Developing small Cas9 hybrids using molecular modeling

Antoine Mangin[1], Vincent Dion[1*] , and Georgina Menzies[2*]

Affiliations:

1: UK Dementia Research Institute at Cardiff University, Cardiff CF24 4HQ, UK

2: School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK

*Corresponding authors: dionv@cardiff.ac.uk, menziesg@cardiff.ac.uk

Keywords: Cas9, molecular dynamics, gene editing.

Abstract:

The RNA-guided CRISPR-Cas9 from *Streptococcus pyogenes* is the best characterized enzyme for gene editing. Its large size, however, precludes it from being packaged together with its single guide (sg)RNA into a single adeno-associated virus, limiting *in vivo* applications. Here, we developed smaller Cas9 hybrids, made of the PAM interacting domain (PID) of *S pyogenes* and the catalytic domains of the smaller Cas9 orthologues, as well as sgRNA cognate hybrids. Molecular modeling revealed that the presence of a sgRNA stabilizes Cas9. Making the D10A mutation to turn Cas9 into a nickase dramatically alters its binding energy to the sgRNA, showing that the approach can identify functionally relevant changes. However, we found that the four Cas9/sgRNA hybrid pairs tested in human cells failed to edit target sequences. We conclude that *in silico* approaches can identify functional changes caused by point mutations but they may not be well suited for designing Cas9/sgRNA hybrids.

Introduction:

The clustered regularly interspaced short palindromic repeats (CRISPR) Cas system evolved as an immunity mechanism against phage invasion[1,2] . But once Cas9 from *Streptococcus pyogenes* was found to be a programmable RNA-guided nuclease creating DNA double-strand breaks, gene editing at high efficiency in mammalian cells became possible[3,4]. Type II CRISPR/Cas complexes are formed of a Cas9 nuclease guided to a target sequence using a CRISPR (cr)RNA. This contains a customizable sequence (~20 nucleotides), and a trans-activating crRNA (tracrRNA) formed of one or more stem loops and are unique to each Cas9 orthologue[5]. The two RNAs can be fused together to produce a convenient single guide (sg)RNA[3]. The sgRNA-Cas9 complex recognizes its complementary sequence in genomic DNA using base pairing when the target sequence is followed by a short (3 to 8 bp), species-specific, protospacer adjacent motif (PAM). Upon binding, the HNH and RuvCI domains of Cas9 are activated and induce a DNA double-strand break, with the HNH cutting the complementary DNA strand while the RuvCI cleaves the noncomplementary strand[3]. The double-strand break is repaired by the cell's endogenous machinery, leading to insertions and deletions[4,6]. Homologous recombination can also occur, provided that a template for repair is present[4,6].

Due to its high cutting efficiency in mammalian cells, the Cas9 enzyme from *Streptococcus pyogenes* (Sp) is the most widely studied of the Cas9 orthologues[4,7], and has been used in the clinic[8]. SpCas9 is most efficient when the PAM sequence is composed of 5'-NGG (with N being any nucleotide), but also tolerates 5'-NAGs and 5'-NTGs[9,10]. This ability to accommodate mismatches between the target sequence and the sgRNA means that some off-target sequences are more accessible and unwanted mutations can occur[11,12]. This has led to a flurry of studies aiming to modify Cas9 to make it more specific or to change the compatibility of its PAM sequence. These studies used a variety of methods, including structure-based rational design[13–18] and screening[19–21]. Luan et al[22], for example, used molecular dynamics simulations and free energy perturbation to shorten the PAM recognition sequence of *Staphylococcus aureus* (Sa) Cas9. They found that free energy perturbation correlated well with gene editing efficiency in human cells. These results suggest that molecular dynamics simulations can assist the design of Cas9 variants quickly and cheaply.

Here we tested whether a similar approach could be used to design larger hybrids with extensive interacting interfaces. Indeed, whilst several variants with a single or a few amino acid changes have been used on the SpCas9 to change in the PAM preference of Cas9[17–19] or improve on-target accuracy[13–16,20,21], it has not yet been possible to reduce the size of the Cas9 enzyme. This is desirable because, SpCas9, with its 1368 amino acids, is too large to fit into an adeno-associated viral (AAV) vector with its sgRNA for *in vivo* delivery. AAVs are one of best delivery systems because they display

low immunoreactivity, have a wide tropism, and their viral DNA does not integrate easily within the host genome[23–25]. The size of SpCas9 means that two AAVs are required, one encoding the Cas9 enzyme, the other the sgRNA, resulting in increased cost, reduced efficiency, and an enhanced risk of an adverse immune reaction. To get around this problem, smaller Cas9 orthologues, like SaCas9 or *Campylobacter Jejuni* (Cj)Cas9, have been used in animal studies as they are small enough to be packaged into a single AAV with their sgRNAs[26–28]. However, they are less efficient than the SpCas9 and use different PAM sequences. SaCas9 recognizes NNGRRT (where R is a purine) and CjCas9 recognizes NNNNACA[28,29]. This means that most sequences targeted by SpCas9 are inaccessible to these smaller orthologues.

To overcome these issues, we aimed to develop new compact Cas9 hybrids by replacing the PAM interacting Domain (PID) of SaCas9 and CjCas9 with that of SpCas9. Because sgRNAs are species-specific[30], we also had to develop new sgRNA hybrids and ensure they interacted with the Cas9 hybrids protein. We used a two-pronged approach, taking advantage of *in silico* modeling to select four sgRNA/Cas9 pairs to test in human cells. We found that small point mutations or the presence of the sgRNA can drastically change the binding energy and the stability of the system, respectively. However, we found that the *in silico* approach did not predict the editing efficiency of the designed Cas9/sgRNA hybrid pairs in human cells, suggesting that molecular modelling and binding energy may not be applicable to large chimeric systems.

Materials and methods:

Data and software Availability

Molecular dynamics simulations datasets are available here: https://github.com/DionLab/Cas9-hybrids, and as a version controlled here: 10.5281/zenodo.10029983.

In silico models

The crystal structures required to run molecular dynamics were obtained from https://www.rcsb.org. PDB ID:4OO8 was used for SpCas9 in complex with its sgRNA and target DNA, this structure is at a 2.5Å resolution[5]. PDB ID:5CZZ was used for SaCas9 in complex with its sgRNA and target DNA this structure is at a 2.6Å resolution[29]. We note that the SaCas9 structure lacked stem loop 2 of the sgRNA. PDB:5X2G was used for the CjCas9 in complex with its sgRNA and a target DNA which was available at a 2.4Å resolution[31]. In this structure, the CjCas9 protein lacked the HNH domain, which was replaced with a GGGSGG linker. The SaSpCas9 hybrid is the hybrid between the N-terminal of SaCas9 (1-910AA) and the PID of SpCas9 (1100-1368AA). Similarly, the CjSp Cas9 hybrid is a fusion between the N-terminal of the CjCas9 (1-828AA) and the same Sp PID. To make the nickase mutations, we used the D10A

mutation for the SaSp Cas9 and the D8A for the CjSpCas9 (Supplementary Table 1), and created the change using the protein editing function in Molecular Operating Environment (MOE) 2019. Cas9 hybrids and sgRNA hybrids (Supplementary Table 1 and 2) were made using MOE 2019 and Pymol v2.5.4. An energy minimization was run on MOE to avoid any clashes between the added PID and rest of the protein prior to molecular dynamics simulations.

Molecular dynamics simulation:

All the preparation steps and analysis for molecular dynamics simulation were carried out using GROMACS version 2018.2[32]. The forcefield AMBER03 was employed to create a stable environment for the system[33]. The protein-RNA-DNA complex was set in a 0.9 nm box filled with TIP3 water molecules. The system was neutralized either by adding Na+ or Cl- ions depending on the overall charge. An integration time step of 0.001 ps was used. The complex was energy minimized to find the best position for the protein to avoid any clashes between the different structures. The energy minimization was run for a maximum of 3000 steps and a maximum energy barrier of 1000 kJ/mol/nm. During the molecular dynamics simulation, the v-rescale coupling method regulated the temperature to 310 K. A Berendsen barostat was coupled to the system to regulate the pressure with a reference of 1 bar. The whole simulation was composed of 5 different groups: protein, ions, solution, DNA, and RNA. The molecular dynamics simulation was run for a total of 100 ns.

Stability data analysis:

The stability of every complex was studied by analyzing the Root Mean Square Deviation (RMSD) and the Root Mean Square Fluctuation (RMSF). Each of these are modules built-in GROMACS. The RMSD was extracted for every structure of the complex (protein-RNA-DNA) using the rms command and given every 10 ps. An average of the RMSD was made only including data from 10 to 100 ns as the first nanoseconds serve to warm up the system. The RMSF was extracted for every residue of every structure present in the complex using the rmsf command.

Binding energy:

The Molecular Mechanics Poisson-Boltzmann surface area (MMPBSA) method was used to precisely determine the interaction between each base of the sgRNA hybrid and the protein hybrid. Recently, a GROMACS integrated tool was developed to perform these calculations[34]. The files and instructions needed to run it were found on: https://rashmikumari.github.io/g_mmpbsa/. This method combines calculations of potential energy in a vacuum, polar solvation energy, and non-polar solvation energy. The binding energy with g MMPBSA, even if less computationally hungry than other methods, remains time-consuming. To reduce the computational burden and make the analysis possible, the last 100

frames of the molecular dynamics simulations were extracted and used to calculate the binding energy between the sgRNA and the protein.

Cell lines plasmids and culture conditions:

HEK293-derived GFP$(CAG)_0$ and GFP$(CAG)_{101}$ cells were a gift from John H. Wilson[35]. They were maintained at 37°C with 5% CO_2 in DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 15 µg ml^{-1} blasticidin, 150 µg ml^{-1} hygromycin, and, during the experiments, 1 µg ml^{-1} of doxycycline diluted in water. Both lines were regularly tested for mycoplasma using a service from Eurofins. The cells remained mycoplasma-free throughout the experiments. They were also genotyped using the Mycrosynth AG service and were determined to be HEK293.2sus as previously determined[36].

Transfections were done as before[37] by seeding 400,000 cells in a 12-well plate well on day 0 and transfecting 1 µg of plasmid using lipofectamine 2000. GFP intensities were measured using an Attune NxT flow cytometer and analyzed using the FlowJo software version 10.8.1. The plasmids used in this study (Supplementary table 3) will be available via Addgene shortly.

