**Supplementary file 1**

**Allele-specific gene editing approach for vision loss restoration in**

***RHO*-associated Retinitis Pigmentosa**

Xiaozhen Liu, Jing Qiao, Ruixuan Jia, Fan Zhang, Xiang Meng, Liping Yang

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| **Supplementary file 1a.** **Results of TA cloning and Sanger Sequencing in 293T cells.** |
| **RHO17-Sg1** |
| **Nucleotide changes** | **Amino acid changes** | **Number of clones** |
| c.41\_50del | p.S14Wfs\*31 | 1 |
| c.43\_44del | p.N15Cfs\*18 | 1 |
| c.44\_45del | p.N15Sfs\*18 | 1 |
| c.44\_45insA | p.N15Kfs\*19 | 1 |
| c.44\_45ins341bp | p.A16Cfs\*9 | 1 |
| c.44\_45ins156bp | p.A16Qfs\*41 | 1 |
| c.44\_45ins45bp | insertion of more than 2 AA | 1 |
| Total  | 7 |
| T>C | p.T17 | 5 |
| **RHO17-Sg2** |
| **Nucleotide changes** | **Amino acid changes** | **Number of clones** |
| c.24\_50del | deletion of more than 2 AA | 1 |
| c.43\_46del | p.N15Rfs\*32 | 1 |
| c.45\_46del | p.A16Dfs\*17 | 2 |
| c.46del | p.A16Rfs\*32 | 2 |
| c.47\_51del | p.A16Gfs\*16 | 1 |
| c.47\_52del | deletion of 1 or 2 AA | 1 |
| c.47\_57del | p.A16Gfs\*14 | 1 |
| c.49\_62del | p.T17Qfs\*12 | 1 |
| c.46\_47 ins75bp | p.A16Efs\*16 | 1 |
| c.37\_47delinsA | p.F13Rfs\*32 | 1 |
| c.43\_46del4bpinsCA | p.N15Hfs\*18 | 1 |
| c.47\_51delins19bp | deletion of 1 or 2 AA | 1 |
| c.50delT ins 44bp | p.T17Nfs\*31 | 1 |
| Total | 15 |

