Supplementary Material

Rationally Engineered Cas9 Nucleases with Improved Specificity

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SUPPLEMENTARY MATERIALS AND METHODS

Structural analysis

Structures of SpCas9 (PDB ID 4UN3 and 4OO8) were analyzed using Pymol (Schrödinger). DNA was hand modeled into the non-complementary strand groove (nt-groove) with the phosphate backbone making hydrogen bond distance contacts with specificity conferring mutations when possible. Electrostatics were calculated using the APBS plugin as part of Pymol. Amino acid sequences were visualized using Geneious 2 (Geneious version (8.0.3) (www.geneious.com(*1*)).

Cloning

Mutants were cloned using the Golden Gate strategy (*2*). Briefly, wild-type SpCas9 (pX330) or wild-type SaCas9 (pX601) were used as template to amplify two PCR fragments, using primers that incorporated BsaI (pX330) or BbsI (pX601) restriction sites. BsaI or BbsI digestion results in distinct 5' overhangs which are either compatible to the AgeI or EcoRI overhangs of the recipient vector or will reconstitute the desired point mutation at the junction of the two Cas9 DNA pieces.

Cell culture and transfections

Human embryonic kidney (HEK) cell lines 293T and 293FT (Fisher Scientific) were maintained in Dulbecco's modified Eagle's medium DMEM (Life technologies) supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2. Cells were plated one day prior to transfection in 24- or 96-well plates (Corning) at a density of approximately 120,000 cells per 24-well or 30,000 cells per 96-well. Transfections were performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer's recommended protocol. Unless otherwise specified, cells were transfected with a total of 400ng of Cas9 plasmid with 100-200ng sgRNA plasmid per 24-well, or 100ng Cas9 with 25-50ng sgRNA plasmid per 96-well. For each transfection, an equal amount of plasmid was delivered to all samples. For cytotoxicity experiments, HEK293T cells were transfected with WT or eSpCas9(1.1) and incubated for 72 hours before measuring cell survival using the CellTiter-Glo (Promega) viability assay, which fluoresces in response ATP production by live cells.

Indel analysis by next-generation sequencing (NGS)

Cells were harvested approximately 3 days post transfection. Genomic DNA was extracted using a QuickExtract DNA extraction kit (Epicentre) by resuspending pelleted cells in QuickExtract (80µL per 24-well, or 20µL per 96-well), followed by incubation at 65°C for 15min, 68°C for 15min and 98°C for 10-15min. PCR fragments for targeted deep sequencing were generated in two step PCR reactions as previously described (*3*). Briefly, primers with PCR handles for second round amplification were used to amplify genomic regions of interest (table.S2), followed by a fusion PCR method to attach Illumina P5 adapters as well as unique sample-specific barcodes to the first round PCR product.

BLESS

Cells were harvested at approximately 24 hr post-transfection, and BLESS was carried out as described previously (*4, 5*). Briefly, a total of 10 million cells were fixed for nuclei isolation and permeabilization and then treated with Proteinase K for 4 min at 37 °C before inactivation with PMSF. Deproteinated nuclei DSBs were labeled with 200 mM of annealed proximal linkers overnight. After Proteinase K digestion of labeled nuclei, chromatin was mechanically sheared with a 26G needle before sonication (BioRuptor, 20 min on high, 50% duty cycle). A total of 20 μg of sheared chromatin was captured on streptavidin beads, washed, and ligated to 200 mM of distal linker. Linker hairpins were then cleaved off with I-SceI digestion for 4 h at 37 °C, and products were PCR-enriched for 18 cycles before proceeding to library preparation with a TruSeq Nano LT Kit (Illumina). For the negative control, cells were mock transfected with Lipofectamine 2000 and pUC19 DNA and were parallel processed through the assay.

The calculation of the DSB score to separate the background DSBs from the bona fide Cas9-induced ones was done as previously described (Ran et al, Nature 2015), and sorting the loci on the DSB score revealed the top off-target sites as had been previously identified for these sgRNA targets. In order to provide additional detection capability beyond these top off-targets, we found from the previous Cas9-BLESS data that a homology-search algorithm could help further identify true Cas9-induced DSBs. The homology-search algorithm searched for the best matched guide sequence within a region of the genome 50nt on either side of the median of a DSB cluster identified in BLESS for all NGG and NAG PAM sequences. A score based on the homology was calculated with the following weights: a match between the sgRNA and the genomic sequence scores +3, a mismatch is -1, while an insertion or deletion between the sgRNA and genomic sequence costs -5. Thereby, an on-target sequence with the full 20bp guide + PAM would score 69. The final homology score for a DSB cluster was identified as the maximum of the scores from all possible sequences. Using these weights, we empirically found that bona fide off-targets (for which indels were identified on targeted deep sequencing) and background DSBs were separated fully when a threshold of >50 was used for the homology score. Using this homology criterion on the top 200 BLESS DSB loci allowed us to further identify off-targets from the background DSBs.

SUPPLEMENTARY FIGURES

Figure S1

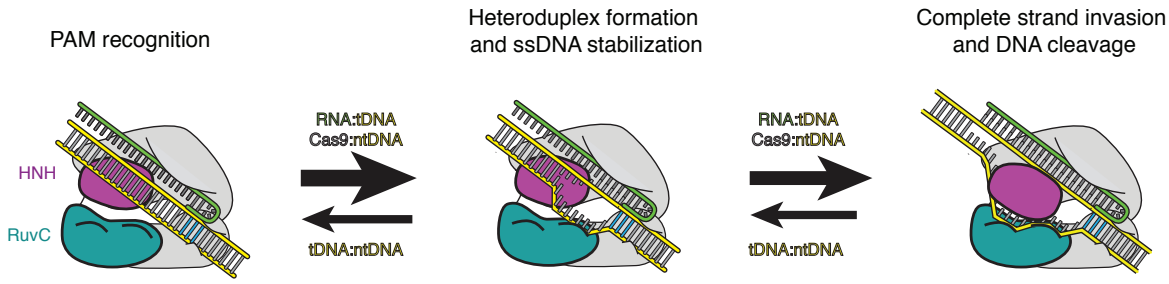

Fig. S1. Schematic sgRNA guided targeting and DNA unwinding.

Cas9 cleaves target DNA in a series of coordinated steps. First, the PAM-interacting domain recognizes an NGG sequence 5' of the target DNA. After PAM binding, the first 10-12 nucleotides of the target sequence (seed sequence) are sampled for sgRNA:DNA complementarity, a process dependent on DNA duplex separation. If the seed sequence nucleotides complement the sgRNA, the remainder of DNA is unwound and the full length of sgRNA hybridizes with the target DNA strand. We hypothesized the nt-groove between the RuvC (teal) and HNH (magenta) domains stabilizes the non-targeted DNA strand (ntDNA) and facilitates unwinding through non-specific interactions with negative charges of the DNA phosphate backbone. In this model, RNA:target-strand DNA (tDNA) and Cas9:ntDNA interactions drive DNA unwinding (top arrow) in competition against tDNA:ntDNA rehybridization (bottom arrow).

Figure S2

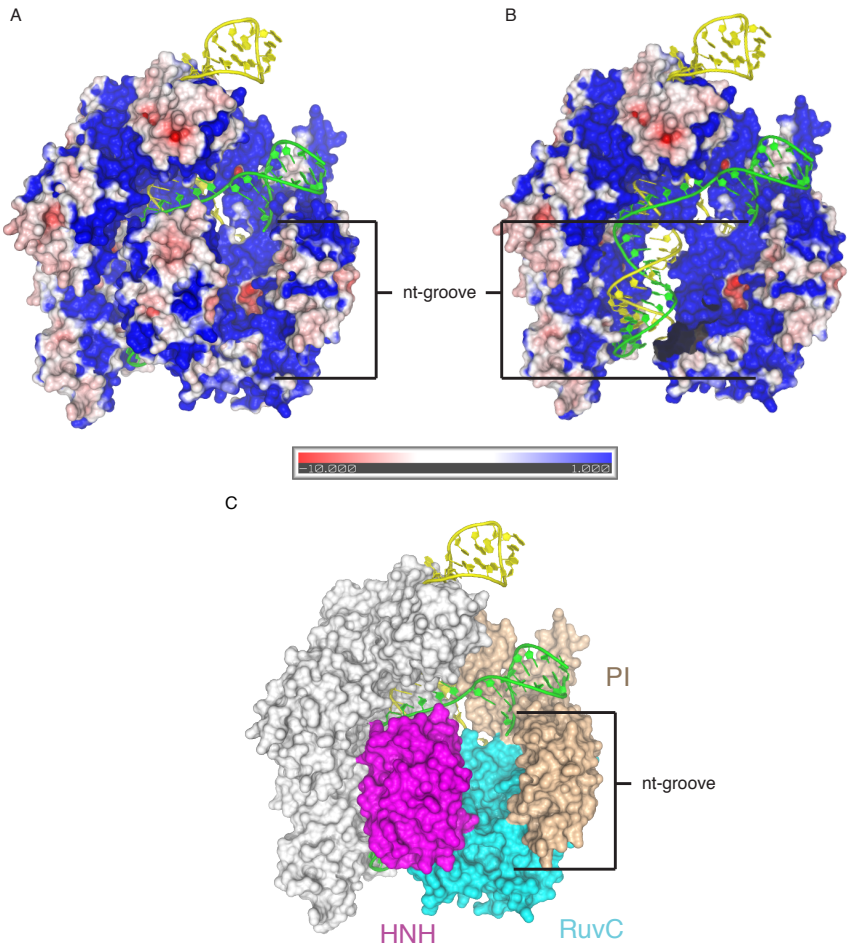

Fig S2. Electrostatics of SpCas9 reveal non-target strand groove.