For the GFP editing assays, we used GFP$(CAG)_0$ cells along with 4 well-characterized sgRNAs against GFP[38] (Supplementary Table 4). The GFP levels in the cells were measured at day 6, 8, and 10 post-transfection. The flow cytometry data were analyzed using FlowJo v.10.8.1

The results were confirmed using a T7 endonuclease I assay using GFP$(CAG)_0$ cells transfected with GFP sgRNAs target 1 or 3 (Supplementary Table 4). The targeted regions were amplified using the primers oVIN-3474 and oVIN-3475 for sgRNA 1, 3 and 4 (Supplementary Table 5). The target region for sgRNA 2 was amplified using oVIN-3476 and oVIN-3477 (Supplementary Table 5). The PCR protocol used was 95°C for 5min, followed by 35 cycles at 95°C for 30'', 55°C for 30'' and 72°C for 1 min followed by a final step at 72°C for 10 min. The annealing of PCR products was performed by mixing 200 ng of DNA in 1x NEBuffer2. After annealing, 10 U of T7 endonuclease I (NEB) was added and incubated for 15 min at 37°C. For repeat instability assays using the GFP reporter, GFP$(CAG)_{101}$ cells were cultured as above, but with dialyzed FBS (Merck). The cells were transfected on days 0, 4, and 8 before being analyzed for GFP intensity by flow cytometry on day 12. The resulting datasets were analyzed using FlowJo v.10.8.1 The plasmids used for transfection can be found in Supplementary Table 2.

Protein quantification and western blot:

Proteins were extracted using commercial RIPA buffer (Fisher Scientific) and quantified using Pierce BCA protein assay (ThermoFisher Scientific). The proteins were separated on a 4-12% Bis tris gel (ThermoFisher Scientific) and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane

was blocked for 1 h in 5% milk in PBS-T then incubated overnight with an anti-flag antibody (Sigma) or actin antibody (Sigma) at 4°C. The secondary blotting was performed after 1 h incubation in an Alexa Fluor 680 anti-mouse antibody (Invitrogen). The blot was imaged using Licor Odyssey CLX.

Statistics

Two-tailed Mann-Whitney U tests were performed to determine statistical significance between the RMSD values of the hybrids and their relevant wild type Cas9 orthologues. We used Graphpad Prism (version 10.0.0) to calculate the p-values.

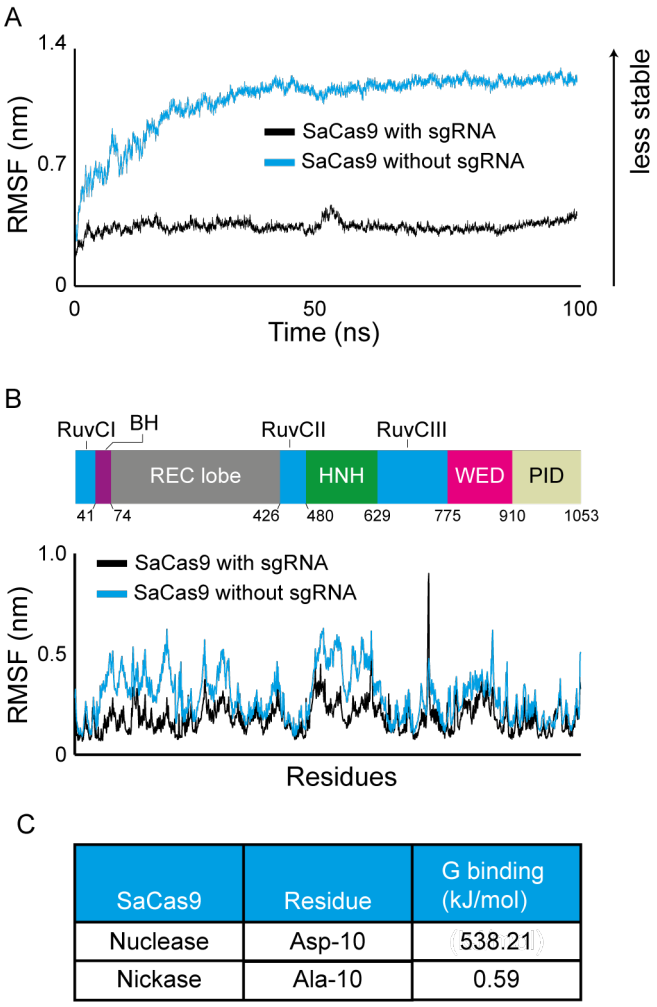
Results:

Molecular dynamics and binding energy identify functional changes in Cas9

To test whether our *in silico* approach could identify meaningful functional changes, we ran molecular dynamics simulations of the SaCas9 complex with and without its sgRNA. We reasoned that the presence of the sgRNA would have a large impact on the dynamics of the Cas9 enzyme. RMSD and RMSF values were extracted from the simulations. RMSD is a measure of the average distance for a group of atoms between a reference structure and the resulting structure after a specified amount of time. The RMSF relates to the movement of each individual residue over the time of the simulation. The SaCas9 without its sgRNA reached a maximum RMSD of 1.22 nm but remained stable beyond 40 ns (Figure 1A), with an overall RMSD average of 1.1 nm. In contrast, the SaCas9 in complex with its sgRNA was more stable with an average RMSD of 0.33 nm, and a maximum of 0.37 nm (Figure 1A). Consistent with these data, the RMSF analysis showed that every domain of the protein is stabilized by the presence of its sgRNA (Figure 1B). The REC lobe, the bridge helix, RuvCI, and HNH domains, were the most impacted. We conclude that changes in RMSD and RMSF values reflect large scale changes in SaCas9.

To determine whether we could find functional differences using binding energy measurements, we compared SaCas9 to the D10A mutant, which turns the nuclease into a nickase [3]. To do so, we used the MMPBSA approach[34,39,40], which works by calculating the overall energy for Cas9 in solution compared to that of Cas9 in a vacuum. The same is done for the sgRNA. From these data, we can calculate the energy difference between Cas9 and its sgRNA in solution as well as in a vacuum, which gives us an approximation of the binding affinity between the two molecules for each amino-acid (Supplementary Figure 1A). We reasoned that the binding energy for the mutated residue should change if this method is predictive of a change in function. Moreover, the rest of the protein should change very little, if at all, by this amino acid change. To test this directly, we calculated the binding

energy associated with every residue extracted from the SaCas9 simulation and compared them to that of the SaCas9 D10A variant. We found that the G binding of D10 was 912 times greater than that of A10 (Figure 1C). This is in contrast to the other residues, which on average changed by 0.39 kJ/mol (Supplementary Figure 1B). Together, these analyses show that the molecular dynamics simulation together with the binding energy calculations robustly predicts functional changes.



SaCas9	Residue	G binding (kJ/mol)
Nuclease	Asp-10	538.21
Nickase	Ala-10	0.59

Figure 1: Molecular dynamics simulations and energy binding calculation identify functionally relevant changes in Cas9 activity. A) Root mean square deviation (RMSD) of SaCas9 with or without its sgRNA over 100 ns simulations. B) Root mean square fluctuation (RMSF) of SaCas9 with or without its sgRNA. (BH = bridge helix, WED = wedge domain, PID = PAM interacting domain). C) G binding values at position 10 of SaCas9 comparing the nuclease (D10) to the nickase (A10).

The Cas9 hybrid complexes are stable in silico

Next we tested whether we could use this computational approach to design multiple compact Cas9/sgRNA hybrid pairs to be tested in human cells. This was done in two steps. First, we performed

molecular dynamics simulations and determined the RMSD and RMSF of the hybrid pairs and then determined which ones to take forward using energy binding calculations. We initially designed 9 such pairs *in silico*, 7 SaSp pairs and 2 CjSp hybrid pairs (Figure 2A-B). For all these, as well as for the wild type versions of SpCas9, SaCas9, and CjCas9, we performed molecular dynamics simulations and analyzed the stability of these hybrids (Figure 2A-J and supplementary figure 2A-B). We found that in the presence of a sgRNA the RMSD for the SaSpCas9 or CjSpCas9, regardless of the sgRNA hybrid used, was systematically higher than the SaCas9, SpCas9 or CjCas9 coupled to their wild type sgRNAs (Figure 2C,D – P value < 0.0001). However, for both hybrids, a plateau was reached, showing that the proteins arrived at a stable conformation.

When comparing the average protein RMSD for the 7 SaSp models, we found that Large Sam (LSam) showed the highest value with a maximum of 0.93 nm whereas Pippin had the lowest value with 0.60 nm (Figure 2E). For the CjSp, Rosy had the lowest value with 0.73 nm. The same protein hybrids showed different RMSD values (P<0.0001) presumably, because of how they interacted with their respective sgRNAs. Analysis of the RNA RMSD showed that Pippin had the highest value with 1.37 nm whereas the lowest values were seen with Merry (0.40 nm, Figure 2F). For the CjSp hybrids, again, Rosy had the lowest value with 0.33 nm. The DNA RMSD did not vary significantly between the SaSp systems (P<0.0001) and ranged between 0.36 nm (Msam) and 0.41 nm (Merry; Figure 2G). For the CjSp, Rosy had the lowest value of 0.52 nm. Altogether, the RMSD data show that the hybrids had consistently higher values than the three wild types, yet they were below what we observed for SaCas9 without the presence of a sgRNA (Figure 1).

To complement these findings, we determined the RMSF values of each residue in the proteins and sgRNAs for each hybrid pair. We found that the N-terminal of the SaSpCas9 coupled to Merry (Figure 2H) and to Gollum (Supplementary Figure 2A) matched closely the wild type SaCas9 coupled to its sgRNA, with the HNH domain being the most fluctuating domain for both proteins. The PID of the SaSp hybrids (Figure 2I and Supplementary Figure 2A) were more mobile than the Sp wild type pair, especially for some atoms around residue 1200. This may be explained by the energy minimization step that moved the initial position of these residues away from other atoms, giving it more space to move. The RMSF for SaSp Merry sgRNA was closest to that of the Sp and Sa sgRNAs (Figure 2J) with the exception of stem loop 2 from Sp wild type showing more fluctuations. Similar to the SaSpCas9 hybrids, we found that the CjSpCas9 hybrids were consistently more mobile than the wild types (Supplementary Figure 2B). Altogether, the RMSD and RMSF data suggest that the Cas9 hybrids that we designed are stable during molecular dynamics simulations.

