2 x6

TCAGACAGGACATTCTGACACCCCAGGA*TG*TCCTCCTCCCTCCATCCCACAAATCCGTCCTTAGATGTG +2

TCAGNCAGGACATTNNGNCACCCCAGG*AAACNNGAGTTTTCGNTNCNNNGANNGTCAGACCCAGN*AGCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG +38

TCAGACAGGACATTNTGACACCCCAGGA*GTNTGCACNTCAGTTTTCTTTANTATGTNGNNNNGGGGCANGNACAAANNTTTN*GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG +54

TCAGACAGGACATTCTGACACCCCAGGATGTTTGTTTGAGTCAGAGTCTCTCTTTTGTCACCCAGGCTGGAGTGCAGTGGAAACCTGTGCCTTTTGTATATCCTCTTTGAAGGTTAAAGAGTCATCATGGATCANCNNCATAAAGCAAACTCCNTCCATCC +116

TCAGACAGGACATTCTGACACCCCAGGA*TAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTAGGGGAGTTCTGAGCTCTGACGCCGGGCGTGTTAGGAGATAGCAGGCCGTTAATGACCATCCCAGCCGAATTCCTCACTGTGCAGATGAGGAAGTGAGCTCAGGGAGGCTGAGTGTCCCAGGCCTGTTGCCAGATGAGGCCACGCTGAGACTGT*GCAAACTCCCTCCATCCCACAAATCCGTCCTTAGATGTG +247

**OT1-6**
TGGAGAGAGGCTCCCATCACGGGGGAGGGAGTTTGCTCCTGGGGAACCTGTGATCCCCACAGGGAAC Wild-type x88
TGGAGAGAGGCTCCCATCACGGGGGAGGGAGTTTG-----------CCTGTGATCCCCACAGGGAAC Δ11
TGGAGAGAGGCTCCCATCACGGGGGAGGGAGTTT-------GGGAACCTGTGATCCCCACAGGGAAC Δ7

**OT1-11**
AGCATCGCTGGACTCTACCCACTGAATGCCAGGAGCAAACTTCCCCTCCCCGAGTTGTGACAGCAAA Wild-type x84
AGCATCGCTGGACTCTACCCACTGAATGCCAGGA--------------CCCGAGTTGTGACAGCAAA Δ14
AGCATCGCTGGACTCTACCCACTGA---------GCAAACTTCCCCTCCCCGAGTTGTGACAGCAAA Δ9
AGCATCGCTGGACTCTACCCACTGAATGCCAG--GCAAACTTCCCCTCCCCGAGTTGTGACAGCAAA Δ2

*AGCATCGCTGGACTCTACCCACTGAATGCCAGGAGTTCAGACGATTGAATGTATCAACTTGGCACATTGCCTATCAACTGGTGAGTGCTCAAAAATATCCATTGCTGTGATCAGTAATGCCACAGGGTGACCATTTAAGGACAGAGTCCATGTTTTATCCATCCTTAGCAAACTTCCCCTCCCCGAGTTGTGACAGCAAA +133*

AGCATCGCTGGACTCTACCCACTGAATGCCAG*AGCCCTTCCTTCTCCCTCTCTTCCTCCAGAGGTCCTGCCGAGATCAGGTTGGAGGTCCTCTTTGTTCTTATGCCCATTCCTCCCCCAGGCACTTGGAGGAGGGCACTGTTTTTGAGTGTGCAAGTCTTTCTCTGTTACTGTT*GGGCAAACTTCCCCTCCCCGAGTTGTGACAGCAAA +*142*

AGCATCGCTGGACTCTACCCACTGAATGCCAGGA*TGTTTTGTTTGCGACGGANTCTCACTCTGTCGNCCGGGCTGGAGTGCANNG*GCACANTTCCTC*ANCTGACTGCNATGTCCGCCTCCCGGATTCAAGTGATTCTCCTGCCCCAGCCTCCCGAGTAGCTGGGATTATAGGTGCCTGCCACCATGCCTGGCTAATTTTTTTTTTTTTTTTAAATGGAGTCTCACTCTGTTG*CCCCCGAGTTGTGACAGCAAA +186