(A) Crystal structure (4UN3) of SpCas9 paired with sgRNA and target DNA colored by electrostatic potential to highlight positively charged regions. Scale is from -10 to 1 keV. (B) Identical to panel (A) with HNH domain removed to reveal the sgRNA:DNA heteroduplex. (C) Crystal structure (in the same orientation as (A)) colored by domain: HNH (magenta), RuvC (teal), and PAM-interacting (PI) (beige).

Figure S3

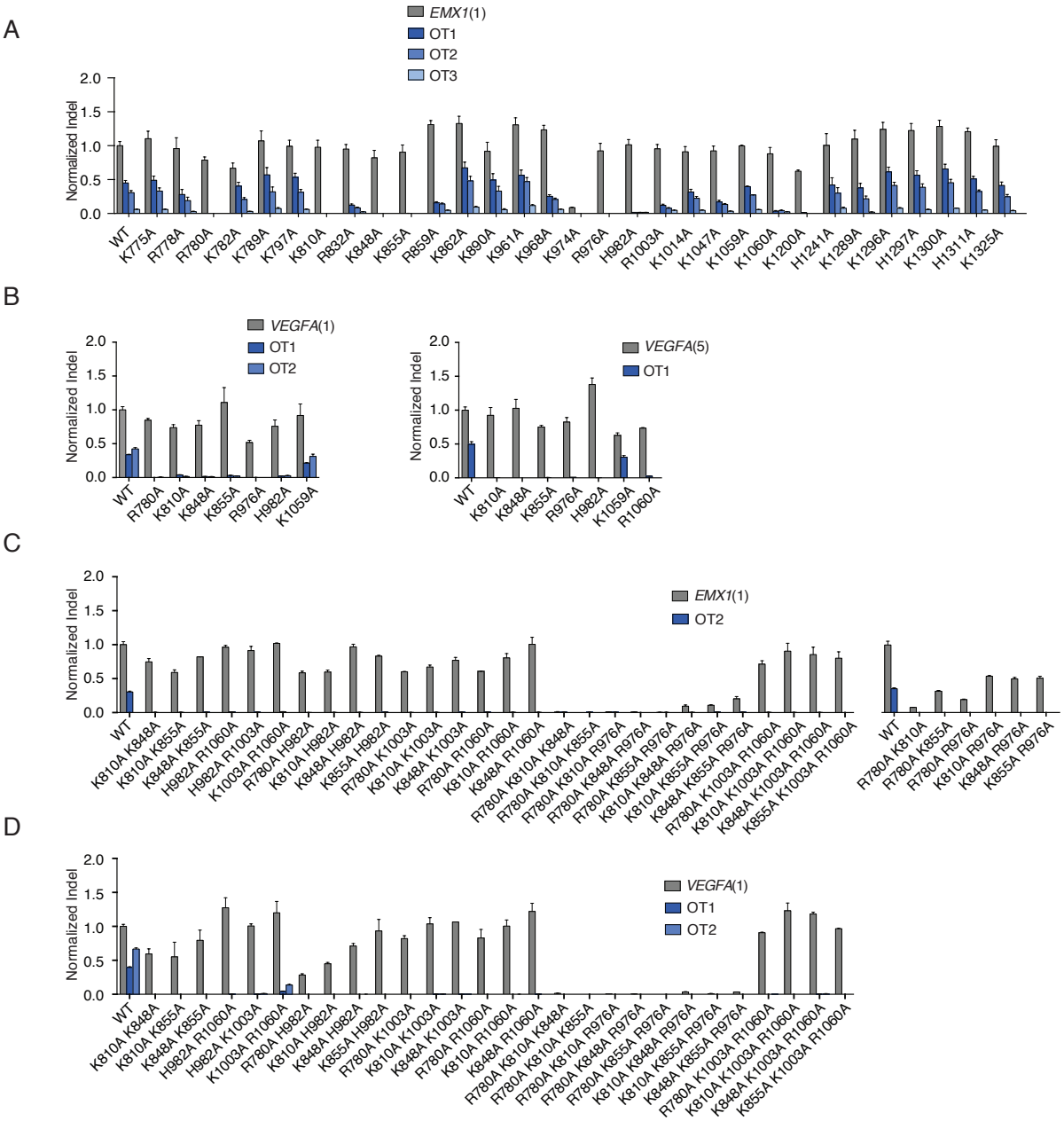

Fig S3. Off-target analysis of generated mutants.

Thirty-one SpCas9 single amino acid mutants were generated and tested for specificity at (A) an *EMX1* target site and (B) two *VEGFA* target sites. Mutants combining the top residues that improved specificity were further tested at (C) *EMX1* and (D) *VEGFA*.

Figure S4

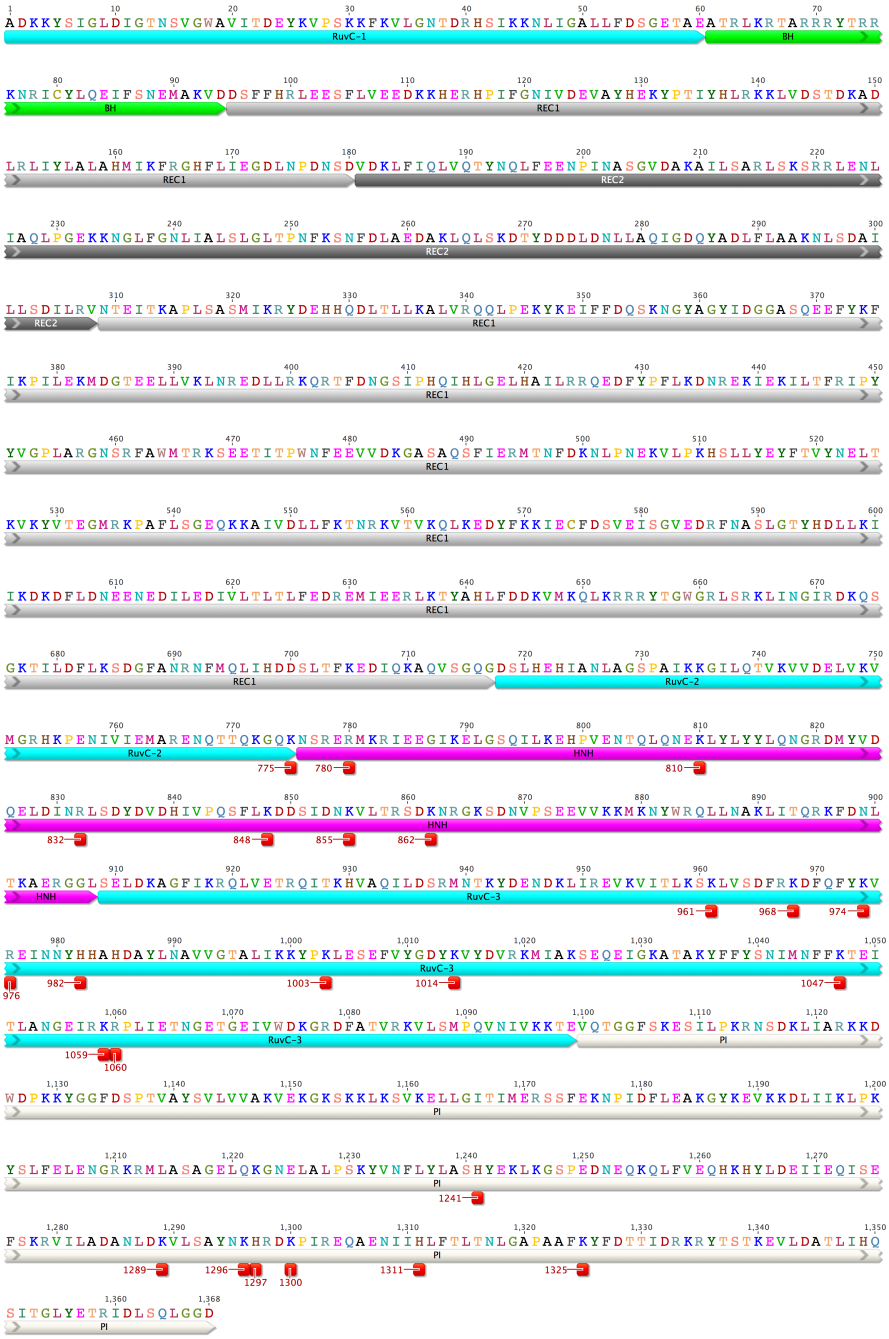

Fig. S4. Annotated SpCas9 amino acid sequence.