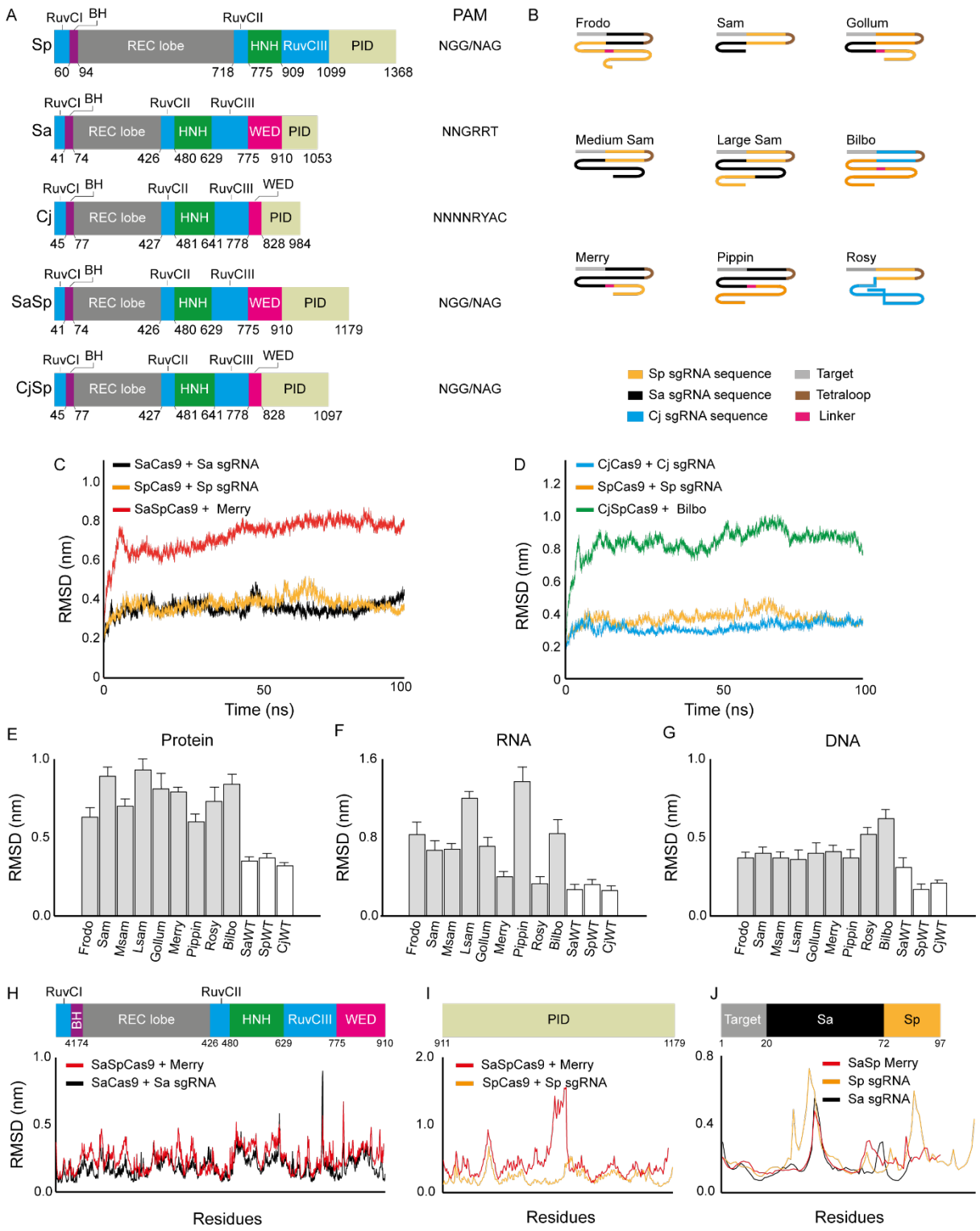


Figure 2: Molecular dynamics simulations of Cas9 hybrids. A) Domain organization of the Sp, Sa, and Cj Cas9 as well as the hybrids tested here. The PAM sequence is either the reported ones or, for the hybrids, the expected PAM. The numbers indicated the amino acids at domain boundaries (BH = bridge helix, WED = wedge domain, PID = PAM interacting domain). B) Schematic representation of the sgRNA hybrids tested here. C) RMSD of SpCas9, SaCas9, and SaSpCas9 with their specific sgRNAs during 100 ns. The SaSp sgRNA presented is Merry. D) RMSD of SpCas9, CjCas9, and CjSpCas9 with their specific sgRNAs over a simulation of 100 ns. The CjSp sgRNA presented is Bilbo. E-G) Average RMSD for the protein (E), sgRNA (F), and DNA (G) in all 12 systems modeled. H) RMSF of SaCas9 with its sgRNA (black) and SaSp Cas9 in combination with Merry (red) for the N-terminal

common to both proteins. I) RMSF of the PID of SpCas9 with its sgRNA (yellow) and SaSpCas9 in combination with Merry (red). J) RMSF of SpCas9 sgRNA (yellow), SaCas9 sgRNA (black) and SaSpCas9 sgRNA (red).

Binding energy of the Cas9/sgRNA hybrids closely match that of wild type complexes

To determine which hybrid to test in cellular systems, we analyzed the binding energy of every SaSp hybrid pair. The analysis of the SaCas9 complex could readily identify nearly all residues previously shown to interact with the sgRNA in the crystal structure[29], as well as additional ones (Figure 3A and Supplementary Figure 3A). This added confidence that the residues with the smallest binding energy were indeed structurally important. Comparing the binding energy in the residues interacting with the sgRNA, we found that some SaSp sgRNAs, especially, Merry and Gollum, showed very similar binding energy compared to the SaCas9 and its sgRNA (Figure 3A and Supplementary Figure 3A). By contrast, Frodo showed more extreme values (Figure 3B). Similarly, the CjSp complexes Rosy and Bilbo showed similar values as the CjCas9 complex, but Rosy had one amino acid that interacted with the sgRNA in CjCas9, Arg-63, which lost its binding affinity (Supplementary Figure 3B). We conclude from these binding energy calculations that the best designs as defined as those that most closely resemble the structural characteristics of SaCas9, SpCas9, and CjCas9, were Merry and Gollum for the SaSpCas9 hybrid and Bilbo for the CjSpCas9 hybrid. We also tested Frodo in cells as an example of a less optimal design.

The Cas9 hybrids failed to edit DNA in human cells

Next we wondered whether the designed Cas9 hybrids could edit DNA in cells. We cloned our SaSpCas9 together with either Merry, Gollum, or Frodo. We transfected them into GFP$(CAG)_0$ cells and showed that they were all expressed to comparable levels (Supplementary Figure 4A,B). Then, we cloned each sgRNA hybrid with four different target sequences against the GFP gene (Figure 4A and Supplementary table 4). This is convenient because gene editing produces insertions and deletions that inactivate GFP. We could then readily detect the drop in GFP intensity using flow cytometry. Using this approach, we confirmed that the SpCas9 was active, leading to a loss of GFP intensity of up to 15% for three of the four target sequences (Figure 4C). By contrast, we found that the SaSpCas9/sgRNA hybrid pairs changed GFP expression very little, if at all, suggesting that they were not functional.

The GFP assay is an indirect measure of gene editing. Thus, we used a second assay, the T7 endonuclease I assay, which detects edits at the DNA level. The insertions and deletions, once amplified and hybridized to unedited strands, form mismatches. These mismatches are then cut by the T7 endonuclease I, producing smaller DNA fragments that can be resolved on an agarose gel. We extracted DNA from the GFP assay at day 10 and performed the T7 endonuclease I assay for targets 1

and 3 as they showed the best results in editing the GFP gene. The results confirmed that whereas mismatches were generated upon SpCas9 transfection, the SaSpCas9 with Merry or Gollum induced no detectable edits (Figure 4D), suggesting they are not functional.

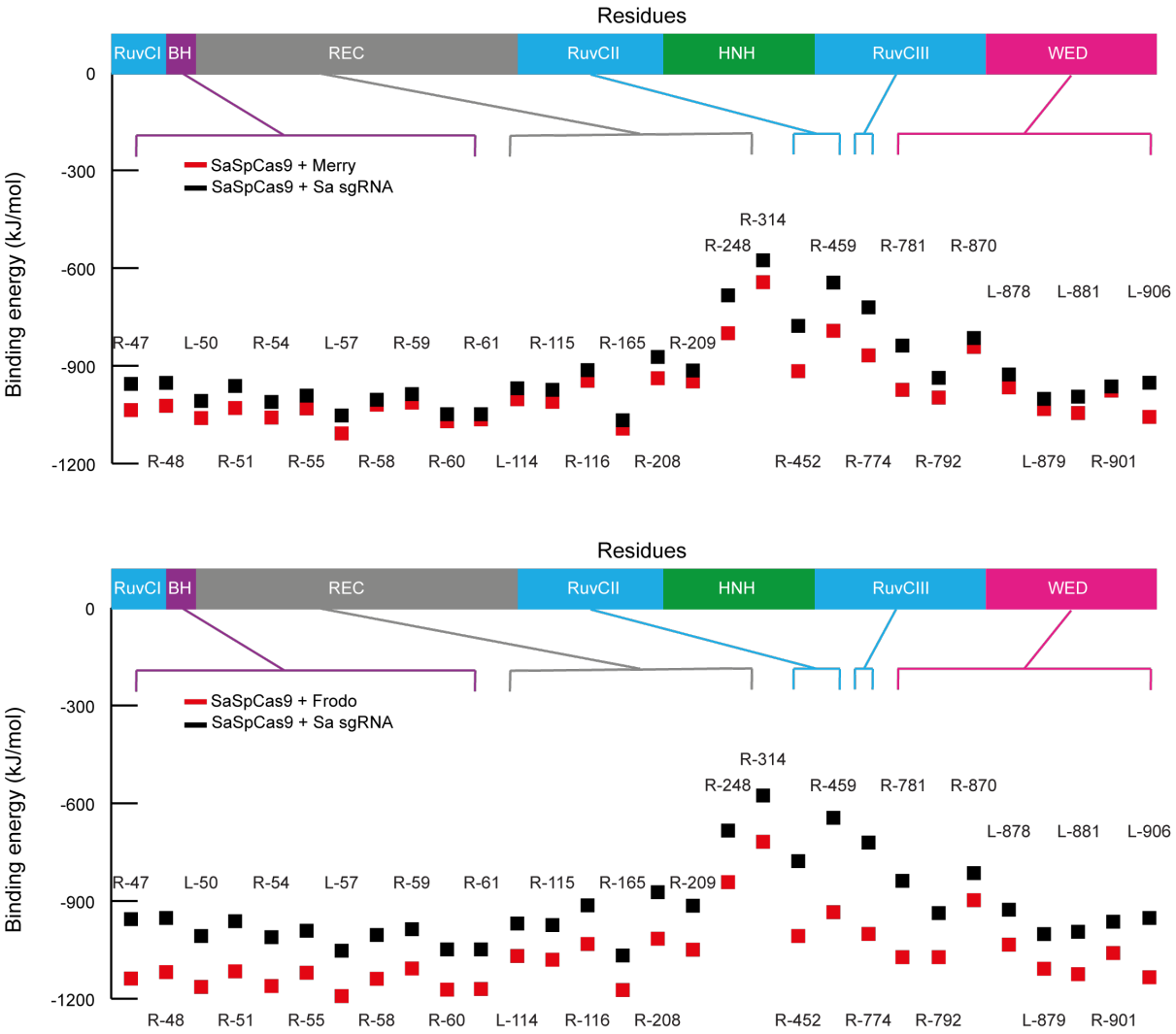


Figure 3: Binding energy calculations for SaSp hybrids. A-B) Binding energy for each amino acid known to interact with the sgRNA for SaCas9 with its sgRNA (black) versus SaSpCas9 with Merry (A) or Frodo (B) (red). BH = bridge helix, WED = wedge domain, PID = PAM interacting domain.

