Supplementary Figure 1

Yeast screening for high-specificity SpCas9 variants

(a) Top panel: scheme of SpCas9 domains. The REC3 domain is part of the recognition lobe. BH: bridge helix. PI: PAM-interacting domain. Bottom panel: hybrid surface/ribbon structure (PDB ID: 4UN3) of SpCas9 in complex with sgRNA and target DNA. The REC3 alpha helical domain is highlighted in blue, while the rest of the structure is coloured in grey. RNA is represented in orange, DNA in red.

(b) Distribution and frequency of amino acid substitutions obtained from the yeast screening. Green bars indicate substitutions belonging to mutants which showed more than 75% of residual on-target activity and more than 3-fold increase in specificity compared to wild-type SpCas9, while grey bars indicate substitutions included in variants failing to meet these requirements. Red bars indicate the subset mutations belonging to highly active and specific variants that are also in close proximity to the target DNA:sgRNA duplex.
Supplementary Figure 2

Evaluation of R661 residue to improve on-target activity of the VNEL SpCas9 variant

(a) SpCas9 crystal structure (PDB ID: 4OO8) showing arginine 661 and its predicted interactions with the target DNA:guide RNA heteroduplex. While the R661L substitution likely introduces clash interactions (indicated by red disks) with the sgRNA backbone in all its rotamers (a representative rotamer is shown), the R661Q and R661S mutations are predicted to preserve one and two polar contacts with the guide, respectively, and simultaneously disrupt bonding interaction with the target DNA backbone. (b) 293multiEGFP were transfected with wild-type SpCas9, evoCas9 or the VNEL variant together with sgRNAs targeting different regions of the EGFP coding sequence. The loss of EGFP fluorescence (reported as mean percentage) was measured by FACS analysis 7 days post-transfection. (c) Editing of five genomic loci was evaluated in 293T cells after transfection with wild-type SpCas9, the VNEQ (evoCas9) variant or the VNEL variant together with the corresponding sgRNA. Indel formation (reported as mean percentage) was measured using the TIDE tool 7 days post-transfection. In panels (b,c) individual biologically independent samples are represented as overlaid circles.
Supplementary Figure 3

evoCas9 intracellular expression and titration of activity

(a) Representative western blot of lysates from 293T cells transfected with wild-type SpCas9, evoCas9 or the other high-fidelity variants, as indicated, together with the sgGFPon sgRNA. The graph below the blot reports the mean densitometric quantification of SpCas9 expression normalized on tubulin levels. Tubulin was used as a loading control. SpCas9 was detected using an anti-FLAG antibody. (b) Titration curve of evoCas9 activity in 293multiEGFP cells. Different amounts of evoCas9 or wild-type SpCas9 were transfected together with a fixed quantity of a sgRNA targeting the EGFP CDS (sgGFPon). Loss of EGFP fluorescence (reported as...
mean percentage) was measured by FACS at 7 days post-transfection. (c) Representative western blot of lysates from 293T cells transduced with lentiviral vectors expressing either wild-type SpCas9, evoCas9 or SpCas9-HF1, as indicated, and the sgGFPon sgRNA. The graph below the blot reports the mean densitometric quantification of SpCas9 expression normalized on actin levels. Actin was used as a loading control. SpCas9 was detected using an anti-FLAG antibody. Lysates were collected 20 days post-transduction and selection for stable SpCas9 expression. In all panels n=2 biologically independent samples (reported as overlaid circles).
Supplementary Figure 4