Mutations of SpCas9 that altered non-targeted strand groove charges were primarily in the RuvC and HNH domains (highlighted in yellow). RuvC (cyan), bridge helix (BH, green), REC (grey), HNH (magenta), and PI (beige) domains are annotated as in Nishimasu et al. (*6*).

Figure S5

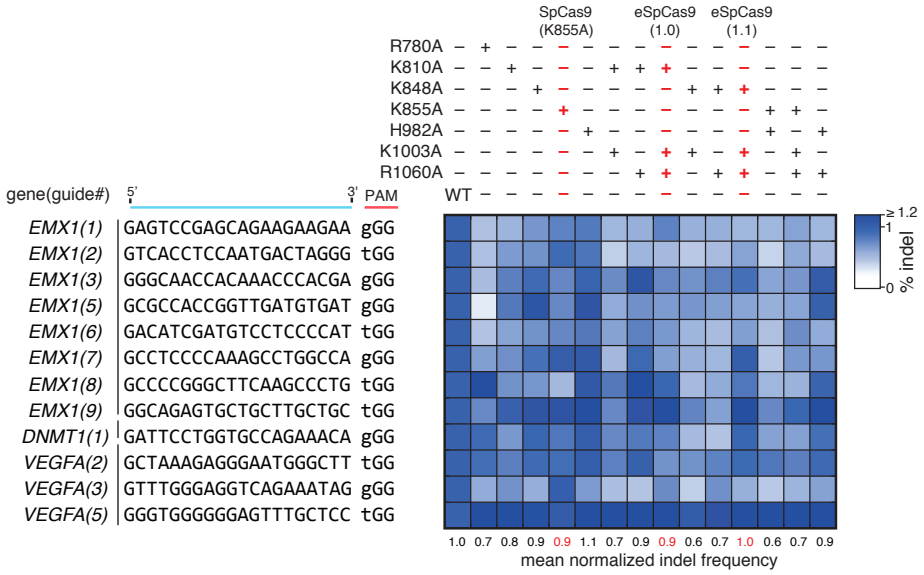

Fig. S5. On-target efficiency screen of SpCas9 mutants.

Screen of top single amino acid mutants and combination mutants at 10 target loci for on-target cleavage efficiency. SpCas9 (K855A), eSpCas9(1.0), and eSpCas9(1.1) are highlighted in red.

Figure S6

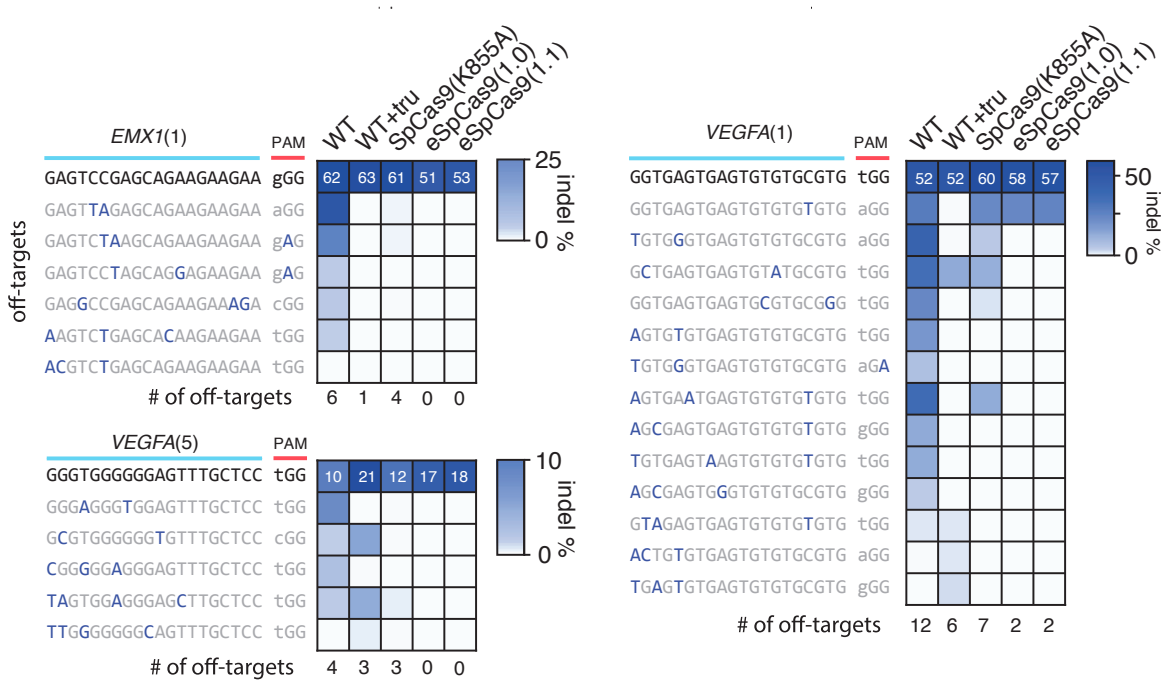

Fig. S6. eSpCas9(1.0) and eSpCas9(1.1) outperforms truncated sgRNAs as a strategy for improving specificity.

Comparison of the specificity of SpCas9(K855A), eSpCas9(1.0) and eSpCas9(1.1) with truncated sgRNAs (tru). Indel frequencies at three loci (*EMX1*(1), *VEGFA*(1) and *VEGFA*(5)) were tested at major annotated off-target sites. For both *VEGFA* target sites, tru-sgRNAs increased indel frequency at some off-target sites and generated indels at off-targets not observed for WT SpCas9. On target indel percentages are labeled in white. The number of off-target sites detectable by targeted deep sequencing for each SpCas9 mutant are listed below the heat map (>0.2% indel).

Figure S7

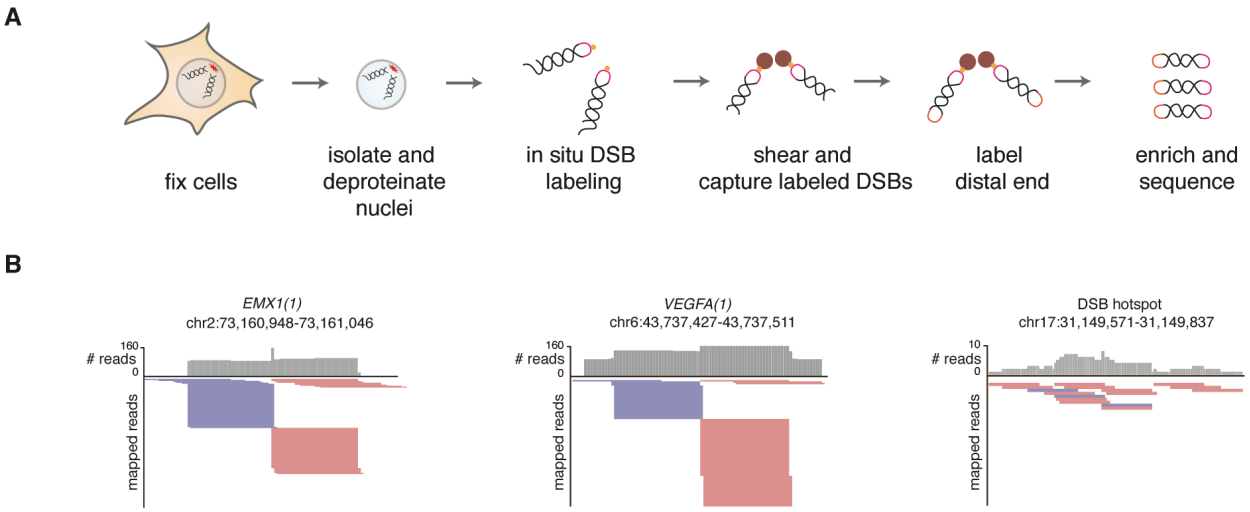

Fig. S7. Diagram of BLESS workflow and reads mapped to an on-target cut site.

