Supplemental Figures

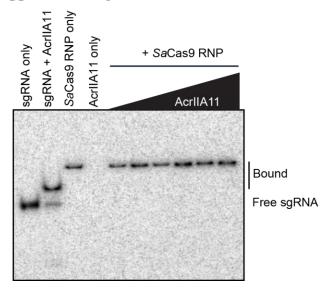


Figure S1. AcrIIA11 binds but does not degrade sgRNA.

Native PAGE gel of ³²P-labeled sgRNA bound by *Sa*Cas9 and incubated with various concentrations of AcrIIA11 for 30 minutes at 37°C. AcrIIA11 weakly binds sgRNA in the absence of *Sa*Cas9.

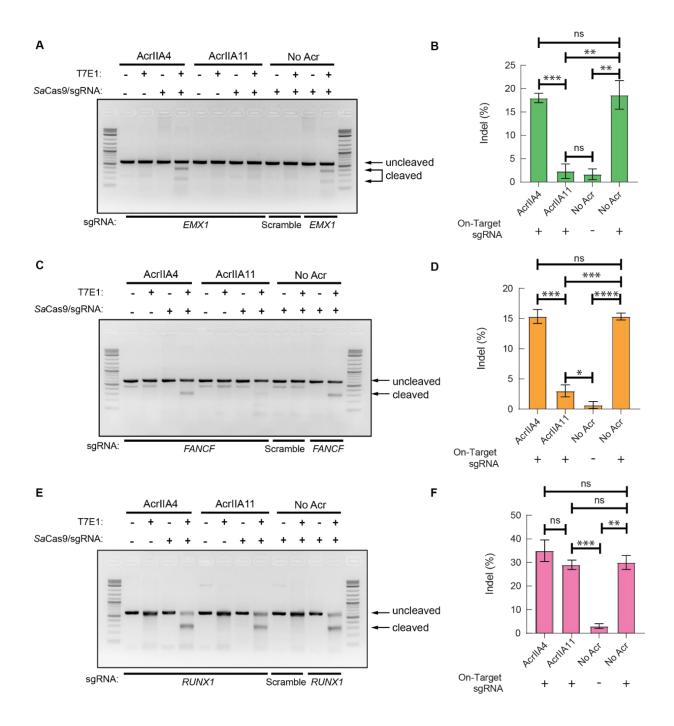


Figure S2. AcrIIA11 inhibits SaCas9 cleavage in human cells.

Representative agarose gels showing SaCas9 genome editing and the quantification of three replicates at the (A, B) EMXI, (C, D) FANCF, and (E, F) RUNXI sites with or without AcrIIA11. Error bars are standard deviation of three replicates. P-values (not significant [ns], p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001; ****p < 0.0001) were determined using a Student's t-test.

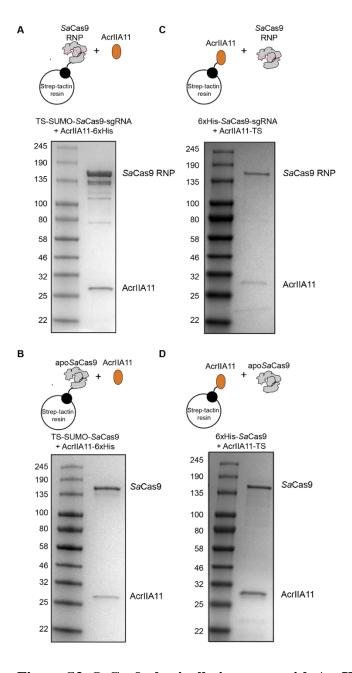


Figure S3. SaCas9 physically interacts with AcrIIA11.

(A, B) Schematic and Coomassie-stained SDS-PAGE gel of AcrIIA11 pulldown. *Sa*Cas9 (A) RNP or apo*Sa*Cas9 (B) were immobilized on Strep-Tactin resin. All AcrIIA11:*Sa*Cas9 complexes were then purified on a size exclusion column (SEC). (C,D) Strep-Tactin-immobilized AcrIIA11 can also pulldown *Sa*Cas9 RNP (C) and apo*Sa*Cas9 (D).

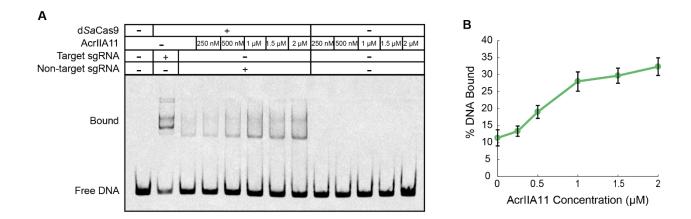


Figure S4. AcrIIA11 induces non-specific binding of SaCas9 on DNA.