As an independent method to confirm the lack of activity of the hybrids in cells, we used a well characterized assay for the contraction of CAG repeats in GFP$(CAG)_{101}$ [35]. The assay works because CAG repeats interfere with the splicing of the GFP reporter in a size-dependent manner with longer repeats producing less GFP (Figure 4E). The SpCas9 nickase (D10A mutant) together with a sgRNA that targets the repeat tract itself induces contractions of the CAG/CTG repeat, thereby increasing GFP levels in these cells[41,42]. We therefore transfected SaSpCas9 together with Frodo, Merry, or Gollum, as well as CjSpCas9 with Bilbo and measured GFP levels (Figure 4F). As expected, the SpCas9 enzyme increased

GFP intensities, indicating contraction of the CAG repeat, whereas the Cas9 hybrids did not (Figure 4G). Together, these results show that the SaSp and CjSp hybrid pairs are not functional in human cells.

Discussion:

In this study, we tested the hypothesis that we could use molecular dynamics simulation and binding energy data to design *in silico* Cas9 hybrids small enough to fit within an AAV. In addition, we find that the binding energy of the hybrids closely mirrored that of wild type Cas9 enzymes, thereby predicting edits in cells. This was not the case. Most other variants of SpCas9 and SaCas9 in the literature differed from the wild type by just a few amino acids and were designed to decrease the frequency of binding off-targets or to change their PAM recognition[13–21]. Similarly, three chimeric Cas9 enzymes have been created that change PAM sequence requirements[43,44] . At first glance these studies appear similar to what we have done here in that the PID of one orthologue was fused to the N-terminal of another. However, the fusions from those previous studies were between SpCas9 N-terminal and the PID of either *Streptococcus macacae* or *Streptococcus thermophilus* Cas9. Both of these orthologues are closely related to SpCas9 and their sgRNAs are interchangeable. Moreover, these hybrids do not reduce the overall size of the resulting proteins, which was our primary goal. Our results suggest that although powerful enough to predict the functional impact of one or a few residues, molecular dynamics simulation and binding energy values are not well suited for predicting function when the changes to the systems are on a larger scale.

It might be possible to further optimize the hybrid pairs by changing specific amino acids while minimizing the RMSD and RMSF. This may help improve the Cas9/sgRNA interactions, stabilize the hybrids further, and lead to a functional outcome. But this approach is predicated on the hypothesis that the parameters are predictive of function, which we have no evidence for here. It is also possible that the sgRNA hybrids were not expressed well enough to lead to robust gene editing.

Alternatively, the approach was perhaps successful, but the PAM requirements from the SpCas9 were not retained. Indeed, some SpCas9 with PAM variants are due to changes lying outside the PID[18,19]. If this was the case, then the target sequences we used here would not be recognized by our hybrids, explaining the lack of edits.

Further work will be required to make small Cas9 variants suitable for *in vivo* work that retain the

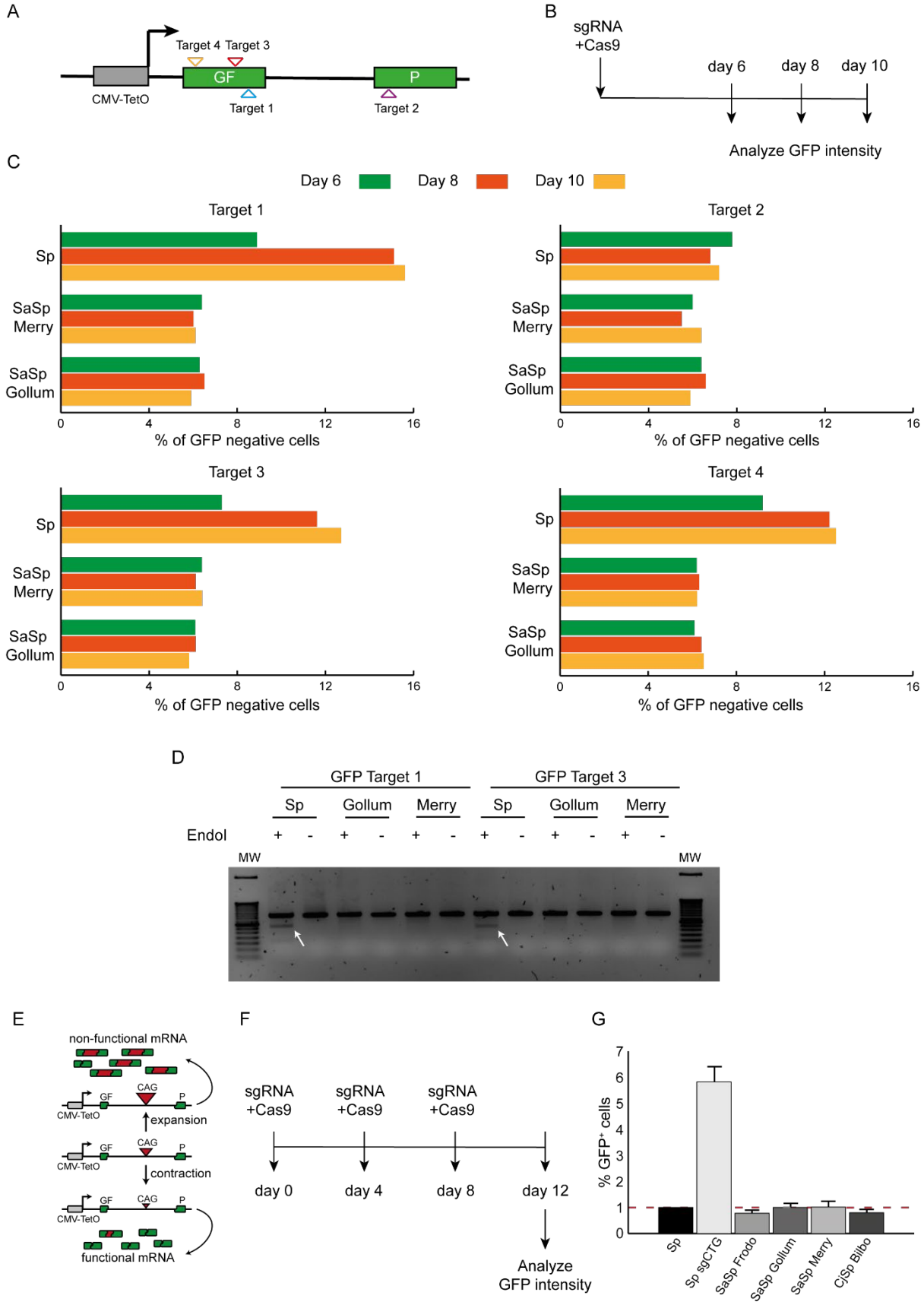


Figure 4: The Cas9 hybrids fail to edit DNA in cells. A) Schematic representation of the GFP gene construction used for the editing assay. Each position targeted by the sgRNA used are shown with different triangles. This representation is not to scale. CMV-TetON is a doxycycline-inducible promoter. B) Timeline of experiment used

for the GFP assay. C) Percentage of GFP negative cells for the 4 targets used at day 6 (green), 8 (orange) and 10 (yellow) for Sp, SaSp Merry and SaSp Gollum. D) T7 endo I assay results for targets 1 and 3. The white arrows indicate the cleavage products indicative of efficient editing. E) GFP-based assay used to detect contraction of the CAG repeats length. The repeat interferes with the splicing of the reporter in a size-dependent manner. F) Timeline experiment for the GFP-based assay to detect CAG contractions. G) Percentage of GFP positive cells for each condition. Dashed lines represent the gate containing the brightest 1% of the cells transfected with Cas9 only. Previous work[42] has shown that this population is enriched in shorter repeats.

SpCas9 PAM interacting properties. This may include taking the Cas9 hybrids and screening empirically for sgRNAs hybrids that bind to the enzyme or adapting the PAM recognition of smaller orthologues. Improving the structure-function prediction of Cas9 variants would benefit a wide range of applications.

Acknowledgements

We thank members of the Dion group for critical reading of the manuscript. This research was undertaken using the supercomputing facilities at Cardiff University operated by Advanced Research Computing at Cardiff (ARCCA) on behalf of the Cardiff Supercomputing Facility and the HPC Wales and Supercomputing Wales (SCW) projects. We acknowledge the support of the latter, which is part-funded by the European Regional Development Fund (ERDF) via the Welsh Government.

Funding:

This work is supported by the UK Dementia Research Institute (DRI-TAP2021-01 and UK DRI-3006) which receives its funding from UK DRI Ltd, funded by the UK Medical Research Council, Alzheimer's Society and Alzheimer's Research UK. VD is further supported by a professorship from the Academy of Medical Sciences (AMSPR1\1014) and the Moondance Foundation lab.

Author contributions

AM performed all the experiments and designed them with the help of VD (for cell work) and GM (for *in silico* work). AM generated all figures and the manuscript was written in collaboration between all three authors.

References

1. Sontheimer, E. J. & Marraffini, L. A. CRISPR intereference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* **11**, 181–190 (2010).

2. Boyaval, P., Moineau, S., Romero, D. a & Horvath, P. CRISPR provides acquired resistance against viruses in Prokaryotes. *Science (1979)* **315**, 1709–1712 (2007).

3. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. **337**, 816–822 (2012).

4. Cong, L. *et al.* Multiplex genome engineering Using CRISPRCas systems. **343**, 881–886 (2013).

5. Nishimasu, H. *et al.* Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949 (2014).

6. Joy Y Wang & Jennifer A Doudna. CRISPR technology A decade of genome editing is only the beginning. *Science (1979)* (2023).

7. Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res* **24**, 1012–1019 (2014).

8. Sharma, G., Sharma, A. R., Bhattacharya, M., Lee, S. S. & Chakraborty, C. CRISPR-Cas9: A Preclinical and Clinical Perspective for the Treatment of Human Diseases. *Molecular Therapy* vol. 29 571–586 Preprint at https://doi.org/10.1016/j.ymthe.2020.09.028 (2021).

9. Tsai, S. Q. *et al.* GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* **33**, 187–198 (2015).

10. Mojica, F. J. M., Dı, C. & Garcı, J. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. 733–740 (2009) doi:10.1099/mic.0.023960-0.

11. Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* **31**, 822–826 (2013).

12. Zhang, X. H., Tee, L. Y., Wang, X. G., Huang, Q. S. & Yang, S. H. Off-target effects in CRISPR/Cas9-mediated genome engineering. *Molecular Therapy - Nucleic Acids* vol. 4 e264 Preprint at https://doi.org/10.1038/mtna.2015.37 (2015).