(A) Schematic outline of the BLESS workflow. (B) Representative BLESS sequencing for forward (red) and reverse (blue) reads mapped to the genome. Reads mapping to Cas9 cut sites have distinct shape compared to DSB hotspots.

Figure S8

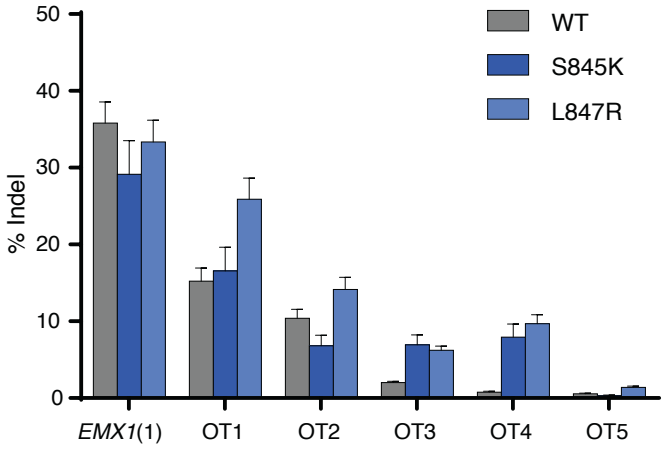

Fig. S8. Increasing positive charge in the nt-groove generates can result in increased cleavage at off-target sites.

Single amino acid mutants SpCas9(S845K) and SpCas9(L847R) exhibited less specificity than WT SpCas9 at the *EMX1*(1) target site.

Figure S9

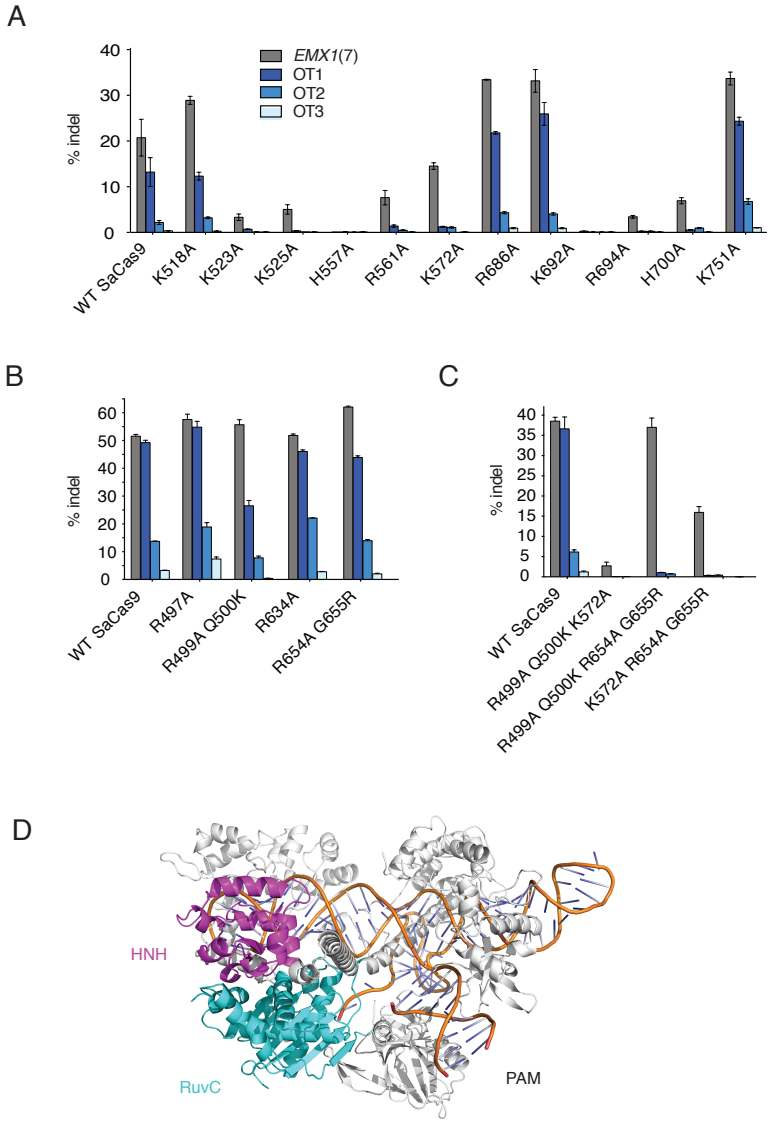

Fig. S9. Generation of eSaCas9 through mutagenesis of the nt-groove.

An improved specificity variant of *S. aureus* Cas9 (SaCas9) was generated similarly to eSpCas9. (A,B) Single and double amino acid mutants of residues in the groove between the RuvC and HNH domains were screened for decreased off-target cutting. (C) Mutants with improved specificity were combined to make a variant of SaCas9 that maintained on-target cutting at *EMX1*(7) and had significantly reduced off-target cutting. (D) Crystal structure of SaCas9 showing the groove between the HNH and RuvC domains.

Figure S10

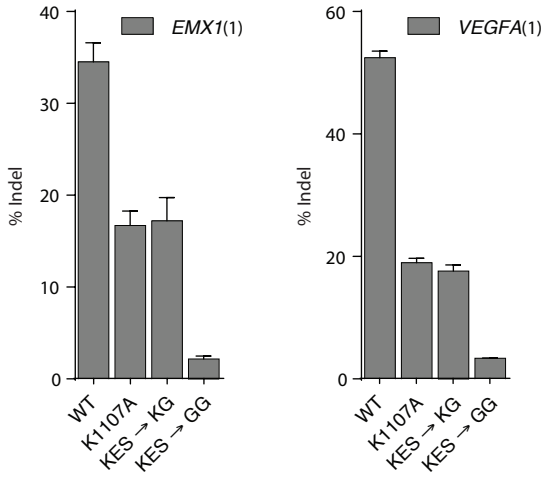

Fig. S10. Characterization of on-target efficiency for specificity-enhancing mutants identified in Anders et al.

Anders et al. previously reported three SpCas9 mutants at the phosphate lock loop (Lys1107, Glu1108, Ser1109) in the PI domain which confer specificity to bases 1 and 2 of the sgRNA proximal to the PAM (*7*). These consisted of a single amino acid mutant (K1107A) and two mutants in which the Lys-Glu-Ser (KES) sequence was replaced with the dipeptides Lys-Gly (KG) and Gly-Gly (GG), respectively. Targeted deep sequencing indicated that these phosphate lock mutants can reduce on-target cleavage efficiency.

Figure S11

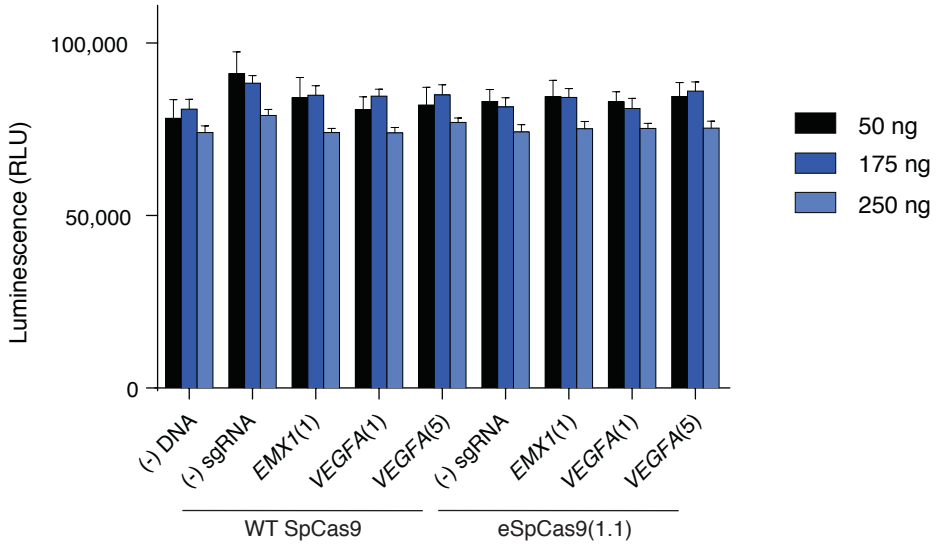

Fig. S11. eSpCas9(1.1) is not cytotoxic to human cells.