(A) EMSA of dSaCas9 non-specific binding at increasing concentrations of AcrIIA11. AcrIIA11 alone does not stably bind the DNA at these concentrations. (B) Quantification of three EMSA replicates. Error bars are S.E.M.

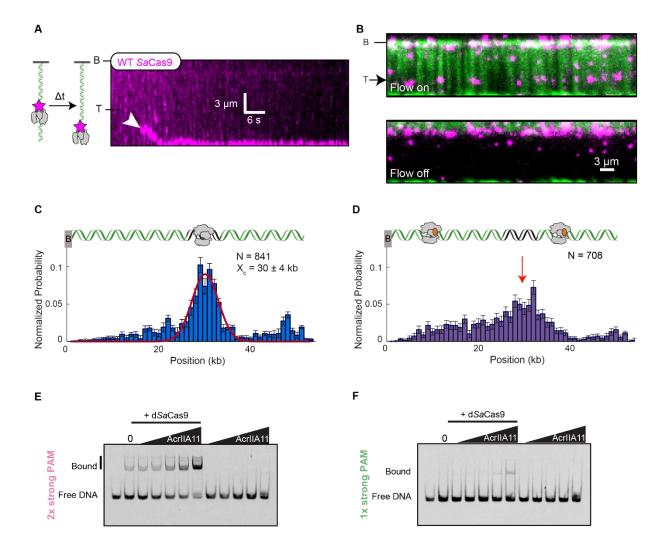


Figure S5. AcrIIA11 sequesters SaCas9 at off-target PAMs sites.

(A) Schematic and kymograph showing WT SaCas9 sliding down to the DNA end. The white arrow indicates SaCas9 binding. (B) Images of dSaCas9 at the target sequence. Top: buffer flow is on. Bottom: buffer flow is off. DNA retracts to the barrier. (C) Binding histogram of dSaCas9 binding to the target site at 29.4 kb. Fit to a single Gaussian (center and SD are indicated). The second peak indicates molecules that slide to the free DNA end, as shown in panel (A). (D) Binding histogram of AcrIIA11:dSaCas9. The red arrow indicates the target site. (E, F) Representative EMSAs of AcrIIA11:dSaCas9 binding to the (E) 2x strong and (F) 1x strong PAM DNA.

Table S1. Oligonucleotides, sgRNA, and gBlocks used in this study.