13. Kleinstiver, B. P. *et al.* High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**, 490–495 (2016).

14. Kulcsár, P. I. *et al.* Blackjack mutations improve the on-target activities of increased fidelity variants of SpCas9 with 5'G-extended sgRNAs. *Nat Commun* **11**, (2020).

15. Slaymaker, I. M. *et al.* Rationally engineered Cas9 nucleases with improved specificity. *Science* **351**, 84–8 (2016).

16. Chen, J. S. *et al.* Enhanced proofreading governs CRISPR–Cas9 targeting accuracy. *Nature* **550**, 407–410 (2017).

17. Nishimasu, H. *et al. Engineered CRISPR-Cas9 nuclease with expanded targeting space*. http://science.sciencemag.org/.

18. Walton, R. T., Christie, K. A., Whittaker, M. N. & Kleinstiver, B. P. Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science* **368**, 290–296 (2020).

19. Hu, J. H. *et al.* Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**, 57–63 (2018).

20. Lee, J. K. *et al.* Directed evolution of CRISPR-Cas9 to increase its specificity. *Nat Commun* **9**, 3048 (2018).

21. Casini, A. *et al.* A highly specific SpCas9 variant is identified by in vivo screening in yeast. *Nat Biotechnol* **36**, 265–271 (2018).

22. Luan, B., Xu, G., Feng, M., Cong, L. & Zhou, R. Combined Computational–Experimental Approach to Explore the Molecular Mechanism of SaCas9 with a Broadened DNA Targeting Range. *J Am Chem Soc* **141**, 6545–6552 (2019).

23. Lee, C. S. *et al.* Adenovirus-mediated gene delivery: Potential applications for gene and cell-based therapies in the new era of personalized medicine. *Genes Dis* **4**, 43–63 (2017).

24. Haggerty, D. L., Grecco, G. G., Reeves, K. C. & Atwood, B. Adeno-Associated Viral Vectors in Neuroscience Research. *Mol Ther Methods Clin Dev* **17**, 69–82 (2020).

25. Wang, D., Tai, P. W. L. & Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov* **18**, 358–378 (2019).

26. Ran, F. A. *et al.* In vivo genome editing using Staphylococcus aureus Cas9. *Nature* **520**, 186–191 (2015).

27. Tan, Y. *et al.* Rationally engineered Staphylococcus aureus Cas9 nucleases with high genome-wide specificity. *Proc Natl Acad Sci U S A* **116**, 20969–20976 (2019).

28. Kim, E. *et al.* In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. *Nat Commun* **8**, (2017).

29. Nishimasu, H. *et al.* Crystal Structure of Staphylococcus aureus Cas9. *Cell* **162**, 1113–1126 (2015).

30. Fonfara, I. *et al.* Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. **42**, 2577–2590 (2014).

31. Yamada, M. *et al.* Crystal Structure of the Minimal Cas9 from Campylobacter jejuni Reveals the Molecular Diversity in the CRISPR-Cas9 Systems. *Mol Cell* **65**, 1109-1121.e3 (2017).

32. Abraham, M. J. *et al.* Gromacs: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1–2**, 19–25 (2015).

33. Duan, Y. *et al.* A Point-Charge Force Field for Molecular Mechanics Simulations of Proteins Based on Condensed-Phase Quantum Mechanical Calculations. *J Comput Chem* **24**, 1999–2012 (2003).

34. Kumari, R., Kumar, R. & Lynn, A. G-mmpbsa -A GROMACS tool for high-throughput MM-PBSA calculations. *J Chem Inf Model* **54**, 1951–1962 (2014).

35. Santillan, B. A., Moye, C., Mittelman, D. & Wilson, J. H. GFP-based fluorescence assay for CAG repeat instability in cultured human cells. *PLoS One* **9**, e113952 (2014).

36. Yang, B. *et al.* Expanded CAG/CTG repeats resist gene silencing mediated by targeted epigenome editing. *Hum Mol Genet* **31**, 386–398 (2022).

37. Cinesi, C., Yang, B. & Dion, V. GFP Reporters to Monitor Instability and Expression of Expanded CAG/CTG Repeats. in *Trinucleotide Repeats: Methods and Protocols* (ed. Richard, G.-F.) 255–268 (Springer New York, 2020). doi:10.1007/978-1-4939-9784-8_16.

38. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. & Joung, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* **32**, 279–284 (2014).

39. Genheden, S. & Ryde, U. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. *Expert Opinion on Drug Discovery* vol. 10 449–461 Preprint at https://doi.org/10.1517/17460441.2015.1032936 (2015).

40. Miller, B. R. *et al.* MMPBSA.py: An efficient program for end-state free energy calculations. *J Chem Theory Comput* **8**, 3314–3321 (2012).

41. Aeschbach, L. & Dion, V. Minimizing carry-over PCR contamination in expanded CAG/CTG repeat instability applications. *Sci Rep* **7**, (2017).

42. Cinesi, C., Aeschbach, L., Yang, B. & Dion, V. Contracting CAG/CTG repeats using the CRISPR-Cas9 nickase. *Nat Commun* **7**, 13272 (2016).

43. Chatterjee, P. *et al.* A Cas9 with PAM recognition for adenine dinucleotides. *Nat Commun* **11**, (2020).

44. Zhao, L. *et al.* PAM-flexible genome editing with an engineered chimeric Cas9. *Nat Commun* **14**, 6175 (2023).

Supplementary Materials

Developing small Cas9 hybrids using molecular modeling

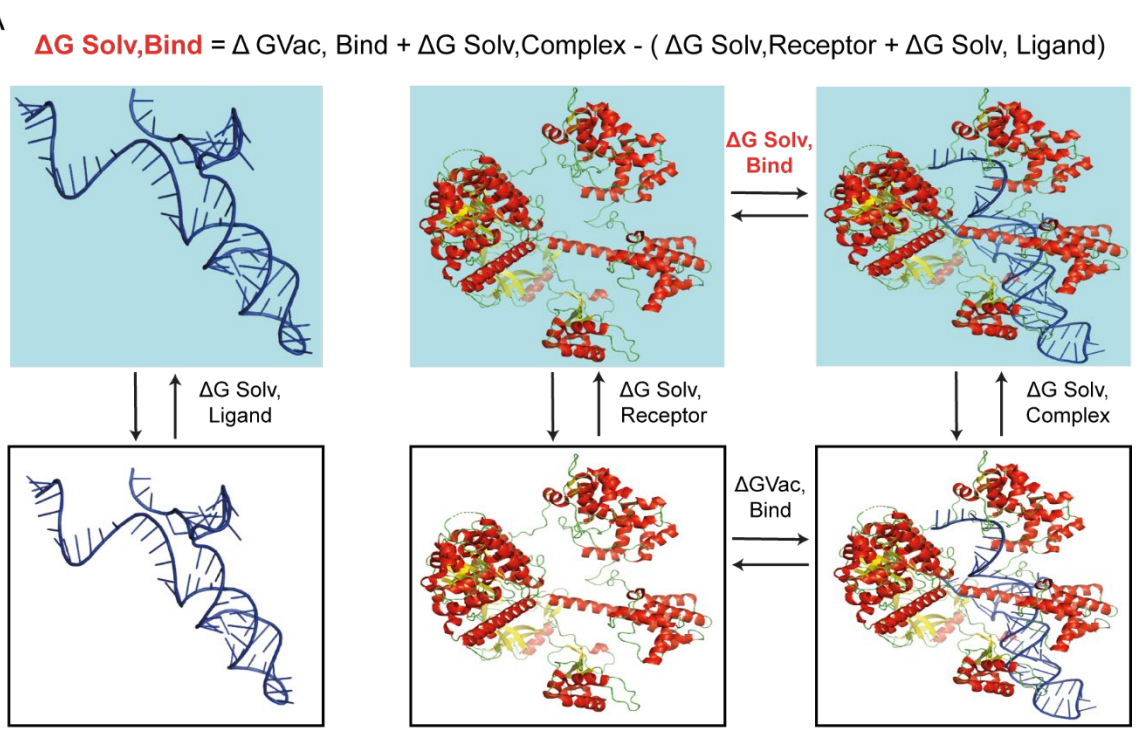
Antoine Mangin[1], Vincent Dion[1*] , and Georgina Menzies[2*]