HEK293T cells were transfected with plasmid encoding WT SpCas9 or eSpCas9(1.1) and incubated for 72 hours before measuring cell survival using the CellTiter-Glo assay, which fluoresces in response ATP production by live cells.

Figure S12

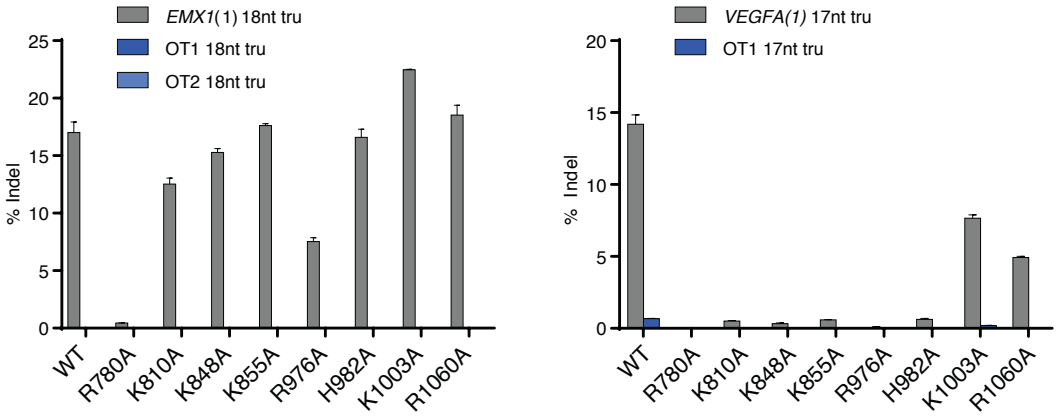

Fig. S12. Nt-groove mutants are not broadly compatible with truncated guide RNAs.

Truncated guide RNAs (tru) were combined with single amino acid SpCas9 mutants and targeted to (A) *EMX1*(1) or (B) *VEGFA*(1). While most mutants targeted to *EMX1*(1) with an 18nt guide retained on-target efficiency, those targeted to *VEGFA*(1) with a 17nt guide were compromised. This indicates that truncated guides are not generally compatible with nt-groove mutants.

Table S1. Golden Gate primers for mutant generation.

SpCas9 primer name	Sequence
SPCAS9-N	ATGGTCTCACCGGTGCCACCATGGACTATAAG
K775A_F	ATGGTCTCAGGCGAACAGCCGCGAGAGAATGAAGCGGAT
R778A_F	ATGGTCTCACGCCGAGAGAATGAAGCGGATCGAAGAGGG
R780A_F	ATGGTCTCAGGCGATGAAGCGGATCGAAGAGGGCATCA
K782A_F	ATGGTCTCAGGCCCGGATCGAAGAGGGCATCAAAGAGCT
K789A_F	ATGGTCTCACGCCGAGCTGGGCAGCCAGATCCTGAAAGA
K797A_F	ATGGTCTCAGGCCGAACACCCCGTGGAAAACACCCAGCT
K810A_F	ATGGTCTCAGGCGCTGTACCTGTACTACCTGCAGAATGG
R832A_F	ATGGTCTCAGCCCTGTCCGACTACGATGTGGACCATATC
K848A_F	ATGGTCTCAGCCGACGACTCCATCGACAACAAGGTGCTGACC
K855A_F	ATGGTCTCAGCAGTGCTGACCAGAAGCGACAAGAACCGGG
R859A_F	ATGGTCTCACGCAAGCGACAAGAACCGGGGCAAGAG
K862A_F	ATGGTCTCACGCGAACCGGGGCAAGAGCGACAAC
K890A_F	ATGGTCTCACGCCCTGATTACCCAGAGAAAGTTCGACAA
K961A_F	ATGGTCTCAGCGCTGGTGTCCGATTTCCGGAAGGATTTC
K968A_F	ATGGTCTCAGCGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAAC
K974A_F	ATGGTCTCACGCAGTGCGCGAGATCAACAACTACCACCA
R976A_F	ATGGTCTCATGGCCGAGATCAACAACTACCACCACGCC
H982A_F	ATGGTCTCACGCCCACGCCCACGACGCCTAC
K1003A_F	ATGGTCTCAGCGCTGGAAAGCGAGTTCGTGTACGGC
K1014A_F	ATGGTCTCAGCGGTGTACGACGTGCGGAAGATGATCG
K1047A_F	ATGGTCTCACGCGACCGAGATTACCCTGGCCAACG
K1059A_F	ATGGTCTCAGCGCGGCCTCTGATCGAGACAAACGG
R1060A_F	ATGGTCTCAGGCGCCTCTGATCGAGACAAACGGCG
K1107A_F	ATGGTCTCACGCCGAGTCTATCCTGCCCAAGAGGAACAG
K1200A_F	ATGGTCTCATGCCTACTCCCTGTTCGAGCTGGAAAACGG
H1241A_F	ATGGTCTCACGCCTATGAGAAGCTGAAGGGCTCCCC
K1289A_F	ATGGTCTCACGCAGTGCTGTCCGCCTACAACAAGCAC
K1296A_F	ATGGTCTCACGCGCACCGGGATAAGCCCATCAGAG
H1297A_F	ATGGTCTCAAGGCCCGGGATAAGCCCATCAGAGAGC
K1300A_F	ATGGTCTCAGCGCCCATCAGAGAGCAGGCCGAG
H1311A_F	ATGGTCTCACGCCCTGTTTACCCTGACCAATCTGGGAG
K1325A_F	ATGGTCTCACGCGTACTTTGACACCACCATCGACCGG
KES_KG_F	ATGGTCTCAAGGCATCCTGCCCAAGAGGAACAGCGATAA
KES_GG_F	ATGGTCTCACGGCATCCTGCCCAAGAGGAACAGCGATAA
SPCAS9-C	ATGGTCTCAAATTCTTACTTTTTCTTTTTTGCCTGGCC
K775A_R	ATGGTCTCACGCCTGTCCCTTCTGGGTGGTCTGG

R778A_R	ATGGTCTCAGGCGCTGTTCTTCTGTCCCTTCTGGGTGGT
R780A_R	ATGGTCTCACGCCTCGCGGCTGTTCTTCTGTCCCT
K782A_R	ATGGTCTCAGGCCATTCTCTCGCGGCTGTTCTTCTGTCC
K789A_R	ATGGTCTCAGGCGATGCCCTCTTCGATCCGCTTCATTCT
K797A_R	ATGGTCTCAGGCCAGGATCTGGCTGCCCAGCTCTTTGAT
K810A_R	ATGGTCTCACGCCTCGTTCTGCAGCTGGGTGTTTTCCA
R832A_R	ATGGTCTCAGGGCGTTGATGTCCAGTTCCTGGTCCAC
K848A_R	ATGGTCTCACGGCCAGAAAGCTCTGAGGCACGATATGGTCCAC
K855A_R	ATGGTCTCACTGCGTTGTCGATGGAGTCGTCCTTCAGAAAGCTCTG
R859A_R	ATGGTCTCATGCGGTCAGCACCTTGTTGTCGATGGAGTC
K862A_R	ATGGTCTCACGCGTCGCTTCTGGTCAGCACCTTGTTG
K890A_R	ATGGTCTCAGGCGGCGTTCAGCAGCTGCCGCCAGTAGTT
K961A_R	ATGGTCTCAGCGCGGACTTCAGGGTGATCACTTTCACTTC
K968A_R	ATGGTCTCACCGCCCGGAAATCGGACACCAGCTTG
K974A_R	ATGGTCTCATGCGTAAAACTGGAAATCCTTCCGGAAATCGGACAC
R976A_R	ATGGTCTCAGCCACTTTGTAAAACTGGAAATCCTTCCGGAAATCGG
H982A_R	ATGGTCTCAGGCGTAGTTGTTGATCTCGCGCACTTTGTAAAACTG
K1003A_R	ATGGTCTCAGCGCAGGGTACTTTTTGATCAGGGCGGTTC
K1014A_R	ATGGTCTCACCGCGTAGTCGCCGTACACGAACTCG
K1047A_R	ATGGTCTCACGCGAAAAAGTTCATGATGTTGCTGTAGAAGAAGTACTTGG
K1059A_R	ATGGTCTCAGCGCCCGGATCTCGCCGTTGGC
R1060A_R	ATGGTCTCACGCCTTCCGGATCTCGCCGTTGGC
K1107A_R	ATGGTCTCAGGCGCTGAAGCCGCCTGTCTGCACCTCGGT
K1200A_R	ATGGTCTCAGGCAGGCAGCTTGATGATCAGGTCCTTTTT
H1241A_R	ATGGTCTCAGGCGCTGGCCAGGTACAGGAAGTTCAC
K1289A_R	ATGGTCTCATGCGTCCAGATTAGCGTCGGCCAGGATC
K1296A_R	ATGGTCTCACGCGTTGTAGGCGGACAGCACTTTGTCC
H1297A_R	ATGGTCTCAGCCTTGTTGTAGGCGGACAGCACTTTGTCC
K1300A_R	ATGGTCTCAGCGCATCCCGGTGCTTGTTGTAGGCG
H1311A_R	ATGGTCTCAGGCGATGATATTCTCGGCCTGCTCTCTGATG
K1325A_R	ATGGTCTCACGCGAAGGCGGCAGGGGCTCC
KES_KG_R	ATGGTCTCAGCCTTTGCTGAAGCCGCCTGTCTGCACCTC
KES_GG_R	ATGGTCTCAGCCGCCGCTGAAGCCGCCTGTCTGCACCTC
SaCas9 primer name	**Sequence**
SACAS9-N	ATGAAGACTACCGGTGCCACCATGGCCC
R497A_F	ATGAAGACTAGCCAACCGGCAGACCAACGAGCG
K518A_F	ATGAAGACTAGCGTACCTGATCGAGAAGATCAAGCTGCA
K523A_F	ATGAAGACTAGCGATCAAGCTGCACGACATGCAGGA
K525A_F	ATGAAGACTAGCGCTGCACGACATGCAGGAAGGC
H557A_F	ATGAAGACTAGCCATCATCCCCAGAAGCGTGTCCTTC