**Supplementary file 1b. A comprehensive summary of mouse experiments.**

|  |
| --- |
| **Step 1** |
| Mouse strain  | Treatment age | Eye | Treatment | Injection volume(totally) | AAV dose(vg/eye) | T7E1 assay | TA and Sanger sequencing | Hi-Tom sequencing  | ERG | Cryosection | IF | HE | RT-PCR | WB |
| *Rho wt/wt* | P2m | Both(*n*=4) | AAV2/8-EFS-EGFP | 1μl  | 1×109 | n/a | n/a | n/a | n/a | 1m p.i.(*n*=2) | 1m p.i.(*n*=2) | n/a | n/a | n/a |
|  |  |  |  |  |  |  |  |  |  | 11m p.i.(*n*=2) | 11m p.i.(*n*=2) |  |  |  |
| *Rho wt/wt* | P2m | Both(*n*=4) | AAV2/8-EFS-EGFP | 1μl  | 3×109 | n/a | n/a | n/a | n/a | 1m p.i.(*n*=2) | 1m p.i.(*n*=2) | n/a | n/a | n/a |
|  |  |  |  |  |  |  |  |  |  | 11m p.i.(*n*=2) | 11m p.i.(*n*=2) |  |  |  |
| **Step 2** |
| Mouse strain  | Treatment age | Eye | Treatment | Injection volume (totally) | AAV dose(vg/eye) | T7E1 assay | TA&Sanger sequencing | Hi-Tom sequencing  | ERG | Cryosection | IF | HE | RT-PCR | WB |
| *Rho wt/hum* | P1.5-1.8m | Both(*n*=4) | AAV2/8-EFS-EGFP and AAV2/8-EFS-SaCas9-U6-17-Sg2 | 1μl  | 5×108 each | 3m *p.i.*(*n*=2) | 3m *p.i.*(*n*=2) | 3m *p.i.*(*n*=2) | n/a | n/a | n/a | n/a | 3m *p.i.*(*n*=2) | n/a |
| *Rho wt/hum* | P1.5-1.8m | Both(*n*=8) | 1μl | 1×109 each | 3m *p.i.*(*n*=3) | 3m *p.i.*(*n*=3) | 3m *p.i.*(*n*=3) | n/a | n/a | n/a | n/a | 3m *p.i.*(*n*=2) | 3m *p.i.*(*n*=3) |
| *Rho wt/hum* | P1.5-1.8m | Both(*n*=10) | 2μl | 3×109 each | 3m *p.i.*(*n*=3) | 3m *p.i.*(*n*=3) | 3m *p.i.*(*n*=3) | n/a | n/a | n/a | n/a | 3m *p.i.*(*n*=3) | 3m *p.i.*(*n*=4) |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=3) | 1μl | 5×108 each | 3m *p.i.*(*n*=3) | 3m *p.i.*(*n*=1) | 3m *p.i.*(*n*=1) | n/a | n/a | n/a | n/a | n/a | n/a |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=4) | 1μl | 1×109 each | 3m *p.i.*(*n*=4) | 3m *p.i.*(*n*=4) | 3m *p.i.*(*n*=4) | n/a | n/a | n/a | n/a | n/a | n/a |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=5) | 2μl | 3×109 each | 3m *p.i.*(*n*=5) | 3m *p.i.*(*n*=5) | 3m *p.i.*(*n*=5) | n/a | n/a | n/a | n/a | n/a | n/a |
| Mut-*Rho hum/hum* | P1.5-1.8m | Both(*n*=2) | 1μl | 1×109 each | 3m *p.i.*(*n*=2) | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Mut-*Rho hum/hum* | P1.5-1.8m | Both(*n*=2) | 2μl | 3×109 each | 3m *p.i.*(*n*=2) | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| **Step 3** |
| Mouse strain  | Treatment age | Eye | Treatment | Injection volume (totally) | AAV dose(vg/eye) | T7E1 assay | TA&Sanger sequencing | Hi-Tom sequencing  | ERG | Cryosection | IF | HE | RT-PCR | WB |
| *Rho hum/m-hum* | P1.5-1.8m | Both(*n*=4) | AAV2/8-EFS-EGFP and AAV2/8-EFS-SaCas9-U6-17-Sg2 | 1μl | 1×109 each | 3m *p.i.*(*n*=4) | 3m *p.i.*(*n*=4) | 3m *p.i.*(*n*=4) | n/a | n/a | n/a | n/a | 3m *p.i.*(*n*=3) | n/a |
| *Rho hum/m-hum* | P1.5-1.8m | Both(*n*=4) | 2μl | 3×109 each | 3m *p.i.*(*n*=4) | 3m *p.i.*(*n*=4) | 3m *p.i.*(*n*=4) | n/a | n/a | n/a | n/a | 3m *p.i.*(*n*=4) | n/a |
| **Step 4** |
| Mouse strain  | Treatment age | Eye | Treatment | Injection volume (totally) | AAV dose(vg/eye) | T7E1 assay | TA&Sanger sequencing | Hi-Tom sequencing  | ERG | Cryosection | IF | HE | RT-PCR | WB |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=5) | AAV2/8-EFS-EGFP and AAV2/8-EFS-SaCas9-U6-CTRL | 1μl | 1×109 each | n/a | n/a | n/a | 3m *p.i*.(*n*=5) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | n/a | n/a |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=5) | 2μl | 3×109 each | n/a | n/a | n/a | 3m *p.i*.(*n*=5) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | n/a | n/a |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=7) | AAV2/8-EFS-EGFP and AAV2/8-EFS-SaCas9-U6-17-Sg2 | 1μl | 1×109 each | n/a | n/a | n/a | 3m *p.i*.(*n*=7) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | n/a | n/a |
| Mut-*Rho wt/hum* | P1.5-1.8m | Both(*n*=9) | 2μl | 3×109 each | n/a | n/a | n/a | 3m *p.i*.(*n*=9) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | 4m *p.i.*(*n*=3) | n/a | n/a |
| 9m *p.i.*(n=3) | 9m *p.i.*(n=3) | 9m *p.i.*(n=3) | n/a | n/a |

*n*= the number of the treated mouse.