1: UK Dementia Research Institute at Cardiff University, CF24 4HQ, UK

2: School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK

*Corresponding authors: dionv@cardiff.ac.uk, menziesg@cardiff.ac.uk

A

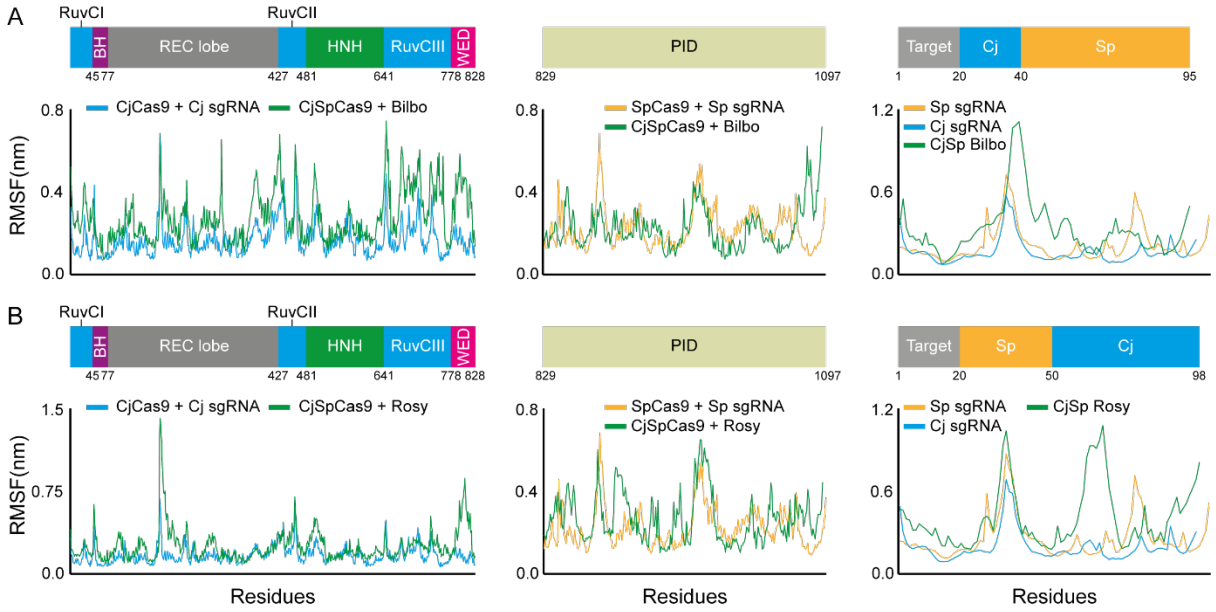


B

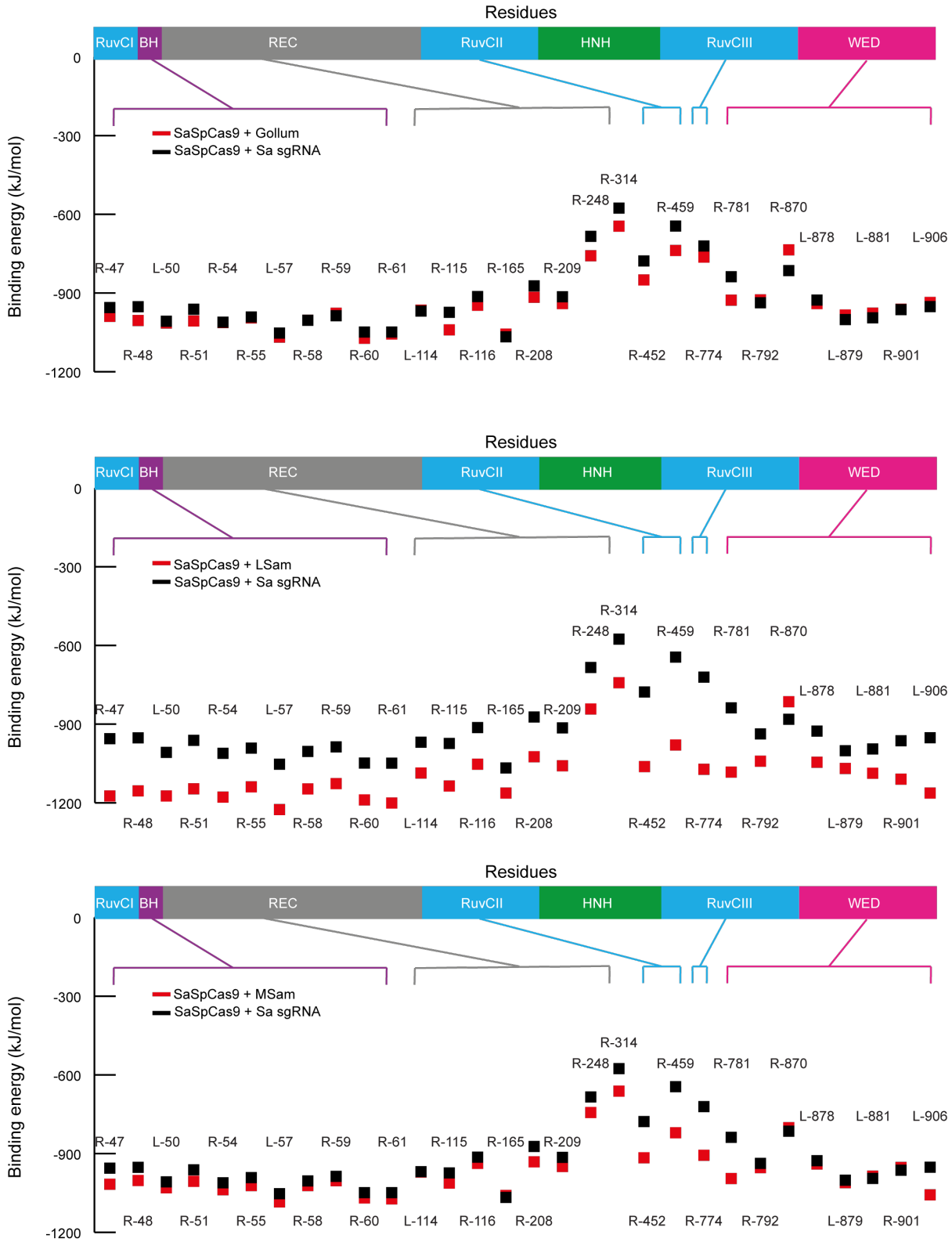
Residue 10	Average ΔG (kJ/mol)
Included	0.91
Not included	0.39

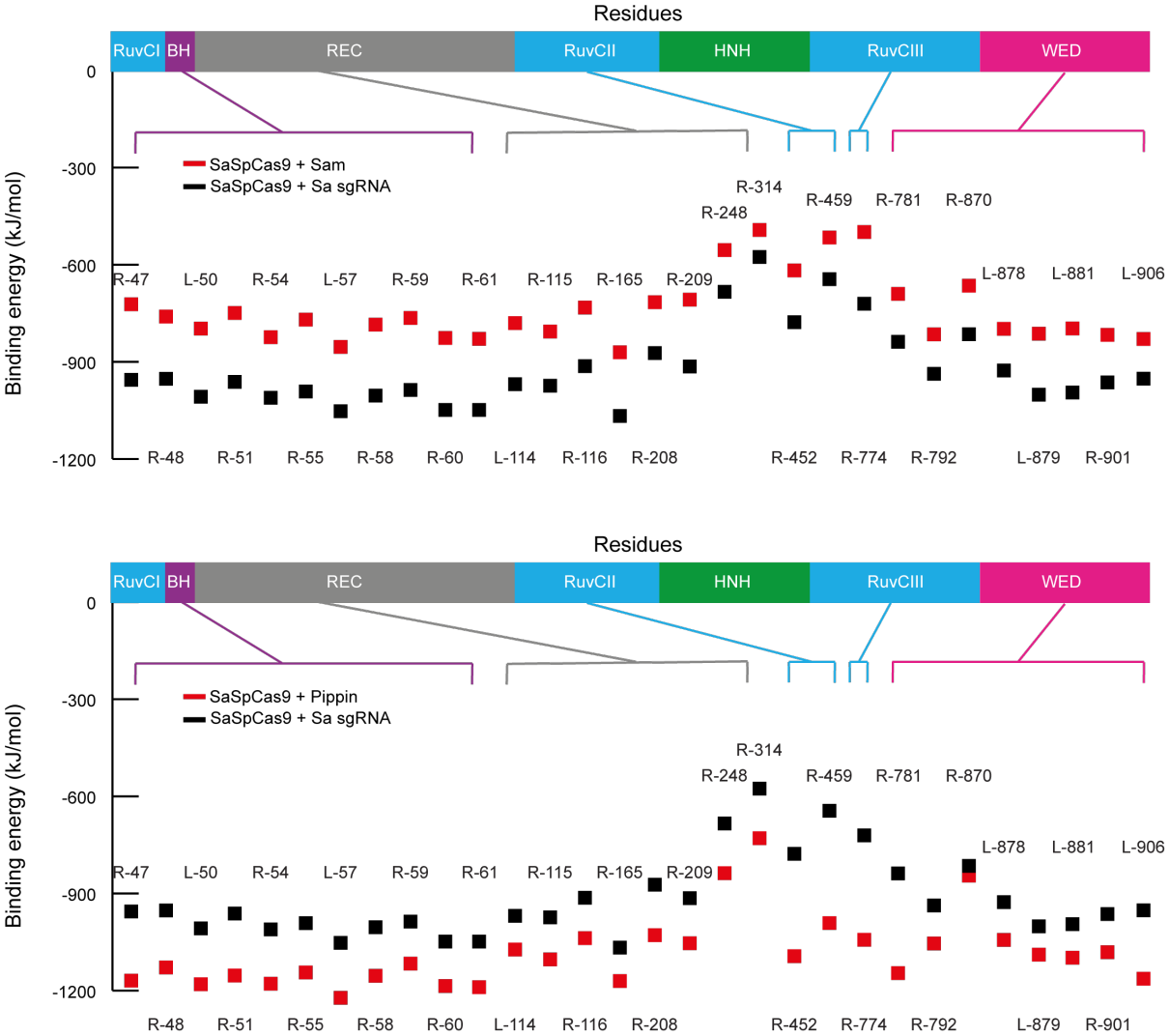
Supplementary figure 1: Binding energy calculations between the Cas9 protein and its sgRNA. A) Principle behind binding energy calculations. The blue boxes represent the system in water whereas the white boxes are in vacuum. B) An average of the ΔG binding for each residue between the nickase and nuclease. 'Included' means that the average ΔG was calculated for every residue in the Cas9 protein, whereas 'not included' excludes the amino acid that changes between the nuclease (D10) and the nickase (A10). This shows an outsize contribution of this residue to the overall binding energy of the protein.

Supplementary figure 2: RMSF analysis from molecular dynamics simulations for the SaSp hybrids: A - F) RMSF of SaSp hybrids (red), SaCas9 (black), and SpCas9 (orange) together with their cognate sgRNAs. A) Gollum, B) LSam, C) MSam, D) Sam, E) Frodo, F) Pippin. Left: N-terminal, middle: PID, right sgRNA. This figure shows that all the hybrid pairs are expected to be stable.