## Target 2 (*VEGFA* Site 2):

**OT2-2**
CACCTCCCTATCCTCAAAACTTGGCCAGAGGCGGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTGGGA
CACCTCCCTATCCTCAAAACTTGGCCAGAGGCGGGGT-------------GGAGCGCCTTGGTGGGA Δ13
CACCTCCCTATCCTCAAAACTT-------GGCGGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTGGGA Δ7
CACCTCCCTATCCTCAAAACTTG------GACGGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTGGGA Δ6
CACCTCCCTATCCTCAAAACTTGGCC--AGGCGGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTGGGA Δ2

CACCTCCCTATCCTCAAAACTTGGCCAGAGACTTANACCTAANACCTCAAACTATGAGACTGCTACNAGAGAACATCANAAAAACTTTCCAGGACATTCTTCTGGNGGGGTGGAGGGGCCCCTANGAGCGCCTTGNNGGGA +74

CACCTCCCTATCCTCAAAACTTGGCCAGAGTCTACAGATTTATAAAATATTACCAGTTAATCATGACACATATTGTTTATTTTCAAATATTTTTTCTAGTTAAACCCACCATTTATATAACCAATTATATTTGATATTATTTAAAATTTTTGTATTAACACCCCACCAAATCATTTTACAGC

GGGGTGGAGGGGCCCCTAGGAGCGCCTTGGTGGGA +150

**OT2-15**
GTCGGTGCCCCACATGTGGCAGATGCCCAGAGGCGGGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Wild-type x79
GTCGGTGCCCCAC-------------------GCGGGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ19
GTCGGTGCCCCACATGTGGC------------GCGGGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ12
GTCGGTGCCCCACATGTGGCAGATGCCCAGA-----GGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ5
GTCGGTGCCCCACATGTGGCAGATGCCCA-----GGGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ5
GTCGGTGCCCCACATGTGGCAGATGCCCAGA----GGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ4
GTCGGTGCCCCACATGTGGCAGATGCCC----GCGGGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ4
GTCGGTGCCCCACATGTGGCAGATGCCC--AGGCGGGGTGTGGGGGGTACTTTGTGGGCGTTTTGGG Δ2 x2

**OT2-24**
GTCCCTCTGGGCCCCATCCTCCCCTCCCCACCCACCCCGCCTCAGGCTTGAAGAGGAAAGAAGAGCA
GTCCCTCTGGGCCCC------------------------------------ANAGNANNNANNANNN Δ36
GTCCCTCTGGGCCCCATCCTCCCCTCCCC-------------------TGAAGAGGAAAGAAGAGCG Δ19
GTCCCTCTNNNCNNCNT-----------------CCNCNCCTCAGGCTTGAAGAGGAAAGAAGAGCG Δ17
GTCCCTCTGGGCCCCATCCTCCCCTC-------------CCTCAGGCTTGAAGAGGAAAGAAGAGCG Δ13
GTCCCTCTGGGCCCCATCCTCCCCTCCCCA----------CTCAGGCTTGAAGAGGAAAGAAGAGCG Δ10
GTCCCTCNNNNNNNCCTCCTCCCC--------NNCCCNCNCTCAGGCTTGAAGAGGAAAGAAGAGCG Δ8
GTCCCTCTGGGCCCCATCCTCCCCTCCCCACCCA------CTCAGGCTTGAAGAGGAAAGAAGAGCG Δ6
GTCCCTCTGGGCCCCATCCTCCCCTCCCCACCCA-----CCTCAGGCTTGAAGAGGAAAGAAGAGCG Δ5

GTCCCTCTGGGCCCCATCCTCCCCTCCCCACCCACCCCG*CATCGTACGTGTCCTTACTAAGCTGCAATTTGGCATCTTCAGCTAAGTCGAA*
*GTTCGA*CCTCAGGCTTGAAGAGGAAAGAAGAGCG +58

## Target 3 (*VEGFA* Site 3):

**OT3-2***
GAGAGCGAGTGAGTGAGTGAGTGAGTGTGTGTGTGGGGGGGACTCGGCTTGTTGTTGTCGGTGACTT Wild-type x26

GAGAGCGAGTGAGTGAGTGAGTGAGTGTGNGTNTNNNNATTTCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTAGGGTGCCTA
ATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
CAANNNNNGNNNAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAG
CGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACANAANGGGACTCGGCTTGTTGTTGTCGGTGACTT +280