| Oligonucleotide | Sequence | | |
|-----------------------------|-------------------------------------------------------------------|--|--|
| KD197 | AACTACATTCTGGGGCTGGCCATCGGGATTACAAGCGTG | | |
| KD198 | TGCTGGTCAAGCAGGAAGAGGCATCTAAAAAGGGCAAT | | |
| | GGAC | | |
| KD201 | /5Cy5/tttgggtattgggttttgggttttgggttatttt | | |
| KD202 | aaaataacccaaaacccaatacccaatacccaaa | | |
| KD203 | /5Cy5/tttTCTCattTCTCattTCTCtttTCTCtatttt | | |
| KD204 | aaaataGAGAaaGAGAaatGAGAaatGAGAaa | | |
| KD245 | /5Cy5/ tttgggtattTCTCattTCTCtttTCTCttgggttatttt | | |
| KD246 | aaaataacccaaGAGAaaaGAGAaatGAGAaatacccaaa | | |
| KD247 | /5Cy5/tttTCTCattTCTCattgggttttTCTCttTCTCtatttt | | |
| KD248 | aaaataGAGAaaGAGAaaaacccaatGAGAaatGAGAaaa | | |
| T7 promoter with | gtcgacTAATACGACTCACTATAGGGTAATGAAATAAGATC | | |
| SaCas9 sgRNA | ACTACGTTTTAGTACTCTGGAAACAGAATCTACTAAAACA | | |
| insert for protein | AGGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAG | | |
| expression | ATctcgag | | |
| KD153 | agatetegagTGCGGCCGCACTCGAGCA | | |
| KD154 | attagtcgacAGCTTGTCGACGGAGCTCGAATTCG | | |
| KD155 | tcgacaagctGTCGACTAATACGACTCACTATAG | | |
| KD156 | tgcggccgcaCTCGAGATCTCGCCAACAAG | | |
| KD174 | tccttttcatGGATCCACCAATCTGTTCTCTGTGAGCCTCAATAA | | |
| | TATC | | |
| KD175 | tggtggatccATGAAAAGGAACTACATTCTG | | |
| KD179 | agatetegagGCGGCCGCACTCGAGGCC | | |
| KD180 | gtgcggccgcCTCGAGATCTCGCCAACAAG | | |
| SaCas9 SMART | UAAUGAAAUAAGAUCACUACGUUUUAGUACUCUGGAAA | | |
| target sgRNA | CAGAAUCUACUAAAACAAGGCAAAAUGCCGUGUUUAUC | | |
| | UCGUCAACUUGUUGGCGAGAU | | |
| Sa Cas9 λ target- | GCGAGGAUUGUUAUGUAAUAGUUUUUAGUACUCUGGAAA | | |
| 29.4 kb sgRNA | CAGAAUCUACUAAAACAAGGCAAAAUGCCGUGUUUAUC | | |
| | UCGUCAACUUGUUGGCGAGAU | | |
| IF365 | AAGAACGCCTCGCACACT | | |
| IF460 | /5atto647n/AACCGCCGAATAACAGAGT | | |
| SaCas9 SMART | AAGAACGCCTCGCACACTcttttgacttgatcggcacgtaagaggttccaacttt | | |
| Target gBlock for | caccataatgaaataagatcactacttgggttattttttgagttatcgagattttcagACTCTGT | | |
| non-target DNA | TATTCGGCGGTT | | |
| EMSA | | | |
| FnCas9 sgRNA | GGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGG | | |
| gBlock for IVT | GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGA | | |
| | GCGCGCGTAATACGACTCACTATAGGGataatgaaataagatcactac | | |
| | GTTTCAGTTGCTGAATTATTTGGTAAACAGTACCAAATAA | | |
| | TTAATGCTCTGTAATCATTTAAAAGTATTTTGAACGGACC | | |
| | TCTGTTTGACACGTCTGAATAACTAAAAATTTTTTT | | |