**Supplementary file 1c. Results of TA cloning & Sanger sequencing in AAV-based SaCas9/17-Sg2-treated Mut-*Rhowt/hum* Retinas.**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Nucleotide changes | Amino acid changes | #M1-GFP+ | #M2-GFP+ | #M3-GFP+ | #M4-GFP+ | #M5-GFP+ | #M6-GFP+ | #M7-all | #M8-all | #M9-all | #M10-all | Total |
| c.29\_360+104del436bp | - |  |  |  |  |  |  |  |  |  | 1 | 1 |
| c.38\_46del9bp | deletion of more than 2 AA |  |  |  |  |  |  | 1 |  |  |  | 1 |
| c.40\_47del8bp | p.S14Dfs\*17 |  |  |  |  |  | 1 |  |  |  |  | 1 |
| c.41C>G | p. S14C  |  |  |  |  |  |  |  |  | 1 |  | 1 |
| c.42\_47del6bp | deletion of 1 or 2 AA |  |  |  |  |  |  |  |  | 1 |  | 1 |
| c.43\_46del4bp | p.N15Rfs\*32 |  |  |  |  |  | 2 |  |  |  |  | 2 |
| c.43\_47del5bp | p.N15Dfs\*17 |  |  |  |  | 1 |  |  |  |  |  | 1 |
| c.44\_46del3bp | deletion of 1 or 2 AA |  |  |  | 1 |  | 1 |  |  |  | 1 | 3 |
| c.44\_49del6bp | deletion of 1 or 2 AA |  | 1 |  |  |  |  |  |  |  |  | 1 |
| c.45T>G | p. N15K  |  |  |  | 1 |  |  |  | 1 |  |  | 2 |
| c.45\_45del1bp | p.N15Kfs\*33 |  | 1 |  |  |  |  |  |  |  |  | 1 |
| c.45\_46del2bp | p.A16Dfs\*17 |  |  |  | 1 | 1 |  |  | 1 | 2 | 1 | 6 |
| c.46\_46del1bp | p.A16Rfs\*32 | 1 | 1 | 1 | 1 | 3 | 5 | 2 | 3 | 1 |  | 18 |
| c.47C>G |  p.A16G |  |  | 1 | 2 |  |  |  | 1 | 1 |  | 5 |
| c.47C>G;c.49\_51del3bp |  p.A16G; deletion of 1 or 2 AA |  |  |  |  |  |  |  |  | 1 |  | 1 |
| c.47\_47del1bp | p.A16Gfs\*32 |  | 1 | 1 |  | 1 | 1 | 2 | 1 |  | 2 | 9 |
| c.47\_48 del2bp | p.A16Dfs\*17 |  |  |  |  | 1 | 1 | 1 | 1 | 1 | 1 | 6 |
| c.47\_49del3bp | deletion of 1 or 2 AA |  |  |  |  | 1 |  |  |  |  |  | 1 |
| c.47\_50del4bp | p.A16Gfs\*31 |  | 1 | 1 |  |  |  |  |  |  |  | 2 |
| c.47\_51del5bp | p.A16Gfs\*16 |  |  |  |  | 1 |  |  |  |  |  | 1 |
| c.47\_54del8bp | p.A16Gfs\*15 |  |  |  | 1 |  |  |  |  |  |  | 1 |
| c.47\_55del9bp | deletion of more than 2 AA |  |  |  |  |  |  |  | 1 |  |  | 1 |
| c.47\_57del11bp | p.A16Gfs\*14 |  |  |  |  |  |  |  |  | 1 |  | 1 |
| c.47\_58del12bp | deletion of more than 2 AA |  |  | 1 |  |  |  |  |  |  |  | 1 |
| c.52G>A | p.G18S  |  |  | 1 |  |  |  |  |  |  |  |  |
| c.1-100\_c.66 del166bp | - |  |  |   | 1 |  |  |  |  |  |  | 1 |
| c.1-99\_c.92 del191bp | - |  | 1 |  |  |  |  |  |  |  |  | 1 |
| C.46\_47inA | p.A16Dfs\*18 |  |  |  |  |  |  |  |  |  | 1 | 1 |
| C.46\_47inT | p.A16Vfs\*18 |  |  |  |  |  |  |  |  |  | 1 | 1 |
| C.46\_47inTA | P.A16Vfs\*33 |  |  |  |  |  |  |  | 1 |  |  | 1 |
| c.47\_50delinsGGTCGCCCGACGCCCGG | p.A16Gfs\*22 |  |  |  |  |  |  |  | 1 |  |  | 1 |
| c.48\_49delins43bp | p.M17Pfs\*45 |  |  |  | 1 |  |  |  |  |  |  | 1 |
| c.47\_49delins82bp | p.A16Vfs\*13 |  | 1 |  |  |  |  |  |  |  |  | 1 |
| c.52\_53insATGG | p.G18Dfs\*17 |  |  | 1 |  |  |  |  |  |  |  | 1 |
| c.28\_79delins50bp | p.Y10Qfs\*13 |  |  |  |  | 1 |  |  |  |  |  | 1 |
| c.46\_47insG | p.A16Gfs\*18 |  |  |  |  |  |  | 1 |  |  |  | 1 |
| c.46\_47ins49bp | p.A16Gfs\*34 |  |  |  |  |  |  | 1 |  |  |  | 1 |
| c.46\_47ins55bp | p.A16Vfs\*36 |  |  |  |  |  | 1 |  |  |  |  | 1 |
| c.47\_48ins71bp | p.M17Efs\*55 |  |  |  |  |  |  | 1 |  |  |  | 1 |
| c.47\_57delins70bp | p.A16Gfs\*6 |  |  |  |  |  |  | 1 |  |  |  | 1 |
| c.48\_49insCG | p.M17Rfs\*32 |  |  |  |  | 1 |  |  |  |  |  | 1 |
| c.50\_51insGGCGAT | deletion and insertion of 1 or 2 AA | 1 |  |  |  |  |  |  |  |  |  | 1 |
| c.50\_51insGCGAT | p.G18Rfs\*32 |  |  |  |  |  | 1 |  |  |  |  | 1 |
| **Number of indels** | 2 | 7 | 7 | 9 | 11 | 13 | 10 | 11 | 9 | 8 |  |
| **Number of clones** | 60 | 60 | 72 | 59 | 57 | 63 | 64 | 60 | 60 | 60 |  |
| **Percentage of indels** | 0.0333 | 0.1167 | 0.0972 | 0.1525 | 0.1930 | 0.2063 | 0.15625 | 0.1833 | 0.15 | 0.1333 |  |