sgRNA requirements for evoCas9 activity

(a) The mean on-target cleavage activity of evoCas9, SpCas9-HF1 and eSpCas9(1.1) was compared to wild-type SpCas9 using truncated sgRNAs (17-19 nt) targeting EGFP; (b) a sgRNA targeting EGFP without a 5’-G (site20) and the same sgRNA containing a mismatched 5’-G nucleotide (site20+G). (c) evoCas9 editing activity in combination with sgRNAs targeting the EMX1-R and CXCR4 endogenous loci containing a 5’ mismatched guanine. (d) Evaluation of the effect of spacer length on evoCas9, SpCas9-HF1 and eSpCas9(1.1) editing activity. Mean EGFP knockdown was measured in the presence of sgRNAs with spacers spanning a length of 19-23 nt either containing a mismatched 5’-G or fully annealing with the target, as indicated. A schematic representation of the tested sgRNAs is reported in the panel above. Red squares indicate non-matching guanines; orange squares indicate extra matching nucleotides extending the spacer. (e) evoCas9, SpCas9-HF1 and eSpCas9(1.1) mean cleavage activity compared to wild-type SpCas9 in 293multiEGFP cells using sgRNAs characterized either by fully matching spacers starting with non-G nucleotides or with a mismatched 5’-G. (f) evoCas9 mean activity and specificity using optimized sgRNAs. evoCas9 together with wild-type SpCas9 and two previously published variants (SpCas9-HF1, eSpCas9(1.1)) were tested in an EGFP disruption assay using sgRNA with optimized scaffolds (extended stem-loop and base-flip) including the same spacers used in Fig. 2. For all the experiments loss of EGFP fluorescence was measured by FACS analysis at 7 days post transfection. For endogenous loci indel analysis cells were collected at 7 days post-transfection. Dashed lines indicate the background loss of EGFP fluorescence. Individual biologically independent samples are represented as overlaid circles.
Supplementary Figure 5

Evaluation of evoCas9 genome-wide specificity by GUIDE-seq (CCR5, CXCR4, FANCF2, HEK site4, EMX1, PD1)

GUIDE-seq profiles obtained with eight sgRNAs targeting the CCR5, CXCR4, FANCF2, HEK site4, EMX1, PD1 genomic loci in combination with wild-type SpCas9, SpCas9-HF1, eSpCas9(1.1) and evoCas9. GUIDE-seq read counts for the different SpCas9 are reported on the right of the sequences. (-) indicates that the off-target was not detected in the corresponding sample. Mismatched positions are indicated with colored boxes. Black squares indicate on-target sites. DNA from three biological replicates was mixed before library preparation. Additional information on the identified off-targets is reported in Supplementary Data 1-2.
Supplementary Figure 6

Evaluation of evoCas9 genome-wide specificity by GUIDE-seq (VEGFA2, VEGFA3)

GUIDE-seq profiles obtained with eight sgRNAs targeting the VEGFA2 and VEGFA3 genomic loci in combination with wild-type SpCas9, SpCas9-HF1, eSpCas9(1.1) and evoCas9. GUIDE-seq read counts for the different SpCas9 are reported on the right of the sequences. (-) indicates that the off-target was not detected in the corresponding sample. For the VEGFA2 sgRNA the off-target list is limited to the sites with higher reads than the on-target obtained with wild-type SpCas9 and all the sites detected with evoCas9, SpCas9-HF1 and eSpCas9(1.1). Mismatched positions are indicated with colored boxes. Black squares indicate on-target sites. DNA from three biological replicates was mixed before library preparation. Additional information on the identified off-targets is reported in Supplementary Data 1-2.
GUIDE-seq on-target specificities of high-fidelity SpCas9 variants

Cleavage specificity, expressed by the percentage of on-target reads captured by GUIDE-seq (from Fig. 3a and Supplementary Fig. 5-6) using wild-type SpCas9, SpCas9-HF1, eSpCas9(1.1) and evoCas9, in combination with sgRNAs targeting the VEGFA2, VEGFA3, EMX1, CCR5, CXCR4, FANCF2, PD1 and HEK site4 loci.
Evaluation of evoCas9 specificity by targeted deep-sequencing