| MmeCas9 sgRNA gGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGG | | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|-------------------------------------------------|
| GCGCGCGTAATACGACTCACTATAGGGaccataatgaaataagatca ctacGTTGTAGCTCCCTTTCATTTCGGAAACGAAATGAGA ACCGTTGCTACAATAAGGCCGTCTGAAAAGATGTGCCGC AACGCTCTGCCCCTTAAAGCTTCTGCTTTAAGGGGCATCG TTTA KD142 | NmeCas9 sgRNA | GGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGG |
| CTACGTTGTAGCTCCCTTTCTCATTTCGGAAACGÁAATGAGA ACCGTTGCTACAATAAGGCCGTCTGAAAAGATGTGCCGC ACCGTTGCTCCCCTTAAAGCTTCTGCTTTAAGGGGCATCG TTTA | gBlock for IVT | GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGA |
| CTACGTTGTAGCTCCCTTTCTCATTTCGGAAACGÁAATGAGA ACCGTTGCTACAATAAGGCCGTCTGAAAAGATGTGCCGC ACCGTTGCTCCCCTTAAAGCTTCTGCTTTAAGGGGCATCG TTTA | | GCGCGCGTAATACGACTCACTATAGGGaccataatgaaataagatca |
| AACGCTCTGCCCCTTAAAGCTTCTGCTTTAAGGGGCATCG TTTA KD142 | | |
| KD142 GGGATGTGCTGCAAGGCG KD143 TAAACGATGCCCTTAAAGCAGA KD144 AAAAAAATTTTTAGTTATTCAGACGTGTCAAAC SaCas9 sgRNA for genome editing in HEK293T cells KJ_T0217_CACN AlD_20nt_Fwd KJ_T0221_CACN AlD_20nt_Evd KJ_T0324_EMX1 2lnt_Fwd_3 KJ_T0325_EMX1 2lnt_Rev_3 KJ_T0325_EMX1 CACCGGCAGGCTCCCCAAAGCCTGCCA ID_20nt_Rev KJ_T0325_EMX1 2lnt_Rev_3 KJ_T0327_FANC F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0328_Scramb ble_sgRNA_Fwd KJ_T0288_scramb ble_sgRNA_Fwd KJ_T0286_CACN ACA GAC ACA CAC ACG GTG CT AID_T7E1_Fwd KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT AID_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Fwd KJ_T0231_EMX1 TO230_FANC F_T7E1_Fwd KJ_T0231_EMX1 TO231_EMX1 TTFI Setl_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0231_EMX1 TTFI Setl_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | ACCGTTGCTACAATAAGGCCGTCTGAAAAGATGTGCCGC |
| KD142 GGGATGTGCTGCAAGGCG KD143 TAAACGATGCCCTTAAAGCAGA KD144 AAAAAAATTTTTAGTTATTCAGACGTGTCAAAC SaCas9 sgRNA for genome editing in HEK293T cells KJ_T0217_CACN AlD_20nt_Fwd KJ_T0221_CACN AlD_20nt_Evd KJ_T0324_EMX1 2lnt_Fwd_3 KJ_T0325_EMX1 2lnt_Rev_3 KJ_T0325_EMX1 CACCGGCAGGCTCCCCAAAGCCTGCCA ID_20nt_Rev KJ_T0325_EMX1 2lnt_Rev_3 KJ_T0327_FANC F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0328_Scramb ble_sgRNA_Fwd KJ_T0288_scramb ble_sgRNA_Fwd KJ_T0286_CACN ACA GAC ACA CAC ACG GTG CT AID_T7E1_Fwd KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT AID_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Fwd KJ_T0231_EMX1 TO230_FANC F_T7E1_Fwd KJ_T0231_EMX1 TO231_EMX1 TTFI Setl_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0231_EMX1 TTFI Setl_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| KD142 | | |
| KD143 TAAACGATGCCCCTTAAAGCAGA KD144 AAAAAAATTTTTAGTTATTCAGACGTGTCAAAC SaCas9 sgRNA for genome editing in HEK293T cells KJ_T0217_CACN AAACCACTACTGAAATACTCCTGCC AID_20nt_Fwd AAACCACTACTGAAATACTCCTGCC AID_20nt_Rev KJ_T0324_EMX1 21nt_Fwd_3 GAACTGGCCAGGCTTTGGGGAGGCCC 21nt_Rev_3 GAACTGGCCAGGCTTTGGGGAGGCCC F_21nt_Rev_3 GAACCCACCGTGCCCGCGCACGGTGG F_21nt_Rev_3 GAACCCACCGTGCCCGGCCACGGTGG F_21nt_Rev_3 GAACCCACCGTGCGCCGGGCCTTGCC F_21nt_Rev_3 GAACCCACCGTGCGCCGGGCCTTGCC F_21nt_Rev_3 GAACCCACCGTGCGCCGGGCCTTGCC F_21nt_Rev_3 GAACCCACCGTGCGCCGGGCCTTGCC F_21nt_Rev_3 GAACCCACCGTGCGCCGGGCCTTGCC F_21nt_Rev_3 GAACCCACCGTGCGCCGGGCCTTGCC L_23nt_Fwd_1 GAACAGTGCTTCATGAGAGGAGTACC L_23nt_Rev_1 GAACAGTGCTTCATGAGAGGTGAGTACC L_23nt_Rev_1 GAACAGTGCTTCATGATATTGGTGGG KJ_T028P_Scram GAACCCACCAATATCAGTAATACC L_23nt_Rev_1 GAACCCCACCAATATCAGTAATACC L_23nt_Rev_1 GAACCCACCACAATATCAGTAATACC L_23nt_Rev_1 GA | KD142 | |
| KD144 AAAAAAATTTTTAGTTATTCAGACGTGTCAAAC SaCas9 sgRNA for genome editing in HEK293T cells KJ_T0217_CACN AlD_20nt_Fwd KJ_T0221_CACN AlD_20nt_Rev KJ_T0324_EMX1 _21nt_Fwd_3 KJ_T0325_EMX1 _21nt_Fwd_3 KJ_T0325_EMX1 _21nt_Rev_3 KJ_T0326_FANC F_21nt_Rev_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0328_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Fwd KJ_T0225_CACN AlD_T7E1_Fwd KJ_T0225_CACN AlD_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Fwd KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA GGC CCT AAC CCT ATG TAG CC TTE1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| SaCas9 sgRNA for genome editing in HEK293T cells | | |
| KJ_T0217_CACN AAACCACTACTGAAATACTCCTGCC AID_20nt_Fwd KJ_T0221_CACN AAACCACTACTGAAATACTCCTGCC AID_20nt_Rev AAACCACTACTGAAATACTCCTGCC AID_20nt_Rev AAACCACTACTGAAATACTCCTGCC AID_20nt_Fwd_3 CACCGGGCCTCCCCAAAGCCTGGCCA 2Int_Fwd_3 CACCGGCCAGGCTTTGGGGAGGCCC 2Int_Rev_3 CACCGGCAAGGCCCGGCGCACGGTGG F_21nt_Fwd_3 CACCGGCAAGGCCCGGCGCACGGTGG F_21nt_Rev_3 CACCGGCAAGGCCCGGCGCACGGTGG F_21nt_Rev_3 CACCGGTACTCACCTCTCATGAAGCACT L_23nt_Fwd_1 CACCGGTACTCACCTCTCATGAAGCACT L_23nt_Fwd_1 CACCGGTACTCACCTCTCATGAAGCACT L_23nt_Rev_1 CACCGGTACTACTATATTGGTGGG KJ_T0329_RUNX CACCGGTATTACTGATATTGGTGGG KJ_T0287_Scram GAACCCCACCAATATCAGTAATACC L_23nt_Rev_1 CACCGGTATTACTGATATTGGTGGG KJ_T0288_scramb GAACCCCACCAATATCAGTAATACC L_23nt_Rev_1 CACCGGTATTACTGATATTGGTGGG KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT AID_T7E1_Fwd ACA GAC ACA CAC ACG GTG CT AID_T7E1_Fwd ACC TCT TTG TGT GGC GAA AG KJ_T0229_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Fwd CCA GGC TCT CTT GGA GTG TC F_T7E1_Fwd CCA GGC TCT CTT GGA GTG TC KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| AID_20n_Fwd KJ_T0221_CACN AID_20nt_Rev KJ_T0324_EMX121nt_Fwd_3 KJ_T0325_EMX121nt_Rev_3 KJ_T0326_FANC F_21nt_Rev_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb ble_sgRNA_Rev T7E1 PCR primers KJ_T0226_CACN AID_T7E1_Rev KJ_T0229_FANC ACA GGC TCT CTT GGA GTG TC F_T7E1_Fwd KJ_T0231_EMX1 CCA GGC TCT CTT GGA GTG TC F_T7E1_Fwd KJ_T0231_EMX1 CCA GGC TCT CTT GGA GTG TC F_T7E1_Fwd KJ_T0231_EMX1 CCA GGC TCT CTT GGA GTG TC F_T7E1_Set1_Fwd KJ_T0231_EMX1 TCTE1_Set1_Fwd KJ_T0231_EMX1 TCTE1_Set1_Fwd KJ_T0231_EMX1 TCTE1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| KJ_T0221_CACN AID_20nt_Rev KJ_T0324_EMX1 _21nt_Fwd_3 KJ_T0325_EMX1 _21nt_Rev_3 KJ_T0326_FANC F_21nt_Rev_3 KJ_T0326_FANC F_21nt_Rev_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX GAACAGTGCTCACCTCTCATGAAGCACT L23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN AID_T7E1_Fwd KJ_T0226_CACN AID_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0231_EMX1 CCAC GGC TCT CTT GGA GTG TC TGGAGT TTC TGC TCC CAT TT AID_T7E1_Fwd KJ_T0230_FANC F_T7E1_Fwd KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC F_T7E1_Set1_Fwd KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC KJ_T0231_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC KJ_T0231_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | CACCOGCAGGAGTATTICAGTAGTG |
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| KJ_T0324_EMX1 _21nt_Fwd_3 KJ_T0325_EMX1 _21nt_Rev_3 KJ_T0326_FANC F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramble_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA GGA GAC CCC CCC ATG TAG CCC CCC GGCACGCCACGGGCCCC GCCCCCCCCCC | _ | AAACCACTACTGAAATACTCCTGCC |