R561A_F	ATGAAGACTAGCAAGCGTGTCCTTCGACAACAGCTTC
K572A_F	ATGAAGACTAGCGGTGCTCGTGAAGCAGGAAGAAAACA
R634A_F	ATGAAGACTAGCGTTCTCCGTGCAGAAAGACTTCATCAAC
R686A_F	ATGAAGACTAGCGAAGTGGAAGTTTAAGAAAGAGCGGAACAA
K692A_F	ATGAAGACTAGCAGAGCGGAACAAGGGGTACAAGCAC
R694A_F	ATGAAGACTAGCGAACAAGGGGTACAAGCACCACGC
H700A_F	ATGAAGACTAGCCCACGCCGAGGACGCCCTGA
K751A_F	ATGAAGACTAGCAGAGATCTTCATCACCCCCCACCAG
R499A;Q500K_F	ATGAAGACTAGCAAAGACCAACGAGCGGATCGAGG
R654A;G655R_F	ATGAAGACTAGCCCGCCTGATGAACCTGCTGCGG
SACAS9-C	ATGAAGACTAAATTCTTAAGCGTAATCTGGAACATCGTATGG
R497A_R	ATGAAGACTATGGCCTTCTGCATCTCGTTGATCATTTTCTG
K518A_R	ATGAAGACTAACGCGGCGTTCTCTTTGCCGGTGG
K523A_R	ATGAAGACTATCGCCTCGATCAGGTACTTGGCGTTCTCTT
K525A_R	ATGAAGACTAGCGCGATCTTCTCGATCAGGTACTTGGCGT
H557A_R	ATGAAGACTATGGCGTCCACCTCATAGTTGAAGGGGTTGT
R561A_R	ATGAAGACTATTGCGGGGATGATGTGGTCCACCTCATA
K572A_R	ATGAAGACTACCGCGTTGTTGAAGCTGTTGTCGAAGGACA
R634A_R	ATGAAGACTAACGCGTTGATGTCCCGTTCTTCCAGCA
R686A_R	ATGAAGACTATCGCCCGCAGAAAGCTGGTGAAGCC
K692A_R	ATGAAGACTACTGCCTTAAACTTCCACTTCCGCCGCA
R694A_R	ATGAAGACTATCGCCTCTTTCTTAAACTTCCACTTCCGCC
H700A_R	ATGAAGACTAGGGCCTTGTACCCCTTGTTCCGCTCTTTC
K751A_R	ATGAAGACTACTGCGTACTCCTGCTCGGTTTCGATCTCG
R499A;Q500K_R	ATGAAGACTATTGCGTTCCGCTTCTGCATCTCGTTGA
R654A;G655R_R	ATGAAGACTAGGGCGGTGGCGTATCTGGTATCCACCA