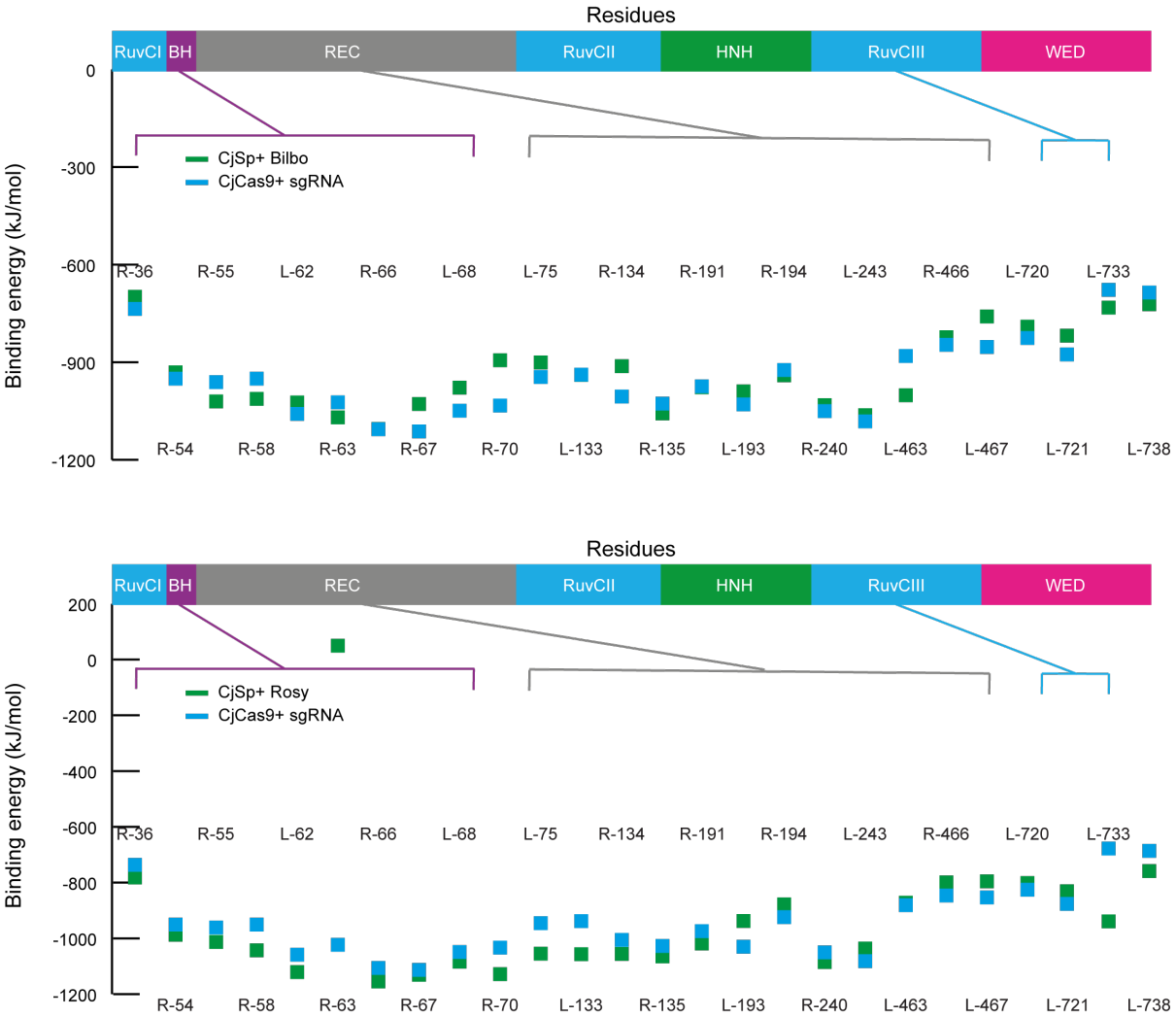


Supplementary figure 3: RMSF analysis from molecular dynamics simulations for the CjSp hybrids: A - B) RMSF of CjSp hybrids (green) **,** CjCas9 (blue) and SpCas9 (orange), together with their cognate sgRNAs. A) Bilbo, B) Rosy. Left: N-terminal, middle: PID, right sgRNA. This figure shows that all the hybrid pairs are expected to be stable.

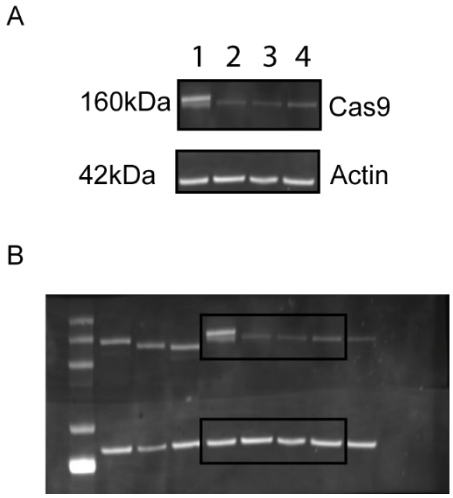

Residues
RuvCI
BH
REC
RuvCII
HNH
RuvCIII
WED
Binding energy (kJ/mol)
0
-300
-600
-900
-1200
SaSpCas9 + Gollum
SaSpCas9 + Sa sgRNA
R-314
R-248
R-459
R-781
R-870
L-878
L-881
L-906
R-47
L-50
R-54
L-57
R-59
R-61
R-115
R-165
R-209
R-48
R-51
R-55
R-58
R-60
L-114
R-116
R-208
R-452
R-774
R-792
L-879
R-901
Residues
RuvCI
BH
REC
RuvCII
HNH
RuvCIII
WED
Binding energy (kJ/mol)
0
-300
-600
-900
-1200
SaSpCas9 + LSam
SaSpCas9 + Sa sgRNA
R-314
R-248
R-459
R-781
R-870
L-878
L-881
L-906
R-47
L-50
R-54
L-57
R-59
R-61
R-115
R-165
R-209
R-48
R-51
R-55
R-58
R-60
L-114
R-116
R-208
R-452
R-774
R-792
L-879
R-901
Residues
RuvCI
BH
REC
RuvCII
HNH
RuvCIII
WED
Binding energy (kJ/mol)
0
-300
-600
-900
-1200
SaSpCas9 + MSam
SaSpCas9 + Sa sgRNA
R-314
R-248
R-459
R-781
R-870
L-878
L-881
L-906
R-47
L-50
R-54
L-57
R-59
R-61
R-115
R-165
R-209
R-48
R-51
R-55
R-58
R-60
L-114
R-116
R-208
R-452
R-774
R-792
L-879
R-901



Supplementary figure 4: Binding energy calculation for the SaSpCas9 hybrids. The binding energy for each amino acid known to interact with the sgRNA from the crystal structures are shown for comparing A) SaCas9 (black) to SaSpCas9 (red). A) Frodo, B) Gollum, C) LSam, D) MSam, E) Pippin, F) Sam. This figures shows that the sgRNAs are expected to make the contacts with the sgRNAs as predicted from the structural information available.



Supplementary figure 5: Binding energy calculation for the CjSPCas9 hybrids. The binding energy for each amino acid known to interact with the sgRNA from the crystal structures are shown for comparing CjCas9 (blue) to CjSpCas9 (green). A) Bilbo, B) Rosy. This figures shows that the sgRNAs are expected to make the contacts with the sgRNAs as predicted from the structural information available.



Supplementary figure 6: Expression of Cas9 in HEK cells: A) Expression of 1) SpCas9 together with its sgRNA, 2) SpCas9 with Gollum, 3) SaSpCas9 plus Frodo, and 4) SaSpCas9 alongside Merry. B) Uncropped western blot for Cas9 expression. Black boxes indicate where the blots were cropped. Note that the membrane was cut prior to blotting but imaged together.

Supplementary Table S1: Amino acid sequence of Cas9 orthologues and of the Cas9 hybrids.

Cas9	Protein sequence (PID in RED, Nickase mutation in BLUE)	Size (AA)
Sp	MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAE ATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFG NIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSD VDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGN LIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAI LLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYA GYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELH AILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEE VVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFL SGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKI IKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWG RLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSL HEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRER MKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDH IVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNL TKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKS KLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRK MIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDF ATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVA YSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPK YSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVE QHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGA PAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD	1368
Sa	MKRNYILGLAIGITSVGYGIIDYETRDVIDAGVRLFKEANVENNEGRRSKRGARRLKRRR RHRIQRVKKLLFDYNLLTDHSELSGINPYEARVKGLSQKLSEEEFSAALLHLAKRRGVHN VNEVEEDTGNELSTKEQISRNSKALEEKYVAELQLERLKKDGEVRGSINRFKTSDYVKEA KQLLKVQKAYHQLDQSFIDTYIDLLETRRTYYEGPGEGSPFGWKDIKEWYEMLMGHCTYF PEELRSVKYAYNADLYNALNDLNNLVITRDENEKLEYYEKFQIIENVFKQKKKPTLKQIA KEILVNEEDIKGYRVTSTGKPEFTNLKVYHDIKDITARKEIIENAELLDQIAKILTIYQS SEDIQEELTNLNSELTQEEIEQISNLKGYTGTHNLSLKAINLILDELWHTNDNQIAIFNR LKLVPKKVDLSQQKEIPTTLVDDFILSPVVKRSFIQSIKVINAIIKKYGLPNDIIIELAR	1053

	EKNSKDAQKMINEMQKRNRQTNERIEEIIRTTGKENAKYLIEKIKLHDMQEGKCLYSLEA IPLEDLLNNPFNYEVDHIIPRSVSFDNSFNNKVLVKQEENSKKGNRTPFQYLSSSDSKIS YETFKKHILNLAKGKGRISKTKKEYLLEERDINRFSVQKDFINRNLVDTRYATRGLMNLL RSYFRVNNLDVKVKSINGGFTSFLRRKWKFKKERNKGYKHHAEDALIIANADFIFKEWKK LDKAKKVMENQMFEEKQAESMPEIETEQEYKEIFITPHQIKHIKDFKDYKYSHRVDKKPN RELINDTLYSTRKDDKGNTLIVNNLNGLYDKDNDKLKKLINKSPEKLLMYHHDPQTYQKL KLIMEQYGDEKNPLYKYYEETGNYLTKYSKKDNGPVIKKIKYYGNKLNAHLDITDDYPNS RNKVVKLSLKPYRFDVYLDNGVYKFVTVKNLDVIKKENYYEVNSKCYEEAKKLKKISNQA EFIASFYNNDLIKINGELYRVIGVNNDLLNRIEVNMIDITYREYLENMNDKRPPRIIKTI ASKTQSIKKYSTDILGNLYEVKSKKHPQIIKKG	
CJ	MARILAFAIGISSIGWAFSENDELKDCGVRIFTKVENPKTGESLALPRRLARSARKRLAR RKARLNHLKHLIANEFKLNYEDYQSFDESLAKAYKGSLISPYELRFRALNELLSKQDFAR VILHIAKRRGYDDIKNSDDKEKGAILKAIKQNEEKLANYQSVGEYLYKEYFQKFKENSKE FTNVRNKKESYERCIAQSFLKDELKLIFKKQREFGFSFSKKFEEEVLSVAFYKRALKDFS HLVGNCSFFTDEKRAPKNSPLAFMFVALTRIINLLNNLKNTEGILYTKDDLNALLNEVLK NGTLTYKQTKKLLGLSDDYEFKGEKGTYFIEFKKYKEFIKALGEHNLSQDDLNEIAKDIT LIKDEIKLKKALAKYDLNQNQIDSLSKLEFKDHLNISFKALKLVTPLMLEGKKYDEACNE LNLKVAINEDKKDFLPAFNETYYKDEVTNPVVLRAIKEYRKVLNALLKKYGKVHKINIEL AREVGKNHSQRAKIEKEQNENYKAKKDAELECEKLGLKINSKNILKLRLFKEQKEFCAYS GEKIKISDLQDEKMLEIDHIYPYSRSFDDSYMNKVLVFTKQNQEKLNQTPFEAFGNDSAK WQKIEVLAKNLPTKKQKRILDKNYKDKEQKNFKDRNLNDTRYIARLVLNYTKDYLDFLPL SDDENTKLNDTQKGSKVHVEAKSGMLTSALRHTWGFSAKDRNNHLHHAIDAVIIAYANNS IVKAFSDFKKEQESNSAELYAKKISELDYKNKRKFFEPFSGFRQKVLDKIDEIFVSKPER KKPSGALHEETFRKEEEFYQSYGGKEGVLKALELGKIRKVNGKIVKNGDMFRVDIFKHKK TNKFYAVPIYTMDFALKVLPNKAVARSKKGEIKDWILMDENYEFCFSLYKDSLILIQTKD MQEPEFVYYNAFTSSTVSLIVSKHDNKFETLSKNQKILFKNANEKEVIAKSIGIQNLKVF EKYIVSALGEVTKAEFRQREDFKK	984
SaSp	MKRNYILGLAIGITSVGYGIIDYETRDVIDAGVRLFKEANVENNEGRRSKRGARRLKRRR RHRIQRVKKLLFDYNLLTDHSELSGINPYEARVKGLSQKLSEEEFSAALLHLAKRRGVHN VNEVEEDTGNELSTKEQISRNSKALEEKYVAELQLERLKKDGEVRGSINRFKTSDYVKEA KQLLKVQKAYHQLDQSFIDTYIDLLETRRTYYEGPGEGSPFGWKDIKEWYEMLMGHCTYF PEELRSVKYAYNADLYNALNDLNNLVITRDENEKLEYYEKFQIIENVFKQKKKPTLKQIA KEILVNEEDIKGYRVTSTGKPEFTNLKVYHDIKDITARKEIIENAELLDQIAKILTIYQS SEDIQEELTNLNSELTQEEIEQISNLKGYTGTHNLSLKAINLILDELWHTNDNQIAIFNR LKLVPKKVDLSQQKEIPTTLVDDFILSPVVKRSFIQSIKVINAIIKKYGLPNDIIIELAR EKNSKDAQKMINEMQKRNRQTNERIEEIIRTTGKENAKYLIEKIKLHDMQEGKCLYSLEA IPLEDLLNNPFNYEVDHIIPRSVSFDNSFNNKVLVKQEENSKKGNRTPFQYLSSSDSKIS YETFKKHILNLAKGKGRISKTKKEYLLEERDINRFSVQKDFINRNLVDTRYATRGLMNLL RSYFRVNNLDVKVKSINGGFTSFLRRKWKFKKERNKGYKHHAEDALIIANADFIFKEWKK LDKAKKVMENQMFEEKQAESMPEIETEQEYKEIFITPHQIKHIKDFKDYKYSHRVDKKPN RELINDTLYSTRKDDKGNTLIVNNLNGLYDKDNDKLKKLINKSPEKLLMYHHDPQTYQKL KLIMEQYGDEKNPLYKYYEETGNYLTKYSKKDNGPVIKKIKYYGNKLNAHLDITDDYPNS RNKVVKLSTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGK SKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRM LASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQI SEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDR KRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD	1179
CjSp	MARILAFAIGISSIGWAFSENDELKDCGVRIFTKVENPKTGESLALPRRLARSARKRLAR RKARLNHLKHLIANEFKLNYEDYQSFDESLAKAYKGSLISPYELRFRALNELLSKQDFAR VILHIAKRRGYDDIKNSDDKEKGAILKAIKQNEEKLANYQSVGEYLYKEYFQKFKENSKE FTNVRNKKESYERCIAQSFLKDELKLIFKKQREFGFSFSKKFEEEVLSVAFYKRALKDFS HLVGNCSFFTDEKRAPKNSPLAFMFVALTRIINLLNNLKNTEGILYTKDDLNALLNEVLK	1097