**OT3-9**
TGGAGGTGTTGGGATGCGGGAGTGGGTGAGTGAGTGCGTGCGGGTGGCGATGCAAGCGTGTGCGAAT Wild-type x101
TGGAGGTGTTGGGATGCGGGAGTGG--------GTGCGTGCGGGTGGCGATGCAAGCGTGTGCGAAT Δ8
TGGAGGTGTTGGGATGCGGGAGTGGGTGA--------GTGCGGGTGGCGATGCAAGCGTGTGCGAAT Δ8

**OT3-18**
CAAAGACAGTAGATCTTAAATGTCCTCACGCACACACTCACCCACACATAAAAGGTGGTAACTGTGT Wild-type x64
CAAAGACAGTAGATCTTAA----------GCACACACTCACCCACACATAAAAGGTGGTAACTGTGT Δ10
CAAAGACAGTAGATCTTAAATGTCCTCACG-------TCACCCACACATAAAAGGTGGTAACTGTGT Δ7
CAAAGACAGTAGATCTTAAATGTC-----GCACACACTCACCCACACATAAAAGGTGGTAACTGTGT Δ5

CAAAGACAGTAGATCTTAAATGTCCTCAC*GCCGACNATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACG*
*GGCCAGATA*GCACACACTCACCCACACATAAAAGGTGGTAACTGTGT +71

CAAAGACAGTAGATCTTAAATGTCCTCAC*GCAAATTTTATTTTGGTTCATGATATGGCTTGGCGTGTATGCTTTTCATTTGTAAAATTGCT*
*GTTCTTTTGACAATTTAAGTGACTGTTTCATTGACTACAAGTTTGAAAATAAAAATTAATTAAGAAAAAAATTCCAATGACTGTGCTGTGG*
*TTGG*GCACACACTCACCCACACATAAAAGGTGGTAACTGTGT +157

CAAAGACAGTAGATCTTAAATGTCCTCAC*GTGGAAACATAGTAGATGAGGTGGCATATCATGAAAAGTACCCAACGATTTATCACCTCANA*
*AAAAAGCTAGTTGACTCAACTGATAAAGCGGACCTGAGGTTAATCTACTTGGCTCTTGCCCATATGATAAAGTTCCGTGGGCACTTTCTCA*
*TTGAGGGTGATCTAAATCCGGACAACTCGGATGTCGA*GCACACACTCACCCACACATAAAAGGTGGTAACTGTGT +190

CAAAGACAGTAGATCTTAAATGTCCTCACG----------------
*TTATTTAGAGACAGAGTCTCACTCTGTTGCCCAGGCTGGGGTGCAGTGGTACGAACTCGGCTCACTGCAACCTCCGTCTCCTGGGCTCAAG*
*TGATTATCCTGCCTCAGCCTCCCGAGTAGCTGGGATTACAGGTGCCCACCACCACACCCGGCTAATTTTTGTATTTTCAGTAGAGCTGGGG*
*TTTCACCATGTTGGCCAGCCTGTTCTCGGCACACACTCACCCACA*CATAAAAGGTGGTAACTGTGT +211 (Δ16 +227)

**Target 4 (*EMX1*):**

**OT4-1**

```
GATTGCCTTTACTCCATGCCTTTCTTCTTCTGCTCTAACTCTGACAATCTGTCTTGCCATGCCATAA Wild-type x74
GATTGCCTTTACTC---------CTTCTTCTGCTCTAACTCTGACAATCTGTCTTGCCATGCCATAA Δ9
GATTGCCTTTACTCCATGCCT------TTCTGCTCTAACTCTGACAATCTGTCTTGCCATGCCATAA Δ6 x2
GATTGCCTTTACTCCATGC------TCTTCTGCTCTAACTCTGACAATCTGTCTTGCCATGCCATAA Δ6
GATTGCCTTTACTCCATGCCT---TTCTTCTGCTCTAACTCTGACAATCTGTCTTGCCATGCCATAA Δ3 x3
```

**Supplementary Fig. 4 Sequences of off-target indel mutations induced by RGENs in human HEK293 cells**

Wild-type genomic off-target sites recognized by RGENs (including the PAM sequence) are highlighted in yellow and numbered as in **Table 1** and **Supplementary Table 2**. Note that the complementary strand is shown for some sites. Deleted bases are shown as red dashes on a grey background. Inserted bases are italicized and highlighted in blue. *Yielded a large number of single bp indels