| ZInt_Fwd_3 KJ_T0325_EMX1 21nt_Rev_3 KJ_T0326_FANC F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0226_CACN AlD_T7E1_Fwd KJ_T0229_FANC KJ_T0229_FANC KJ_T0229_FANC KJ_T0229_FANC KJ_T0220_FANC KJ_T0231_ENV KJ_T0231_ENV CACCGGTATTACTGATATTGGTGGG GAACCCCACCACAATATCAGTAATACC TGG AGT TTC TGC TCC CAT TT AlD_T7E1_Fwd KJ_T0226_CACN ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | S |
| KJ_T0325_EMX1 _21nt_Rev_3 KJ_T0326_FANC F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX I_23nt_Rev_1 KJ_T0329_RUNX GAACAGTGCTCACCTCTCATGAAGCACT I_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | CACCGGGCCTCCCCAAAGCCTGGCCA |
| | | |
| KJ_T0326_FANC F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0328_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0229_FANC ACC TCT TTG TGC TCC CAT TT A1D_T7E1_Rev KJ_T0230_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA GGA GAT TGG AGA CAC GGA GA GGA GAT TGG AGA CAC GGA GA GGA GAT TGG AGA CAC GGA GA GGA GAT TGG AGA CAC GGA GA GGA GAT TGG AGA CAC GGA GA | | GAACTGGCCAGGCTTTGGGGAGGCCC |
| F_21nt_Fwd_3 KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Fwd KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | _21nt_Rev_3 | |
| KJ_T0327_FANC F_21nt_Rev_3 KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC F_T7E1_Rev KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | KJ_T0326_FANC | CACCGGCAAGGCCCGGCGCACGGTGG |
| F_2Int_Rev_3 KJ_T0328_RUNX CACCGGTACTCACCTCTCATGAAGCACT 1_23nt_Fwd_1 KJ_T0329_RUNX KJ_T0329_RUNX GAACAGTGCTTCATGAGAGGTGAGTACC 1_23nt_Rev_1 CACCGGTATTACTGATATTGGTGGG KJ_T0287_Scram CACCGGTATTACTGATATTGGTGGG ble_sgRNA_Fwd GAACCCCACCAATATCAGTAATACC kJ_T0288_scramb GAACCCCACCAATATCAGTAATACC le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd ACA GAT TTC TGC TCC CAT TT KJ_T0229_FANC ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd ACC TCT CTT GGA GTG TC KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev GCC CCT AAC CCT ATG TAG CC KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd GGA GAT TGG AGA CAC GGA GA | F_21nt_Fwd_3 | |
| KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX GAACAGTGCTTCATGAGAGGTGAGTACC 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 LT0231_EMX1 LT0232_EMX1 CCA CGGTATTACTGATATTGGTGGG GAACCCCACCAATATCAGTAATACC TGG AGT TTC TGC TCC CAT TT ACC TCT TTG TGT GGC GAA AG GCC CCT AAC CCT ATG TAG CC TTE1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | KJ_T0327_FANC | GAACCCACCGTGCGCCGGGCCTTGCC |
| KJ_T0328_RUNX 1_23nt_Fwd_1 KJ_T0329_RUNX GAACAGTGCTTCATGAGAGGTGAGTACC 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 LT0231_EMX1 LT0232_EMX1 CCA CGGTATTACTGATATTGGTGGG GAACCCCACCAATATCAGTAATACC TGG AGT TTC TGC TCC CAT TT ACC TCT TTG TGT GGC GAA AG GCC CCT AAC CCT ATG TAG CC TTE1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | F 21nt Rev 3 | |
| 1_23nt_Fwd_1 KJ_T0329_RUNX GAACAGTGCTTCATGAGAGGTGAGTACC 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd CACCGGTATTACTGATATTGGTGGG KJ_T0288_scramb GAACCCCACCAATATCAGTAATACC le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd ACA GACT TTC TGC TCC CAT TT KJ_T0226_CACN TGG AGT TTC TGC TCC CAT TT A1D_T7E1_Rev ACC TCT TTG TGT GGC GAA AG KJ_T0229_FANC ACC TCT CTT GGA GTG TC F_T7E1_Fwd CCA GGC TCT CTT GGA GTG TC KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev GCC CCT AAC CCT ATG TAG CC KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd GGA GAT TGG AGA CAC GGA GA | | CACCGGTACTCACCTCTCATGAAGCACT |
| KJ_T0329_RUNX 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| 1_23nt_Rev_1 KJ_T0287_Scram ble_sgRNA_Fwd CACCGGTATTACTGATATTGGTGGG KJ_T0288_scramb GAACCCCACCAATATCAGTAATACC le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd ACA GAT TTC TGC TCC CAT TT KJ_T0226_CACN TGG AGT TTC TGC TCC CAT TT A1D_T7E1_Rev ACC TCT TTG TGT GGC GAA AG KJ_T0229_FANC ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd CCA GGC TCT CTT GGA GTG TC KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev GCC CCT AAC CCT ATG TAG CC KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd GGA GAT TGG AGA CAC GGA GA | | GAACAGTGCTTCATGAGAGGTGAGTACC |
| KJ_T0287_Scram ble_sgRNA_Fwd KJ_T0288_scramb GAACCCCACCAATATCAGTAATACC le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd KJ_T0226_CACN ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| ble_sgRNA_Fwd KJ_T0288_scramb le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 CCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | CACCGGTATTACTGATATTGGTGGG |
| KJ_T0288_scramb GAACCCCACCAATATCAGTAATACC le_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd KJ_T0226_CACN TGG AGT TTC TGC TCC CAT TT A1D_T7E1_Rev KJ_T0229_FANC ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| Ie_sgRNA_Rev T7E1 PCR primers KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd KJ_T0226_CACN TGG AGT TTC TGC TCC CAT TT A1D_T7E1_Rev KJ_T0229_FANC ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | GAACCCCACCAATATCAGTAATACC |
| KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd KJ_T0226_CACN TGG AGT TTC TGC TCC CAT TT A1D_T7E1_Rev KJ_T0229_FANC ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| KJ_T0225_CACN ACA GAC ACA CAC ACG GTG CT A1D_T7E1_Fwd KJ_T0226_CACN TGG AGT TTC TGC TCC CAT TT A1D_T7E1_Rev KJ_T0229_FANC ACC TCT TTG TGT GGC GAA AG F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | T7E1 PCR nrimers |
| A1D_T7E1_Fwd KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC KJ_T0231_EMX1 CCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | KI T0225 CACN | |
| KJ_T0226_CACN A1D_T7E1_Rev KJ_T0229_FANC KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC CCA GGC TCT CTT GGA GTG TC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | _ = = | Herr one herrene hed did er |
| A1D_T7E1_Rev KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | TGG AGT TTC TGC TCC CAT TT |
| KJ_T0229_FANC F_T7E1_Fwd KJ_T0230_FANC KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 CCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | 100 AOT TIC TOC TCC CAT II |
| F_T7E1_Fwd KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 CCC CCT AAC CCT ATG TAG CC T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| KJ_T0230_FANC F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | ACC ICI IIU IUI UUC UAA AU |
| F_T7E1_Rev KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | |
| KJ_T0231_EMX1 GCC CCT AAC CCT ATG TAG CC _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | CCA GGC TCT CTT GGA GTG TC |
| _T7E1_Set1_Fwd KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | 5.55 C.55 C.55 C.55 C.55 C.55 C.55 C.55 |
| KJ_T0232_EMX1 GGA GAT TGG AGA CAC GGA GA | | GCC CCT AAC CCT ATG TAG CC |
| | | |
| _T7E1_Set1_Rev | | GGA GAT TGG AGA CAC GGA GA |
| | _T7E1_Set1_Rev | |
| KJ_T0320_RUNX CCAGCACAACTTACTCGCACTTGAC | KJ_T0320_RUNX | CCAGCACAACTTACTCGCACTTGAC |
| 1_PCR_Fwd | 1_PCR_Fwd | |