(a) On/off-target ratios for the nine common off-targets associated to the VEGFA2 locus calculated from the data in Fig. 3g and reported for wild-type SpCas9 and each different high-fidelity variant, as indicated in the graph. The bars indicate the interval between the values of the ratios obtained from the two replicate samples. Indel analysis of previously validated off-target sites relative to the EMX1 locus (b) and the VEGFA3 locus (c) performed by targeted deep-sequencing on genomic DNA of 293T cells expressing wild-type SpCas9, SpCas9-HF1, eSpCas9(1.1) or evoCas9 together with each specific sgRNA. Cells not expressing SpCas9 were sequenced to determine background indel levels. Two independent experiments were performed by mixing genomic DNA from three biological replicates before library preparation. The shaded circles represent the two measured editing percentages, while the solid circle indicates the mean.
Supplementary Figure 9

Side-by-side comparison of evoCas9, SpCas9-HF1 and eSpCas9(1.1) specificity on selected therapeutically relevant genes

293T cells were transfected with wild-type SpCas9, SpCas9-HF1, eSpCas9(1.1) or evoCas9 together with sgRNAs targeting the FANCF2 (a) or the CCR5 loci (b). Mean indel formation at the on-targets and at two previously validated off-target sites (one for each locus) was evaluated 7 days post-transfection using the TIDE tool. The sequences of the on- and off-target sites for each locus are reported above the corresponding graphs; the red square indicates the mismatched base. (c) On/off ratios calculated from the mean indel percentages obtained in (a). The dotted lines indicate evoCas9 fold increase in specificity. (d) Schematic representation of the CCR5 locus and its off-target site in the highly homologous CCR2 gene. Simultaneous cleavage of the two sites generates a chromosomal deletion of approximately 16 kb. Semi-quantitative PCR was performed on genomic DNA of 293T cells transfected with wild-type SpCas9, SpCas9-HF1, eSpCas9(1.1) or evoCas9 and the CCR5 sgRNA to assess the amount of chromosomal deletion.
generated in each condition. The FANCF locus was used as an internal normalizer. The amount of deletion was quantified using densitometry with ImageJ. The numbers above the dotted line indicates evoCas9 fold increase in specificity relative to the connected histogram bars. For all panels n=2 biologically independent samples (represented as overlaid circles).
Supplementary Figure 10

Comparison of evoCas9 and HypaCas9 specificity by meta-analysis

Meta-analysis of GUIDE-seq experimental data relative to evoCas9 and HypaCas9 (from Chen et al., Nature 2017). (a) Total number of off-target sites detected by GUIDE-seq for the VEGFA2, VEGFA3 and FANCF2 sgRNAs in combination with evoCas9 and HypaCas9. (b) Radar plot reporting the global distribution of the detected off-target sites for evoCas9 and HypaCas9 according to their number of mismatches (1 to 5). (c) Venn diagram showing the intersection among all the off-targets identified with evoCas9 and HypaCas9. On-target cleavage specificity, measured as the percentage of GUIDE-seq reads captured by the on-target site for the corresponding locus, reported for each tested sgRNA (as indicated in the graphs, d) or for the whole dataset (e) for evoCas9 and HypaCas9. (f) On/off-target ratios calculated from the GUIDE-seq reads obtained for the 9 off-target sites common to evoCas9 and HypaCas9. Statistical significance was assessed using the two-sided Wilcoxon rank sum test, sample size n=9. Summary of data distributions and statistical details are reported in Supplementary Table 5. ns, not significant.
Supplementary Figure 11

evo-dCas9 transcriptional activation

(a) Schematic representation of the Tet Responsive Element (TRE)-EGFP based transcriptional activator reporter. Upon binding of dCas9-VP64 to TetO repeats EGFP expression is activated. (b) EGFP activation was measured in 293T cells transfected with dCas9 or evo-dCas9 based transcriptional activators together with matching sgRNAs (both with or without a 5' mismatched G) or mismatched sgRNA, as indicated. TetO-off6 contains a mismatch in position 6 from the PAM, TetO-off1314 contains two mismatches in positions 13-14 and TetO-off1819 contains two mismatches in positions 18-19. n=2 biologically independent samples are represented as overlaid circles. EGFP expression was measured by FACS analysis at 2 days post-transfection. (c) Fold activation of EGFP expression with respect to the non-targeting control calculated from the data in (b). Individual values are reported as overlaid circles.
**Supplementary Figure 12**