**Supplementary file 1d. Off-target sites of 17-Sg2 obtained from Benchling (<https://www.benchling.com/>).**

Red indicates mismatches of off-target sequence compared to 17-Sg2 sequence.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Sequence | PAM | Score | Gene | Chromosome | Strand | Position | Mismatches | On-target | Forward primer | Reverse primer |
| n/a | **TGCGTACCACACCCaTCGCAT** | **TGGAG** | **100** | **RHO(ENSG00000163914)** | **chr3** | **-1** | **129247623** | **0** | **TRUE** | n/a | n/a |
| OT1 | GACCTACCACACCCGCCACAT | CTGAG | 0.134943182 |  | chr3 | -1 | 192513775 | 5 | FALSE | AGGCCTCTGGACCTTTTTCG | TCACGCAATCTTGACTAGAGC |
| OT2 | GGCCTAACACACCCTTCGCAG | AAGAA | 0.133327096 |  | chr15 | 1 | 95244889 | 5 | FALSE | CACCAGAGGGTGTAACTTAGCG | GTAGCTTCTGTCGGCGTGAC |
| OT3 | CCCGTACCACACCCATCACTT | TAGGA | 0.130208333 |  | chr3 | -1 | 51983185 | 5 | FALSE | AAGGCTGAATGGTACTCTGTTATGT | GAAGAATGCTTGATTCACCTGGC |
| OT4 | GGCCTACCACACCCTTGGCAA | GGGGA | 0.124388966 |  | chr9 | -1 | 137665529 | 5 | FALSE | CGTTCACCCCAAGACTGCTT | CTTTCCACATGGTGTCACGG |
| OT5 | CCCGTACCACAGCCATCCCAT | CAGGG | 0.121670082 | NAV2 (ENSG00000166833) | chr11 | -1 | 20005730 | 5 | FALSE | CCTCTCTCAAAAGCCTGCCTG | TACTGCTGTCAGAAGCACCAC |
| OT6 | CGCCTACCTCACCAGTGGCAT | CTGGA | 0.113023477 |  | chr10 | -1 | 25408992 | 5 | FALSE | AAAGCACATAGCGGCGGAA | GAAACATGATGGGGGTGGGTA |
| OT7 | GCCATTCCACACCCATCGCAT | ATGGG | 0.109682882 |  | chr6 | -1 | 31333868 | 5 | FALSE | TGCATGCCAGCAGCTTCTTA | GCAACATGTTTTTACTCCCCACT |
| OT8 | AGCATACCACACCCACTGCAT | GAGGG | 0.108612805 |  | chr18 | 1 | 35109411 | 5 | FALSE | TGCTTGGATGGGTAACCTGG | AGTAGCCCCTCAGGACCAAA |
| OT9 | TGCGTACCACAGCCGCAGGAT | GAGAG | 0.092388485 |  | chr7 | 1 | 156183789 | 4 | FALSE | TGATGGAACTCAGCCCACG | GCTCATTGTCTTGCCATTTCGT |
| OT10 | TGCGTACATCATACGTCGCAT | CTGAA | 0.089420181 |  | chr8 | -1 | 37075079 | 4 | FALSE | CTTGAATGCACACAGGGTCA | CTCAGGAATCACAGGGAGATGG |

**Supplementary file 1e. Off-target sites of 17-Sg2 obtained from Cas-OFFinder (http://www.rgenome.net/cas-offinder) and NGS results.**