| KJ_T0321_RUNX | CATCACCAACCCACAGCCAAGG |
|-----------------|-----------------------------------------------------------------|
| 1_PCR_Rev | |
| KJ_T0206_AcrIIA | tataggGagacccaagctggctagcATG GCA GAT ATG ACG CTT CGC |
| 11a1 gBlock for | CAG TTC TGCGAG CGA TAT CGC AAG GGT GAC TTC CTC |
| expression in | GCAAAG GAT CGA GAA ACT CAA ATC GAG GCA GGTTG |
| HEK293T cells | G TAC GAT TGG TTT TGT GAT GAC AAA GCCTTG GCG GG |
| | C CGA TTG GCA AAA ATC TGG GGGATT TTG AAG GGG A |
| | TA ACC TCA GAT TAT ATCTTG GAT AAC TAC CGC GTA T |
| | GG TTC AAA AACAAC TGT CCA ATG GTA GGA CCA CTG |
| | TAC GACGAT GTA CGC TTC GAA CCG CTT GAT GAA GAA |
| | CAG CGA GAT GAG CTC TAC TTC GGC GTC GCAATC GAC |
| | GAT AAG AGG AGG GAA AAG AAA TACGTC ATA TTC AC |
| | T GCT CGA AAT GAC TAT GAAAAC GAG TGT GGT TTC A |
| | AC AAC GTG AGA GAAGTA CGC CAA TTT ATA AAT GGA |
| | TGG GAA GACGAA TTG AAG AAC GAA GAG TTC TAT AA |
| | G GCTAGG GAG AAA AAA CGG CAA GAA ATG GAA GAA |
| | GCC AAT AAC AAA TTC GCA GAA ATA ATG CAACGG GC |
| | C GAT GAG ATA TTG TGG AAC CTG AAAGAG GACtccggacc |
| | tccgaagaaaaagcgaaaggtg ggatccagtgga tatccctatgacgtgcccgattatgcc |
| | taaCtcgagcggccgccactgtgctgga |