	NGTLTYKQTKKLLGLSDDYEFKGEKGTYFIEFKKYKEFIKALGEHNLSQDDLNEIAKDIT LIKDEIKLKKALAKYDLNQNQIDSLSKLEFKDHLNISFKALKLVTPLMLEGKKYDEACNE LNLKVAINEDKKDFLPAFNETYYKDEVTNPVVLRAIKEYRKVLNALLKKYGKVHKINIEL AREVGKNHSQRAKIEKEQNENYKAKKDAELECEKLGLKINSKNILKLRLFKEQKEFCAYS GEKIKISDLQDEKMLEIDHIYPYSRSFDDSYMNKVLVFTKQNQEKLNQTPFEAFGNDSAK WQKIEVLAKNLPTKKQKRILDKNYKDKEQKNFKDRNLNDTRYIARLVLNYTKDYLDFLPL SDDENTKLNDTQKGSKVHVEAKSGMLTSALRHTWGFSAKDRNNHLHHAIDAVIIAYANNS IVKAFSDFKKEQESNSAELYAKKISELDYKNKRKFFEPFSGFRQKVLDKIDEIFVSKPER KKPSGALHEETFRKEEEFYQSYGGKEGVLKALELGKIRKVNGKIVKNGTGGFSKESILPK RNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSS FEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYV NFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSA YNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSIT GLYETRIDLSQLGGD	

Supplementary Table 2: Sequence of the WT sgRNAs and hybrid sgRNAs.

sgRNA	DNA sequence (Yellow = Sp sgRNA ; Black = Sa sgRNA ; Blue = Cj sgRNA; Grey= Target sequence)	Size (bp)
Sp sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG CUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU	98
Sa sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGUACUCUGGAAACAGAAUCUACUAAAACA AGGCAAAAUGCCGUGUUUAUCUCGUCAACUUGUUGGCGAGAU	96
Cj sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGUCCCUGAAAAAGGGACUAAAAUAAAGAG UUUGCGGGACUCUGCGGGGUUACAAUCCCCUAAAACCGC	93
Frodo sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGUACUCUGGAAACAGAAUCUACUAAAACA AGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU	100
Sam sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC AAAAUGCCG	63
Medium Sam sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC AAAAUGCCGAUCUCGUCAACUUGUUGGCGAGAU	87
Large Sam sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC AAAAUGCCGAUCUCGUCAACUUGUUGCGAGAUGCACCGAGUCGGUGCUU	103
Pippin sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGUACUCUGGAAACAGAAUCUACUAAAACA AGGCAAAAUGCCGAACUUGAAAAAGUGGCCACCGAGUCGGUGCUU	99
Merry sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGUACUCUGGAAACAGAAUCUACUAAAACA AGGCAAAAUGCCGAACUUGAAAAAGUG	81
Gollum sgRNA	GCUGCUGCUGCUGCUGCUGCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG CAAAAUGCCGUGUUUAACUUGAAAAAGUG	83
Bilbo sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGUCCCUGAAAAGGGACUAAAAUGGCUAGU CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU	92
Rosy sgRNA	GCUGCUGCUGCUGCUGCUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAAGA GUUUGCGGGACUCUGCGGGGUUACAAUCCCCUAAAACCGC	94

Table 3: List of plasmids used.

Plasmid name	Content	Reference
pcDNA3.3-TOPO - Cas9_D10A	Sp Cas9 D10A	Mali et al, 2013[1]
pPN10-gCTG	Sp Cas9 sgRNA sgCTG	Cinesi et al, 2016[2]
pUC57-ITR2-H1- sgCTG Frodo-eCMV-SaSpD10A (Neuron codon optimization)-3XFLAG-SV40 NLS-ITR2	SaSpD10A Cas9+ Frodo sgCTG	This study

pUC57-ITR2-H1- sgCTG Gollum-eCMV-SaSpD10A (Neuron codon optimization)-3XFLAG-SV40 NLS-ITR2	SaSpD10A Cas9+ Gollum sgCTG	This study
pUC57-ITR2-H1- sgCTG Merry-eCMV-SaSpD10A+linker (Neuron codon optimization)-3XFLAG-SV40 NLS-ITR2	SaSpD10A Cas9 + Merry sgCTG	This study
pUC57-ITR2-H1-GFP sgRNA Target 1-eCMV- S.pyoegenes-3XFLAG-SV40NLS-ITR2	Sp Cas9 + Sp guide targeting GFP1	This study
pUC57-ITR2-H1-GFP sgRNA Target 2-eCMV- S.pyoegenes-3XFLAG-SV40NLS-ITR2	Sp Cas9 + Sp guide targeting EGFP2	This study
pUC57-ITR2-H1-GFP sgRNA Target 3-eCMV- S.pyoegenes-3XFLAG-SV40NLS-ITR2	Sp Cas9 + Sp guide targeting EGFP3	This study
pUC57-ITR2-H1-GFP sgRNA Target 4-eCMV- S.pyoegenes-3XFLAG-SV40NLS-ITR2	Sp Cas9 + Sp guide targeting EGFP4	This study
pUC57-ITR2-H1-GFP sgRNA Merry Target 1- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Merry guide targeting EGFP1	This study
pUC57-ITR2-H1-GFP sgRNA Merry Target 2- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Merry guide targeting EGFP2	This study
pUC57-ITR2-H1-GFP sgRNA Merry Target 3- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Merry guide targeting EGFP3	This study
pUC57-ITR2-H1-GFP sgRNA Merry Target 4- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Merry guide targeting EGFP4	This study
pUC57-ITR2-H1-GFP sgRNA Gollum Target 1- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Gollum guide targeting EGFP1	This study
pUC57-ITR2-H1-GFP sgRNA Gollum Target 2- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Gollum guide targeting EGFP2	This study
pUC57-ITR2-H1-GFP sgRNA Gollum Target 3- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Gollum guide targeting EGFP3	This study
pUC57-ITR2-H1-GFP sgRNA Gollum Target 4- eCMV- SaSp-3XFLAG-SV40NLS-ITR2	SaSp Cas9 + Gollum guide targeting EGFP4	This study
pUC57-ITR2-H1- sgCTG Bilbo -eCMV-CjSpD8A-3XFLAG-SV40 NLS-ITR2	CjSpD8A Cas9 + Bilbo sgCTG	This study

Supplementary Table S4: The sgRNA sequence used to target GFP.

Target	Sequence	Reference
GFP Target 1	GGGCACGGGCAGCUUGCCGG	*Fu et al. 2014*[3]
GFP Target 2	GAUGCCGUUCUUCUGCUUGU	*Fu et al. 2014*[3]
GFP Target 3	GGUGGUGCAGAUGAACUUCA	*Fu et al. 2014*[3]
GFP Target 4	GGGCGAGGAGCUGUUCACCG	*Fu et al. 2014*[3]

Supplementary Table S5: The primers used to amplify the region targeted by the GFP sgRNA

Name	Description	Sequence
oVIN 3474	Forward primer to amplify the target 1,3 and 4 for T7 endonuclease I assay	ACGCTGTTTTGACCTCCAT
oVIN 3475	Reverse primer to amplify the target 1,3 and 4 for T7 endonuclease I assay	CGTCGTCCTTGAAGAAGATG
oVIN 3476	Reverse primer to amplify the target 2 for T7 endonuclease I assay	GTCTTTGCTCAGGGCGGACT
oVIN 3477	Forward primer to amplify the target 2 for T7 endonuclease I assay	TTTACCCTAGCACGCATGAAG

References:

1. Prashant Mali *et al.* RNA-Guided Human Genome Engineering via Cas9. *Science (1979)* **339**, 823–826 (2013).

2. Cinesi, C., Aeschbach, L., Yang, B. & Dion, V. Contracting CAG/CTG repeats using the CRISPR-Cas9 nickase. *Nat Commun* **7**, 13272 (2016).

3. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. & Joung, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* **32**, 279–284 (2014).