Red indicates mismatches of off-target sequence compared to 17-Sg2 sequence.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Off-target ID | Sequence | PAM | Chromosome | Position | Strand | Mismatches | On-target | Forward primer | Reverse primer | Product length |
| **N/A** | **TGCGTACCACACCCaTCGCAT** | **TGGAG** | **chr3** | **129247623** | **-1** | **0** | **TRUE** | **ATGAATGGCACAGAAGGCCC** | **GAGCAGGATGTAGTTGAGAGGC** | 231 |
| Off1 | TGAGTACCAGCCCCTGTCACAT | CTGAAT | chr1 | 203955786 | 1 | 4 | FALSE | AAAGGCATTCCTACCCCAAGA | CTGGGATGGGTGTGCAAATTC | 247 |
| Off2 | TGGGTACCACACCAGCTCTGAT | GGGGGT | chr1 | 198213041 | -1 | 4 | FALSE | GACAAAGCCAACACACTAAGGC | CCCTAAAGGCCAGACTACTGT | 223 |
| Off3 | GGCCTGTCCCACACCACTCGCAT | CAGGAT | chr1 | 111368178 | -1 | 4 | FALSE | ACAAAACAAAGAGGAGTGGAGAGT | TGCTTGCTGGATCCCAGTTT | 207 |
| Off4 | GGCCTCAGCCACACCCTTCGCAT | TAGAGT | chr2 | 7694251 | 1 | 4 | FALSE | TACCCATCCACGAGGATATACAGT | TGCAAGTTTCCTTTGGGGAAGT | 239 |
| Off5 | TGCCTGCCACACCCTGGAGGCAT | TCGGGT | chr2 | 121961972 | -1 | 4 | FALSE | AGGCATGCAGCAGTCTGG | AAGGCTGAACTTGGTCCTCG | 234 |
| Off6 | GGCGTGCCCCACCCTTCTGGCAT | ATGGAT | chr2 | 51489418 | 1 | 4 | FALSE | GAGTTCAAAGCACAGGAACTTC | TGTGTGACCAGACAGTTGGAAC | 240 |
| Off7 | TGAGCAACCACACACGTCGGAT | GTGAGT | chr6 | 11382305 | 1 | 4 | FALSE | GCTCATGTTTCCCTCTCCATGC | GAACAAGCTTTCTTACCTGTCCC | 249 |
| Off8 | TGCGTACCACAGCCGCAGGAT | GAGAGT | chr7 | 156391076 | 1 | 4 | FALSE | GGCAGCCAAGAAGCACCG | AGAATCCATCTCGTGGCTCG | 247 |
| Off9 | TGCCAGGGCCACTCCTGTCGCAT | CTGAGT | chr9 | 108166315 | 1 | 4 | FALSE | TGGTGTGGGGCCGTATCT | GTTAACTGTGTTTTACATCCTCTGG | 203 |
| Off10 | TGCTGTAACACACCAGCCTCAT | GTGAAT | chr10 | 75341442 | 1 | 4 | FALSE | GCACCCATAGGAAAAGTCTGTG | AATTCTCAGCCAACCCACAAGT | 222 |
| Off11 | TGCCTCTCCCACACCTGTGGCAT | CCGAGT | chr10 | 128566018 | 1 | 4 | FALSE | TGGCGGGAATATTTATCTACACCAT | CTTCCGTGGCAGAAAGCTACA | 216 |
| Off12 | TGCCTGCCACACCAGGGTGGCAT | TGGGGT | chr11 | 446447 | 1 | 4 | FALSE | GACCCCCAGCCTGAAGC | CTCTGGCTTGTTGGAGGGAG | 243 |
| Off13 | TGAGTACCACACCCACCACACT | GTGAAT | chr14 | 61376057 | -1 | 4 | FALSE | CTCCAGCCTGGGCAACAAGA | GCTACCCTGAGCAGACCTTC | 232 |
| Off14 | TGCTTGGCCACACCCGTCCCAT | CTGGGT | chr15 | 100384583 | -1 | 4 | FALSE | GGCAGAGACTCCACCAATCC | GAGCTTTGCTTCCTGGGACT | 246 |
| Off15 | TGCGTCACCACACCTGGCTAAT | TTGAAT | chr16 | 861503 | 1 | 3 | FALSE | GCTCACTGTAACCTCTGCCTC | TTATGAAGCGAGGCCGGG | 239 |
| Off16 | TGCGTCACCACACCTGGCCCAT | GTGAGT | chr16 | 10438533 | 1 | 4 | FALSE | CCCATGCTTCCCTCAAACTCC | GCCGATATTGCTAAGCTATAGT | 246 |
| Off17 | TGCGAACCAGACCCGTCACACTC | CGGGGT | chr17 | 60090564 | -1 | 4 | FALSE | GAGGTTGTGAAAACAAGCCTGA | CCCTGTGCCAGGGGTATGTA | 241 |
| Off18 | TGCGTGACACACCCATACCCAT | TAGGAT | chr19 | 51877543 | 1 | 4 | FALSE | ACCACAGCATTTGCACTTTGA | GTCAGTACCATTTTACATTCCCACC | 232 |
| Off19 | TGGGCACTCCACGCCCATCGCAT | GAGAGT | chr20 | 60038317 | 1 | 4 | FALSE | ATTGCAGACTCTTTTCAGATGGGA | GCAGAATCTGGCCTTCCGTC | 204 |
| Off20 | TAGGTACCACACCCAAGTCACCT | CTGGGT | chr20 | 4400103 | -1 | 4 | FALSE | CCATTTTTATACAGTTGGATTAGCAA | GTCCATTTGTCTGATCATTTTCTTAG | 231 |