Reproducibility of wild-type SpCas9 GUIDE-seq experiments across independent studies

Scatter plots reporting the relative abundance of each common off-target site in corresponding datasets obtained for different tested sgRNAs, as indicated. Boxed numbers indicate the Pearson’s correlation coefficient for the corresponding pair of experiments and the associated p-value (paired two-sided Wilcoxon signed-rank test). Sample sizes, corresponding to common off-target sites: VEGFA2=97, VEGFA3=12, EMX1=9, FANCF2=11. The Venn diagrams show the intersection among the datasets of the off-target sites detected for each tested sgRNA in association to wild-type SpCas9, as indicated. Previously published datasets included in these analyses are Tsai et al.9, Kleinstiver et al.1 and Chen et al.10. R1 and R2 indicate two replicate datasets generated in the present study for the VEGFA3 site in combination with wild-type SpCas9.
Supplementary Notes

**In vivo yeast screening for high-specificity SpCas9 variants**

To generate the reporter yeast strains (yACMO-off1/-off4) we modified the *TRP1* (chromosome IV) and *ADE2* (chromosome XV) genomic loci. An EGFP-derived on-target sequence (**Supplementary Table 2**) for Cas9 was introduced in the *TRP1* gene, while similar sequences containing single mismatches (off-targets, **Supplementary Table 2**) were incorporated into the *ADE2* locus. A stop codon was added immediately after each on/off-target site to ensure premature interruption of translation, while a 100bp duplication was positioned on both sides to favour homology-driven scarless repair after cleavage of the target sequence. The knockout of the two genes by reporter cassette insertion produces defects in the tryptophan and adenine metabolic pathways. This metabolic alteration leads to growth arrest in the absence of tryptophan and to the accumulation of a red pigment in low adenine medium leading to the formation of red colonies. Following double strand breaks induced by Cas9, each locus can be efficiently repaired by single strand annealing favoured by the two homology regions, thus restoring prototrophy for the two nutrients (corresponding to cell growth and the generation of white colonies, for *TRP1* and *ADE2* targeting, respectively) ([Fig. 1a](#)). Therefore, the successful editing event at each of the two loci can be visualized using appropriate reporter plates: no tryptophan and low adenine concentrations (SDluta₅ plates). The assay readout consists in a two-step process: first, on-target cleavage efficiency can be measured by dividing the total number of colonies (red + white) obtained on reporter plates without tryptophan and with low adenine (SDluta₅ plates) over those recovered on plates selecting for total transformants incorporating Cas9 and sgRNA plasmids (SDlu plates); second, on- vs. off-target activity can be evaluated by counting the number of red (*TRP1⁺/ade2⁺*) and white (*TRP1⁺/ADE2⁺*, corresponding to off-target cleavages) colonies in the same reporter plates.
To perform the screening, the yACMO-off4 reporter strain was stably transformed with a plasmid coding for a sgRNA perfectly matching the on-target site (TRP1 locus). The off-target sequence (off4) located in the ADE2 locus is characterized by a single mismatch with the sgRNA spacer in position 15 from the PAM. The library of mutated SpCas9 molecules was generated by co-transformation of PCR-mutated fragments deriving from the open reading frame of the REC3 domain (Supplementary Figure 1a) and a plasmid encoding a galactose-inducible REC3-deleted SpCas9. The mutagenized REC3 fragments and the REC3-deleted SpCas9 plasmid reconstitute in vivo the full-length SpCas9 open reading frame by homologous recombination. The transformed colonies containing the new nuclease variants were recovered after an overnight incubation in non-selective medium followed by SpCas9 induction and plating on several reporter plates. We employed a short induction time (5 hours) to select against variants with poor on-target activity which might emerge with longer induction periods (>5 hours). Red colonies were streaked on reporter plates containing galactose to maintain SpCas9 constantly expressed and exacerbate any off-target cleavage. After 48 hours, the DNA coding for the selected SpCas9 variants was recovered from the most red-pigmented streaks and subsequently used for re-challenging in yACMO-off4, in order to eliminate false positive clones containing non-sense mutations or frameshifts (see schematics in Fig. 1c). In addition, this allowed to measure more precisely the cleavage activity of each variant, discard those less catalytically active compared to wild-type SpCas9 and rank the remaining ones according to on-target cleavage efficiency and their ability to discriminate off-targets (Fig. 1d).