**Supplementary Table 1 Sequences of oligonucleotides used to generate expression plasmids encoding sgRNAs/variant sgRNAs targeted to sites in the *EGFP* reporter gene and sgRNAs targeted to six endogenous human gene targets**

***See accompanying file***

**Supplementary Table 2 Sequences and characteristics of genomic on- and off-target sites for six RGENs targeted to endogenous human genes and primers and PCR conditions used to amplify these sites**

***See accompanying file***

**Supplementary Table 3 Numbers of off-target sites in the human genome for six RGENs targeted to endogenous human genes and three RGENs targeted to the *EGFP* reporter gene**

| Target Site | Number of mismatches to on-target site | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** | **1** | **2** | **3** | **4** | **5** | **6** |
| **Target 1 (*VEGFA* Site 1)** | 1 | 1 | 4 | 32 | 280 | 2175 | 13873 |
| **Target 2 (*VEGFA* Site 2)** | 1 | 0 | 2 | 35 | 443 | 3889 | 17398 |
| **Target 3 (*VEGFA* Site 3)** | 1 | 1 | 17 | 377 | 6028 | 13398 | 35517 |
| **Target 4 (*EMX*)** | 1 | 0 | 1 | 18 | 276 | 2309 | 15731 |
| **Target 5 (*RNF2*)** | 1 | 0 | 0 | 6 | 116 | 976 | 7443 |
| **Target 6 (*FANCF*)** | 1 | 0 | 1 | 18 | 271 | 1467 | 9551 |
| **EGFP Target Site #1** | 0 | 0 | 3 | 10 | 156 | 1365 | 9755 |
| **EGFP Target Site #2** | 0 | 0 | 0 | 11 | 96 | 974 | 7353 |
| **EGFP Target Site #3** | 0 | 0 | 1 | 14 | 165 | 1439 | 10361 |

Off-target sites for each of the six RGENs targeted to the *VEGFA*, *RNF2*, *FANCF*, and *EMX1* genes and the three RGENs targeted to EGFP Target Sites #1, #2 and #3 were identified in human genome sequence build GRCh37. Mismatches were only allowed for the 20 nt region to which the sgRNA anneals and not to the PAM sequence.

**Supplementary Table 4 Indel mutation frequencies at on- and off-target genomic sites induced by different amounts of Cas9- and sgRNA-expressing plasmids for the RGEN targeted to *VEGFA* Target Site 3**

| Site | Sequence | 250ng sgRNA/750 ng Cas9 Mean indel frequency (%) ± SEM | 12.5ng sgRNA/250 ng Cas9 Mean indel frequency (%) ± SEM |
|---|---|---|---|
| T3 (On-target) | GGTGAGTGAGTGTGTGCGTGTGG | 49.4 ± 3.8 | 33.0 ± 3.7 |
| OT3-1 | GGTGAGTGAGTGTGTG**T**GTGAGG | 7.4 ± 3.4 | *N.D.* |
| OT3-2 | **A**GTGAGTGAGTGTGTG**T**GTGGGG | 24.3 ± 9.2 | 9.8 ± 4.2 |
| OT3-4 | G**C**TGAGTGAGTGT**A**TGCGTGTGG | 20.9 ± 11.8 | 4.2 ± 1.2 |
| OT3-9 | GGTGAGTGAGTG**C**GTGCG**G**GTGG | 3.2 ± 0.3 | *N.D.* |
| OT3-17 | G**T**TGAGTGA**A**TGTGTGCGTGAGG | 2.9 ± 0.2 | *N.D.* |
| OT3-18 | **T**GTG**G**GTGAGTGTGTGCGTGAGG | 13.4 ± 4.2 | 4.9 ± 0.0 |
| OT3-20 | **A**G**A**GAGTGAGTGTGTGC**A**TGAGG | 16.7 ± 3.5 | 7.9 ± 2.4 |

Amounts of sgRNA- and Cas9-expressing plasmids transfected into U2OS.EGFP cells for these assays are shown at the top of each column. (Note that data for 250 ng sgRNA/750 ng Cas9 are the same as those presented in **Table 1**.) Mean indel frequencies were determined using the T7EI assay from replicate samples as described in Methods. OT = Off-target sites, numbered as in **Table 1** and **Supplementary Table 2**. *N.D.* = none detected

## Supplementary References:

1. Maeder, M.L. et al. Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol Cell* **31**, 294-301 (2008).

2. Reyon, D. et al. FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotech* **30**, 460-465 (2012).