**Supplementary file 1f. Off-target sites of 17-Sg2 obtained from Cas-OFFinder (http://www.rgenome.net/cas-offinder) and NGS results.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample ID | Off-target ID | Deletion\_num | Insertion\_Deletion\_num | Insertion\_num | NHEJ\_num | Unmodified |
| 293T-control | RHO-WT site |  441(0.0936%) |  2154(0.4574%) |  22832(4.8482%) |  25427(5.3992%) |  445513(94.6008%) |
| 293T-17-Sg2 | RHO-WT site |  750(0.0964%) |  3688(0.4743%) |  39864(5.1265%) |  44302(5.6972%) |  733310(94.3028%) |
| 293T-control | off1 |  2414(0.5363%) |  596(0.1324%) |  2370(0.5265%) |  5380(1.1952%) |  444761(98.8048%) |
| 293T-17-Sg2 | off1 |  2533(0.5124%) |  565(0.1143%) |  1735(0.3509%) |  4833(0.9776%) |  489553(99.0224%) |
| 293T-control | off2 |  812(0.2702%) |  664(0.2210%) |  363(0.1208%) |  1839(0.6120%) |  298657(99.3880%) |
| 293T-17-Sg2 | off2 |  690(0.2295%) |  618(0.2055%) |  295(0.0981%) |  1603(0.5331%) |  299103(99.4669%) |
| 293T-control | off3 |  162(0.0881%) |  156(0.0849%) |  8(0.0044%) |  326(0.1774%) |  183470(99.8226%) |
| 293T-17-Sg2 | off3 |  159(0.0903%) |  123(0.0699%) |  16(0.0091%) |  298(0.1693%) |  175698(99.8307%) |
| 293T-control | off4 |  378(0.1363%) |  1111(0.4007%) |  69(0.0249%) |  1558(0.5619%) |  275719(99.4381%) |
| 293T-17-Sg2 | off5 |  444(0.1450%) |  959(0.3133%) |  70(0.0229%) |  1473(0.4812%) |  304659(99.5188%) |
| 293T-control | off5 |  601(0.1010%) |  510(0.0857%) |  52(0.0087%) |  1163(0.1954%) |  593993(99.8046%) |
| 293T-17-Sg2 | off5 |  535(0.0896%) |  420(0.0703%) |  40(0.0067%) |  995(0.1666%) |  596146(99.8334%) |
| 293T-control | off6 |  2507(0.6303%) |  655(0.1647%) |  334(0.0840%) |  3496(0.8789%) |  394268(99.1211%) |
| 293T-17-Sg2 | off6 |  2385(0.6036%) |  616(0.1559%) |  255(0.0645%) |  3256(0.8240%) |  391898(99.1760%) |
| 293T-control | off7 |  1302(0.2401%) |  875(0.1614%) |  148(0.0273%) |  2325(0.4288%) |  539838(99.5712%) |
| 293T-17-Sg2 | off7 |  750(0.2310%) |  491(0.1512%) |  90(0.0277%) |  1331(0.4099%) |  323381(99.5901%) |
| 293T-control | off8 |  275(0.0511%) |  641(0.1191%) |  117(0.0217%) |  1033(0.1919%) |  537225(99.8081%) |
| 293T-17-Sg2 | off8 |  337(0.0502%) |  757(0.1127%) |  202(0.0301%) |  1296(0.1929%) |  670585(99.8071%) |
| 293T-control | off9 |  819(0.2224%) |  312(0.0847%) |  246(0.0668%) |  1377(0.3739%) |  366901(99.6261%) |
| 293T-17-Sg2 | off9 |  271(0.2241%) |  85(0.0703%) |  74(0.0612%) |  430(0.3556%) |  120494(99.6444%) |
| 293T-control | off10 |  20889(5.1968%) |  666(0.1657%) |  11941(2.9707%) |  33496(8.3332%) |  368463(91.6668%) |
| 293T-17-Sg2 | off10 |  23879(4.6622%) |  764(0.1492%) |  16917(3.3029%) |  41560(8.1143%) |  470620(91.8857%) |
| 293T-control | off11 |  719(0.2307%) |  258(0.0828%) |  46(0.0148%) |  1023(0.3283%) |  310578(99.6717%) |
| 293T-17-Sg2 | off11 |  670(0.2664%) |  174(0.0692%) |  36(0.0143%) |  880(0.3499%) |  250605(99.6501%) |
| 293T-control | off12 |  5363(0.9884%) |  657(0.1211%) |  249(0.0459%) |  6269(1.1553%) |  536352(98.8447%) |
| 293T-17-Sg2 | off12 |  5642(0.9764%) |  625(0.1082%) |  179(0.0310%) |  6446(1.1156%) |  571380(98.8844%) |
| 293T-control | off13 |  273376(74.9158%) |  43565(11.9385%) |  14268(3.9100%) |  331209(90.7643%) |  33702(9.2357%) |
| 293T-17-Sg2 | off13 |  300893(75.5530%) |  46414(11.6544%) |  14489(3.6381%) |  361796(90.8455%) |  36458(9.1545%) |
| 293T-control | off14 |  1522(0.2977%) |  591(0.1156%) |  213(0.0417%) |  2326(0.4549%) |  508970(99.5451%) |
| 293T-17-Sg2 | off14 |  1410(0.2728%) |  522(0.1010%) |  174(0.0337%) |  2106(0.4075%) |  514688(99.5925%) |
| 293T-control | off15 |  108(6.9813%) |  14(0.9050%) |  69(4.4602%) |  191(12.3465%) |  1356(87.6535%) |
| 293T-17-Sg2 | off15 |  111(6.7766%) |  13(0.7937%) |  67(4.0904%) |  191(11.6606%) |  1447(88.3394%) |
| 293T-control | off16 |  1870(0.4322%) |  553(0.1278%) |  618(0.1428%) |  3041(0.7028%) |  429666(99.2972%) |
| 293T-17-Sg2 | off16 |  2186(0.3894%) |  678(0.1208%) |  700(0.1247%) |  3564(0.6348%) |  557876(99.3652%) |
| 293T-control | off17 |  1021(0.1968%) |  386(0.0744%) |  2665(0.5136%) |  4072(0.7848%) |  514770(99.2152%) |
| 293T-17-Sg2 | off17 |  1112(0.2032%) |  356(0.0650%) |  3146(0.5748%) |  4614(0.8431%) |  542672(99.1569%) |
| 293T-control | off18 |  4269(0.8299%) |  1741(0.3385%) |  2526(0.4911%) |  8536(1.6594%) |  505853(98.3406%) |
| 293T-17-Sg2 | off18 |  3570(0.7832%) |  1360(0.2984%) |  2405(0.5276%) |  7335(1.6092%) |  448484(98.3908%) |
| 293T-control | off19 |  1988(0.4913%) |  194(0.0479%) |  123(0.0304%) |  2305(0.5696%) |  402372(99.4304%) |
| 293T-17-Sg2 | off19 |  1164(0.4907%) |  133(0.0561%) |  66(0.0278%) |  1363(0.5746%) |  235843(99.4254%) |
| 293T-control | off20 |  1086(0.2307%) |  719(0.1527%) |  488(0.1037%) |  2293(0.4871%) |  468445(99.5129%) |
| 293T-17-Sg2 | off20 |  1033(0.2452%) |  589(0.1398%) |  504(0.1196%) |  2126(0.5046%) |  419231(99.4954%) |