Table S2. Guide sequences and primers for targeted deep sequencing

Name	5' Sequence 3'	PAM	Forward Primer	Reverse Primer
VEGFA(1)	GGTGAGTGAGTGTGTGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcGCGTCTTCGAGAGTGAGGAC	CCTCTCTATGGGCAGTCGGTGATgGGGGAGAGGGACACACAGAT
OT1	GCTGAGTGAGTGTATGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcGCCCATTTCTCCTTTGAGGT	CCTCTCTATGGGCAGTCGGTGATgAGCCACAGAGGTGGAGACTG
OT2	TGTGGGTGAGTGTGTGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcTGTCACCACACAGTTACCACCT	CCTCTCTATGGGCAGTCGGTGATgGGGAATCTAATGTATGGCATGG
	GGTGAGTGAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcAGGGACCCCTCTGACAGACT	CCTCTCTATGGGCAGTCGGTGATgCACACCCACACCCTCATACA
	GGTGAGTGAGTGCGTGCGGG	GG	CCATCTCATCCCTGCGTGTCTCcCCTCCCACAGGAATTTGAAG	CCTCTCTATGGGCAGTCGGTGATgGCACCCCAACACCTACATCT
	AGTGAATGAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcATAAGGGGCAAGTTCTGGGCTAT	CCTCTCTATGGGCAGTCGGTGATgTGTGACCCAAAAGATTCCCACC
	TGTGAGTAAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcTGATGAAGCTGCCTTTCCTAAGC	CCTCTCTATGGGCAGTCGGTGATgCACAGGCACTAACTTCTTCAGCCTA
	ACTGTGTGAGTGTGTGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcTCTGCCAGATCCTTAGGCG	CCTCTCTATGGGCAGTCGGTGATgCCCCAGCAAAACGCACTG
	AGCGAGTGGGTGTGTGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcGACGTCTGGGTCCCGAGC	CCTCTCTATGGGCAGTCGGTGATgCCACACACAGCGTCTTCCG
	AGTGTGTGAGTGTGTGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcTCCTGTGGAACAACCAGACACC	CCTCTCTATGGGCAGTCGGTGATgTCAAAGCTGTATCCCCATTGCCTA
	TGTGGGTGAGTGTGTGCGTG	GA	CCATCTCATCCCTGCGTGTCTCcAAGCTGCTGGCTTTCCTAAG	CCTCTCTATGGGCAGTCGGTGATgAGCAACGAGACGTTAACCC
	AGCGAGTGAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcAGGACCCAGGTTTGCACT	CCTCTCTATGGGCAGTCGGTGATgTTCTGCCACTGGCTTAGCTT
	GTAGAGTGAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcATGATTAGAAACCTGCACTCCCAG	CCTCTCTATGGGCAGTCGGTGATgGTAAGTGAATCTCTGTCTGTCTCAT
	TGAGTGTGAGTGTGTGCGTG	GG	CCATCTCATCCCTGCGTGTCTCcGTGGGCACCAGGAGCGTAG	CCTCTCTATGGGCAGTCGGTGATgCAGGAGGTTAAATCCCTCCTCCA
	AGAGAGTGAGTGTGTGCATG	GG	CCATCTCATCCCTGCGTGTCTCcTTCCCCCATGCTTTTCTTGTGATAG	CCTCTCTATGGGCAGTCGGTGATgTCGGGAAACTTACAATCATGGAAGAAT
	CGTGAGTGAGTGTGTACCTG	GG	CCATCTCATCCCTGCGTGTCTCcCTGCCATTGTGAACAGTGCT	CCTCTCTATGGGCAGTCGGTGATgAGGCATGAGCCACTGAGACT
	TGTGAGTGAGTGTGTGTGTG	GA	CCATCTCATCCCTGCGTGTCTCcTATTTTCCCCACCTGGGAGT	CCTCTCTATGGGCAGTCGGTGATgAACACAGTCCCATCCTTGAAGT
VEGFA(5)	GGGTGGGGGGAGTTTGCTCC	GG	CCATCTCATCCCTGCGTGTCTCcAAGCAACTCCAGTCCCAAAT	CCTCTCTATGGGCAGTCGGTGATgCCCTAGTGACTGCCGTCTG
OT1	GGGAGGGTGGAGTTTGCTCC	GG	CCATCTCATCCCTGCGTGTCTCcCCTGCAGGTGTCTCCTTTTC	CCTCTCTATGGGCAGTCGGTGATgGCCACAGTCGTGTCATCTTG
	CGGGGGAGGGAGTTTGCTCC	GG	CCATCTCATCCCTGCGTGTCTCcACTTCTTGGGCAGTGATGGA	CCTCTCTATGGGCAGTCGGTGATgTACAAGGTGAGCCTGGGTCT
	TAGTGGAGGGAGCTTGCTCC	GG	CCATCTCATCCCTGCGTGTCTCcTGCAAAGCTAAGCAGAGATGC	CCTCTCTATGGGCAGTCGGTGATgGAAAGAAAGCCCCACCCTCG
	GCGTGGGGGGTGTTTGCTCC	GG	CCATCTCATCCCTGCGTGTCTCcGCAGAGATGCCTATGCCTACAT	CCTCTCTATGGGCAGTCGGTGATgCACCCTCGCTCTTTTAGTCTC
	TTGGGGGGCAGTTTGCTCC	GG	CCATCTCATCCCTGCGTGTCTCcACATGCGATTCTGCAGGGAA	CCTCTCTATGGGCAGTCGGTGATgTCAGAGGGTGCTGTCTGTCT
EMX1(1)	GAGTCCGAGCAGAAGAAGAAG	GG	CCATCTCATCCCTGCGTGTCTCcCAAAGTACAAACGGCAGAAGC	CCTCTCTATGGGCAGTCGGTGATgGTTGCCCACCCTAGTCATTG
OT1	GAGTTAGAGCAGAAGAAGAAA	GG	CCATCTCATCCCTGCGTGTCTCcTTCTGAGGGCTGCTACCTGT	CCTCTCTATGGGCAGTCGGTGATgGCCCAATCATTGATGCTTTT
OT2	GAGTCTAAGCAGAAGAAGAAG	AG	CCATCTCATCCCTGCGTGTCTCcCACGGCCTTTGCAAATAGAG	CCTCTCTATGGGCAGTCGGTGATgGGCTTTCACAAGGATGCAGT
OT3	GAGTCCTTAGCAGGAGAAGAAG	AG	CCATCTCATCCCTGCGTGTCTCcCCAGACTCAGTAAAGCCTGGA	CCTCTCTATGGGCAGTCGGTGATgTGGCCCCAGTCTCTCTTCTA
OT4	AAGTCTGAGCACAAGAAGAAA	GG	CCATCTCATCCCTGCGTGTCTCcGTTCTGACATTCCTCCTGAGGGA	CCTCTCTATGGGCAGTCGGTGATgATGGCTTACATATTTATTAGATAAAATGTATTCC
OT5	GAGGCCGAGCAGAAGAAAGAG	GG	CCATCTCATCCCTGCGTGTCTCcTGGGAGAGAGACCCCTTCTT	CCTCTCTATGGGCAGTCGGTGATgTCCTGCTCTCACTTAGACTTTCTC
	ACGTCTGAGCAGAAGAAGAAA	GG	CCATCTCATCCCTGCGTGTCTCcGGCCCTTCCTCTGTACTCTATAC	CCTCTCTATGGGCAGTCGGTGATgTGCCAGTGCCTCAAGAATGTC
EMX1(2)	GTCACCTCCAATGACTAGGG	GG	CCATCTCATCCCTGCGTGTCTCcCCAATGGGGAGGACATCGAT	CCTCTCTATGGGCAGTCGGTGATgTCCAGCTTGGGCCCAC
EMX1(3)	GGGCAACCACAAACCCACGA	GG	CCATCTCATCCCTGCGTGTCTCcCCAATGGGGAGGACATCGAT	CCTCTCTATGGGCAGTCGGTGATgTCCAGCTTGGGCCCAC
EMX1(4)	GCTTGTCCCTCTGTCAATGG	GG	CCATCTCATCCCTGCGTGTCTCcAACCCACGAGGGCAGAGT	CCTCTCTATGGGCAGTCGGTGATgGAGGAGAAGGCCAAGTGGTC
EMX1(5)	GCGCCACCGGTTGATGTGAT	GG	CCATCTCATCCCTGCGTGTCTCcCAAAGTACAAACGGCAGAAGC	CCTCTCTATGGGCAGTCGGTGATgGTTGCCCACCCTAGTCATTG
EMX1(6)	GACATCGATGTCCTCCCCAT	GG	CCATCTCATCCCTGCGTGTCTCcCAAAGTACAAACGGCAGAAGC	CCTCTCTATGGGCAGTCGGTGATgGTTGCCCACCCTAGTCATTG
EMX1(7)	GCCTCCCCAAAGCCTGGCCA	GG	CCATCTCATCCCTGCGTGTCTCcAACCCACGAGGGCAGAGT	CCTCTCTATGGGCAGTCGGTGATgGAGGAGAAGGCCAAGTGGTC
EMX1(8)	GCCCCGGGCTTCAAGCCCTG	GG	CCATCTCATCCCTGCGTGTCTCcAACCCACGAGGGCAGAGT	CCTCTCTATGGGCAGTCGGTGATgGAGGAGAAGGCCAAGTGGTC
EMX1(9)	GGCAGAGTGCTGCTTGCTGC	GG	CCATCTCATCCCTGCGTGTCTCcCCAATGGGGAGGACATCGAT	CCTCTCTATGGGCAGTCGGTGATgTCCAGCTTGGGCCCAC
VEGFA(2)	GCTAAAGAGGGAATGGGCTT	GG	CCATCTCATCCCTGCGTGTCTCcAAGCAACTCCAGTCCCAAAT	CCTCTCTATGGGCAGTCGGTGATgCCCTAGTGACTGCCGTCTG
VEGFA(3)	GTTTGGGAGGTCAGAAATAG	GG	CCATCTCATCCCTGCGTGTCTCcAAGCAACTCCAGTCCCAAAT	CCTCTCTATGGGCAGTCGGTGATgCCCTAGTGACTGCCGTCTG
VEGFA(4)	GTTGGAGCGGGGAGAAGGCC	GG	CCATCTCATCCCTGCGTGTCTCcGCGTCTTCGAGAGTGAGGAC	CCTCTCTATGGGCAGTCGGTGATgGGGGAGAGGGACACACAGAT
Chr8:48997789(1)	GTAGAGTGAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcATGATTAGAAACCTGCACTCCCAG	CCTCTCTATGGGCAGTCGGTGATgTAAGTGAATCTCTGTCTGTCTCAT
Chr6:9118779(1)	GAAGAATGGACAGAACTCTG	GG	CCATCTCATCCCTGCGTGTCTCcGGCCCTTCCTCTGTACTCTATAC	CCTCTCTATGGGCAGTCGGTGATgTGCCAGTGCCTCAAGAATGTC
DNMT1(1)	GATTCCTGGTGCCAGAAACAG	GG	CCATCTCATCCCTGCGTGTCTCcTGTTAAAAACACAACATCAGTGCAT	CCTCTCTATGGGCAGTCGGTGATgCGTGTTCCCCAGAGTGACTT
TPCN2(1)	GTGGGTGAGTGAGTGCGTGCG	GG	CCATCTCATCCCTGCGTGTCTCcCCTCCCACAGGAATTTGAAG	CCTCTCTATGGGCAGTCGGTGATgGCACCCCAACACCTACATCT
TPCN2(2)	GAGGCTGGGGTGGAGGTGTTG	GG	CCATCTCATCCCTGCGTGTCTCcCCTCCCACAGGAATTTGAAG	CCTCTCTATGGGCAGTCGGTGATgGCACCCCAACACCTACATCT
Chr14:106029015(1)	GGTGAGTGAGTGTGTGTGTG	GG	CCATCTCATCCCTGCGTGTCTCcAGGGACCCCTCTGACAGACT	CCTCTCTATGGGCAGTCGGTGATgCACACCCACACCCTCATACA
Chr14:106029024(1)	GTGTGTGTGTGAGGGTGTAAG	GG	CCATCTCATCCCTGCGTGTCTCcAGGGACCCCTCTGACAGACT	CCTCTCTATGGGCAGTCGGTGATgCACACCCACACCCTCATACA
NRG2(1)	GGGCAGTTTGCTCCTGGCAC	GG	CCATCTCATCCCTGCGTGTCTCcACATGCGATTCTGCAGGGAA	CCTCTCTATGGGCAGTCGGTGATgTCAGAGGGTGCTGTCTGTCT
CACNA2D4(1)	GGAGAGAGGCTCCCATCACG	GG	CCATCTCATCCCTGCGTGTCTCcACTTCTTGGGCAGTGATGGA	CCTCTCTATGGGCAGTCGGTGATgTACAAGGTGAGCCTGGGTCT
ADAMTSL1(1)	GAGAAGAGAAGTGGGGTGGGG	GG	CCATCTCATCCCTGCGTGTCTCcAGGACCCAGGTTTGCACT	CCTCTCTATGGGCAGTCGGTGATgTTCTGCCACTGGCTTAGCTT
EMX1(7) (SaCas9)	GGCCTCCCCAAAGCCTGGCCAg g GAGt		CCATCTCATCCCTGCGTGTCTCcAACCCACGAGGGCAGAGT	CCTCTCTATGGGCAGTCGGTGATgGAGGAGAAGGCCAAGTGGTC
OT1 (SaCas9)	GACCTCCCCATAGCCTGGCCAg g GAGg		CCATCTCATCCCTGCGTGTCTCcGAAGCTTTGAGGGGAATGTG	CCTCTCTATGGGCAGTCGGTGATgGTTGTTGCGATTGGTGGATT
OT2 (SaCas9)	GGCCTGCCCAAGGCCTGACCAa g GGAa		CCATCTCATCCCTGCGTGTCTCcGATGCTGCCCTTCTGACCTC	CCTCTCTATGGGCAGTCGGTGATgCTGGAAGAAGCTGGGGAAGA
OT3 (SaCas9)	GGCCT-CCCAAAGCCAGGCCAg g GGGa		CCATCTCATCCCTGCGTGTCTCcGGCCCTTGCTCTTCAGATTT	CCTCTCTATGGGCAGTCGGTGATgATTAGCATGGGGACGCTTTT