Table S2. Plasmids used in this study.

| Plasmid | Description | Primers | Source |
|---------|-------------------------------|-------------------------------|------------|
| pIF592 | p6XHis_NLS-SaCas9 (item | N/A | (Soares |
| | #101086) | | et al., |
| | | | 2017) |
| pIF936 | pMCSG7-Wt-NmeCas9 (item # | N/A | (Zhang et |
| | 71474) | | al., 2015) |
| pIF937 | AcrIIA11-6xHis in pET19 | N/A | This |
| | vector | | study |
| pIF938 | AcrIIA11-TS in pET19 vector | N/A | This |
| | _ | | study |
| pIF939 | TS-SUMO-AcrIIA11 No C- | N/A | This |
| | terminal tags in pET19 vector | | study |
| pIF940 | TS-SUMO-3xFLAG-SaCas9 | N/A | This |
| | | | study |
| pIF941 | TS-SUMO-3xFLAG-dSaCas9 | KD197 and KD198 | This |
| | | | study |
| pIF942 | TS-SUMO-SaCas9-sgRNA | KD174, KD175, KD179, KD180 | This |
| | _ | | study |
| pIF943 | p6xHis_NLS-SaCas9-sgRNA | KD153, KD154, KD155, KD156, | This |
| _ | | T7 promoter with SaCas9 sgRNA | study |
| | | insert | |