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| **Supplementary file 1g. The number of off-target sites of 17-Sg2 obtained from Cas-OFFinder (<http://www.rgenome.net/cas-offinder>).** |
| mismatches\_num | candidate off-target sites\_num | off-target sites validated by WGS\_num |
| 1 | 0 | 0 |
| 2 | 0 | 0 |
| 3 | 6 | 0 |
| 4 | 42 | 0 |
| 5 | 351 | 9 |

**Supplementary file 1h. Whole-genome Sequencing results of off-target activity for SaCas9/17Sg-2.**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| #CHROM | POS | ID | REF | ALT | QUAL | Func | Gene | ExonicFunc | AAchange | 293T-17Sg-2. variant\_Info(HomoHete;Depth;Frequency) | 293T.variant2\_Info(HomoHete;AltDepth;Frequency) | MutType | MutLength | PosInfo | DNA | crRNA | Mismathes | BugleInfo | Distance |
| chr2 | 41773507 | rs70959463 | CGTGTGTGT | C,CGTGTGTGTGTGT | 49.92 | intergenic | SLC8A1;LINC01913 | . | . | Hete;9;0.474 | Hete;8,8;0.364,0.364 | InDel | -8,4 | 41773482;- | caCGTACACACACatATCGCAgATGTGA | TGCGTAC-CACACCCATCGCATNNGYGA | 5 | DNA;1 | 25 |
| chr2 | 176462528 | . | AT | A | 22.13 | intergenic | ATP5MC3;LNPK | . | . | Hete;9;0.600 | Homo;14;0.667 | InDel | -1 | 176462417;- | TGCacACCTACAgCCATCtaATCTGTGA | TGCGTACC-ACACCCATCGCATNNGYGA | 5 | DNA;1 | 111 |
| chr6 | 33110133 | rs9296085 | A | G | 119.21 | upstream | HCG24 | . | . | Homo;24;1.000 | Homo;26;1.000 | SNP | 0 | 33110189;- | TGGgGcACCACACCCAgCcCgTTAGTGA | TG-CGTACCACACCCATCGCATNNGYGA | 5 | DNA;1 | -56 |
| chr6 | 33110141 | rs35402346 | GA | G | 110.18 | upstream | HCG24 | . | . | Homo;21;1.000 | Homo;25;1.000 | InDel | -1 | 33110189;- | TGGgGcACCACACCCAgCcCgTTAGTGA | TG-CGTACCACACCCATCGCATNNGYGA | 5 | DNA;1 | -48 |
| chr8 | 37989344 | . | CTT | CT,C | 83.98 | intronic | ASH2L | . | . | Hete;7,7;0.444,0.444 | Hete;16,7;0.593,0.296 | InDel | -1,-2 | 37989401;- | TGCGTACCACtgCacTCCAGCcTGGGCGA | TGCGTACCACACCCATC--GCATNNGYGA | 5 | DNA;2 | -57 |
| chr9 | 86279838 | . | CAAAA | C,CA,CAA | 86.62 | intronic | UBQLN1 | . | . | Hete;7,7;0.467,0.467 | Hete;3,5;0.333,0.417 | InDel | -4,-3 | 86279787;+ | TGCGCccCCACACtCATaGCcTAGGCGA | TGCG-TACCACACCCATCGCATNNGYGA | 5 | DNA;1 | 51 |
| chr12 | 95426924 | rs200022502 | CAA | CA,C | 78.61 | intronic | NR2C1 | . | . | Homo;18;0.900 | Hete;6,8;0.375,0.500 | InDel | -1,-2 | 95426846;+ | gGgGTAgCACACCATgATtGCATCTGTGA | TGCGTACCACACC--CATCGCATNNGYGA | 5 | DNA;2 | 78 |
| chr13 | 46880587 | . | CTTTT | CTTT,C,CT,CTTTTT | 62.33 | intergenic | LINC00563;RUBCNL | . | . | Hete;6,9;0.333,0.476 | Hete;16,5;0.593,0.222 | InDel | -1,-4 | 46880535;+ | TGaGCcACCACACCCAgCctATATGTGA | TGCG-TACCACACCCATCGCATNNGYGA | 5 | DNA;1 | 52 |
| chr15 | 43834499 | rs35106975 | CG | C | 64.16 | intronic | PPIP5K1 | . | . | Homo;8;0.889 | Hete;7;0.636 | InDel | -1 | 43834526;- | atCGTACCACAgCacTCTAGCATGGGTGA | TGCGTACCACACCCATC--GCATNNGYGA | 5 | DNA;2 | -27 |