Table S3. BLESS DSB, similarity scores and genomic addresses

Target	chr	pos	sequence of homology	DSB	Similarity Score	Indel % (rep 1)	Indel % (rep 2)
WT SpCas9 *VEGFA*(1)	6	43737469	GGTGAGTGAGTGTGTGCGTG tGG	4.98	69	50.92	52.07
	22	37662823	GCTGAGTGAGTGTATGCGTG tGG	1.69	61	35.97	35.46
	5	115434674	TGTGGGTGAGTGTGTGCGTG aGG	1.64	61	44.20	40.93
	5	89440968	AGAGAGTGAGTGTGTGCATG aGG	1.51	58	29.44	34.31
	14	65569158	AGTGAGTGAGTGTGTGTGTG gGG	0.96	62	no data	no data
	14	106029030	GGTGAGTGAGTGTGTGTGTG aGG	0.55	65	30.59	28.61
	11	68851137	GGTGAGTGAGTGCGTGCGGG tGG	0.35	61	24.68	25.33
	20	20178283	AGTGTGTGAGTGTGTGCGTG tGG	0.33	62	20.58	18.90
	14	62078772	TGTGAGTAAGTGTGTGTGTG tGG	0.28	58	15.04	12.06
	2	177463424	GGTGAGTGTGTGTGTGCATG tGG	0.26	61	no data	no data
	10	98760587	GTTGAGTGAATGTGTGCGTG aGG	0.22	61	no data	no data
	19	6109031	GTGAGTGAGTGTGTGTGTGT gAG	0.20	56	no data	no data
	14	74353495	AGCGAGTGGGTGTGTGCGTG gGG	0.17	57	4.85	4.45
SpCas9 (K855A) *VEGFA*(1)	6	43737469	GGTGAGTGAGTGTGTGCGTG tGG	5.10	69	59.73	59.31
	22	37662823	GcTGAGTGAGTGTaTGCGTG tGG	0.68	61	14.73	10.99
	5	115434674	TGTGGGTGAGTGTGTGCGTG aGG	0.51	61	6.33	4.07
	5	89440968	AGAGAGTGAGTGTGTGCATG aGG	0	58	3.05	3.10
	14	65569158	AGTGAGTGAGTGTGTGTGTG gGG	0.81	62	no data	no data
	14	106029030	GGTGAGTGAGTGTGTGtGTG aGG	0.99	65	25.52	22.61
	11	68851137	GGTGAGTGAGTGCGTGCGGG tGG	0.00	61	2.47	1.98
	20	20178283	AGTGTGTGAGTGTGTGCGTG tGG	0.00	62	0.20	0.31

	14	62078772	TGTGAGTAAGTGTGTGTGTG tGG	0.00	58	0.09	0.05
	2	177463424	GGTGAGTGTGTGTGTGCATG tGG	0	61	no data	no data
	10	98760587	GTTGAGTGAATGTGTGCGTG aGG	0	61	no data	no data
	19	6109031	GTGAGTGAGTGTGTGTGTGT gAG	0	56	no data	no data
	14	74353495	AGCGAGTGGGTGTGTGCGTG gGG	0.00	57	0.13	0.03
eSpCas9(1.1) *VEGFA*(1)	6	43737469	GGTGAGTGAGTGTGTGCGTG tGG	5.88	69	58.18	59.37
	22	37662823	GCTGAGTGAGTGTATGCGTG tGG	0.00	61	0.00	0.13
	5	115434674	TGTGGGTGAGTGTGTGCGTG aGG	0.00	61	0.05	0.01
	5	89440968	AGAGAGTGAGTGTGTGCATG aGG	0	58	0.06	0.05
	14	65569158	AGTGAGTGAGTGTGTGTGTG gGG	0.91	62	no data	no data
	14	106029030	GGTGAGTGAGTGTGTGtGTG aGG	1.69	65	27.00	25.11
	11	68851137	GGTGAGTGAGTGCGTGCGGG tGG	0.00	61	0.28	0.41
	20	20178283	AGTGTGTGAGTGTGTGCGTG tGG	0.00	62	0.10	0.09
	14	62078772	TGTGAGTAAGTGTGTGTGTG tGG	0.00	58	0.00	0.00
	2	177463424	GGTGAGTGTGTGTGTGCATG tGG	0	61	no data	no data
	10	98760587	GTTGAGTGAATGTGTGCGTG aGG	0	61	no data	no data
	19	6109031	GTGAGTGAGTGTGTGTGTGT gAG	0	56	no data	no data
	14	74353495	AGCGAGTGGGTGTGTGCGTG gGG	0.00	57	0.00	0.04
WT SpCas9 *EMX1*(1)	2	73160997	GAGTCCGAGCAGAAGAAGAA gGG	6.13	69	63.56	60.46
	5	45359066	GAGTTAGAGCAGAAGAAGAA aGG	1.43	61	52.12	56.83
	15	44109762	GAGTCTAAGCAGAAGAAGAA gAG	0.84	61	30.19	26.75
	5	9227161	AAGTCTGAGCACAAGAAGAA tGG	0.20	57	4.24	4.66

	8	128801257	GAGTCCTAGCAGGAGAAGAA gAG	0.29	61	4.50	5.21
SpCas9 (K855A) *EMX1*(1)	2	73160997	GAGTCCGAGCAGAAGAAGAA gGG	12.85	69	59.30	56.47
	5	45359066	GAGTTAGAGCAGAAGAAGAA aGG	0.00	61	0.99	1.31
	15	44109762	GAGTCTAAGCAGAAGAAGAA gAG	0.00	61	0.68	1.23
	5	9227161	AAGTCTGAGCACAAGAAGAA tGG	0.00	57	0.00	0.11
	8	128801257	GAGTCCTAGCAGGAGAAGAA gAG	0.00	61	0.20	0.35
eSpCas9(1.1) *EMX1*(1)	2	73160997	GAGTCCGAGCAGAAGAAGAA gGG	13.77	69	52.47	49.36
	5	45359066	GAGTTAGAGCAGAAGAAGAA aGG	0.00	61	0.02	0.03
	15	44109762	GAGTCTAAGCAGAAGAAGAA gAG	0.00	61	0.14	0.00
	5	9227161	AAGTCTGAGCACAAGAAGAA tGG	0.00	57	0.00	0.24
	8	128801257	GAGTCCTAGCAGGAGAAGAA gAG	0.00	61	0.00	0.00

SUPPLEMENTARY SEQUENCES

Wild-type SpCas9

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