| pIF944 | pCK002_U6-Sa- | KJ T0217 CACNA1D 20nt Fwd, | (Singer et |
|--------|---------------------------|------------------------------|------------|
| | sgRNA(mod)_EFS-SaCas9-2A- | KJ_T0221_CACNA1D_20nt_Rev, | al., 2016) |
| | Puro_WPRE (item # 85452) | KJ_T0324_EMX1_21nt_Fwd_3, | |
| | | KJ_T0325_EMX1_21nt_Rev_3, | |
| | | KJ_T0326_FANCF_21nt_Fwd_3, | |
| | | KJ_T0327_FANCF_21nt_Rev_3, | |
| | | KJ_T0328_RUNX1_23nt_Fwd_1, | |
| | | KJ_T0329_RUNX1_23nt_Rev_1, | |
| | | KJ_T0287_Scramble_sgRNA_Fwd, | |
| | | KJ_T0288_scramble_sgRNA_Rev, | |
| pIF945 | pAAV-CMV-NLS-AcrIIA4 | N/A | (Bubeck |
| | (item # 113038) | | et al., |
| | | | 2018) |
| pIF946 | pAAV-CMV-NLS-AcrIIA11 | N/A | This |
| | | | study |
| pIF967 | TS-SUMO-SaCas9 | N/A | This |
| | | | study |

Table S3. Single-molecule data analysis.

| Diffusing vs stationary SaCas9 molecules (Figure 3C) | | | | | |
|------------------------------------------------------|--------------------------------------|----------------------|------------|------------------------|--|
| Condition | Stationary | Diffusing | Number of | p-value (Chi- | |
| | molecules | molecules | molecules | squared test) | |
| - AcrIIA11 | 28 | 61 | 89 | 3 x 10 ⁻⁹ | |
| + AcrIIA11 | 70 | 23 | 93 | | |
| | SaCas9 diffu | usion coefficients (| Figure 3E) | | |
| Condition | Mean diffusion co | efficient ± | Number of | p-value | |
| | S.E.M. $(\mu m^2 s^{-1})$ | | molecules | (Mann- | |
| | | | | Whitney U- | |
| | | | | test) | |
| - AcrIIA11 | 0.05 ± 0.01 | | 33 | 9.6 x 10 ⁻⁷ | |
| + AcrIIA11 | 0.006 ± 0.003 | | 33 | | |
| | WT SaCas9 target binding (Figure 4D) | | | | |
| Condition | Target bound | Non-target | Number of | p-value (Chi- | |
| | molecules | bound | molecules | squared test) | |
| | | molecules | | | |
| - AcrIIA11 | 38 | 51 | 89 | 1 x 10 ⁻⁶ | |
| + AcrIIA11 | 13 | 95 | 108 | | |