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| **Supplementary file 1i. List of primers.** |
| Primers | sequence (5`-3`) |
| **Oligos for molecular cloning and site-directed mutagenesis** |
| FUGW-RHO F | CTGCAGGTCGACTCTAGAGGATCCATGAATGGCACAGAA |
| FUGW-RHO R | CGATAAGCTTGATATCGAATTTTAGGCCGGGGCCAC |
| RHO-pEGFPN1 F | GATCTCGAGCTCAAGCTTCGATGAATGGCACAGAAGGCC |
| RHO-pEGFPN1 R | CTTGCTCACCATGGTGGCGATGGCCGGGGCCACCTGGCT |
| c.46del F | CTACGTGCCCTTCTCCAATCGATGGGTGTGGTA |
| c.46del R | CCACACCCATCGATTGGAGAAGGGCACGTAGAAGTT |
| c.45\_46del F | TAACTTCTACGTGCCCTTCTCCAACGATGGGTGTGGTAC |
| c.45\_46del R | GTACCACACCCATCGTTGGAGAAGGGCACGTAGAA |
| c.44\_46del F | TACGTGCCCTTCTCCACGATGGGTGTGGTACGCAGC |
| c.44\_46del R | TACCACACCCATCGTGGAGAAGGGCACGTAGAAGTTAGG |
| c.43\_46del F | TTCTACGTGCCCTTCTCCCGATGGGTGTGGTACGCA |
| c.43\_46del R | TACCACACCCATCGGGAGAAGGGCACGTAGAAGTTAGG |
| c.46\_55del F | TTCTACGTGCCCTTCTCCAATTGGTACGCAGCCCCTTC |
| c.46\_55del R | AGGGGCTGCGTACCAATTGGAGAAGGGCACGTAGAAGTTAG |
| c.46\_47ins49bp F | CAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACGATGGGTGTGGTACGCAGC |
| c.46\_47ins49bp R | CTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCATTGGAGAAGGGCACGTAGAAG |
| **Oligos for humanized mice genotyping** |
| WT-F | GGCAGCAGTGGGATTAGCGTTAGTA |
| WT-R | TGTGTAGAGGGTGGTGGTGAATCCT |
| Mut-R | ACGATCAGCAGAAACATGTAGGCGG  |
| **Oligos for T7E1 cleavage analysis** |
| RHO17-CDS F | GCAGTGCACCCGTACCTTT |
| RHO17-CDS R | TGCAGAGAGGTGTAGAGGGTG |
| RHO17-F | ATTATGAACACCCCCAATCTCC |
| RHO17-R | GGCTTTGGATAACATTGACAGGA |
| WT-F  | GGCAGCAGTGGGATTAGCGTTAGTA  |
| Rho-mR | TCTGAACCCATGTTTCTTGC |
| **Oligos for Hi-Tom sequencing** |
| RHO17-Hi-Tom F | GGAGTGAGTACGGTGTGCATGGCACAGAAGGCCCTAAC |
| RHO17-Hi-Tom R | GAGTTGGATGCTGGATGGAGCGTGAGGAAGTTGATGGG |
| **Oligos for quantitative PCR** |
| SaCas9 F | TACGAAGTGAATAGCAAGT |
| SaCas9 R | TGTAGAAGGAGGCGATAA |
| SgRNA17 F | TACCACACCCATCGCATGT |
| SgRNA R | TTGACGAGATAAACACGGCATTT |
| Human GAPDH F | CTCTGGTAAAGTGGATATTGT |
| Human GAPDH R | GGTGGAATCATATTGGAACA |
| Mouse Gapdh F | AGTGGCAAAGTGGAGATT |
| Mouse Gapdh R | GTGGAGTCATACTGGAACA |
| Macaca GAPDH F | TTGCCCTCAACGACCACTTT |
| Macaca GAPDH R | GAGGGGAGATTCAGTGTGGC |
| hRHO F | GTGCCCTTCTCCAATGCGA |
| hRHO R | TGAGGAAGTTGATGGGGAAGC |
| mRho F | TGAGGTCAACAACGAATC |
| mRho R | CCATAGCAGAAGAAGATGA |