**Effect of extra 5′ guanines in spacers on evoCas9 and evo-dCas9-VP64 activity**

It has been reported that high-fidelity SpCas9 variants are incompatible with the addition of a mismatched guanine at the 5′-end of guide RNA spacers to favor transcription from the Pol III1,2. We thus evaluated evoCas9 activity in these experimental conditions by using
sgRNAs containing 5’ mismatched guanines as well as guides characterized by longer spacers (Supplementary Fig. 4b-d). We observed complete abrogation of cleavage activity in all the experimental conditions where the spacers did not correspond to a canonical 20 bp matching sequence, including those where fully matching spacers longer than 20 nucleotides were employed. Interestingly, the same drop in on-target activity was measured also for a spacer containing 19 matching nucleotides and a mismatched G in the 20th PAM-distal position. This feature may reduce the total number of targetable sites, since spacer sequences natively starting with a guanine can exclusively be used. However, this limitation can be circumvented by using other methods for guide RNAs synthesis (e.g. chemical synthesis and electroporation or expression through tRNA-flanked sgRNA\textsuperscript{3,4}), or by designing sgRNAs starting with cytidine or adenine instead of guanine, which are compatible with U6-driven transcription systems, as demonstrated by our (Supplementary Fig. 4e) and others’ results\textsuperscript{1}.

In light of the current model where mismatches located in more PAM-distal positions are more easily tolerated by SpCas9, this result is unexpected since a mismatch in position 18 of the guide cannot be completely discriminated by evoCas9 (Fig. 2b). This, together with the complete loss of activity observed with spacer sequences longer than 20 bp and containing or not mismatched 5’-Gs, indicates that evoCas9 does not tolerate alterations of the RNA:DNA heteroduplex in positions that are structurally located at the end of the cavity that harbours the duplex itself. In addition, it has been consistently reported that the inclusion of two extra guanines at the 5’-terminus of sgRNAs increases targeting specificity\textsuperscript{5,6}. This effect, still lacking a functional explanation, is possibly justified by the behaviour we observed with our mutant.

Additional data on the effect of 5’ mismatched guanines were obtained using catalytically inactive SpCas9-based transcriptional activators (dCas9-VP64\textsuperscript{7}, Supplementary Fig. 11). This experimental system can be considered a good indicator of the strength of SpCas9
binding to the target DNA, as transcriptional activation will be proportional to the time spent by the activator on the target promoter. Interestingly, we did not observe any important difference in EGFP transcriptional activation when comparing samples transfected with evo-dCas9-VP64 together with the on-target TetO-on guide RNA or the same on-target guide with an added extra 5'-G (Supplementary Fig. 11). This result, together with previous data showing a consistent loss of editing activity when using sgRNA with an additional initial G (Supplementary Fig. 4b-e), suggests that evoCas9 is indeed able to bind target sites using sgRNAs characterized by a mismatched extra 5’-guanine, but is then unable to cut the bound DNA. Accordingly, when we compared the EGFP fold-activation obtained using mismatched guides, the increased specificity observed using evo-dCas9-VP64 was modest when compared with the original dCas9-VP64, further reinforcing the idea that evoCas9 binds to mismatched targets, even though less efficiently, but is then unable to complete the cleavage reaction (Supplementary Fig. 11b-c). Finally, the lower background activation observed in the presence of our high-specificity variant might be due to a lower propensity of evo-Cas9 to bind stably DNA (Supplementary Fig. 11b-c).

Supplementary References


