Conformational control of DNA target cleavage by CRISPR–Cas9

Samuel H. Sternberg1, Benjamin LaFrance2, Matias Kaplan3† & Jennifer A. Doudna1,2,3,4,5

Cas9 is an RNA-guided DNA endonuclease that targets foreign DNA for destruction as part of a bacterial adaptive immune system mediated by clustered regularly interspaced short palindromic repeats (CRISPR)1,2. Together with single-guide RNAs3, Cas9 also functions as a powerful genome engineering tool in plants and animals4–8, and efforts are underway to increase the efficiency and specificity of DNA targeting for potential therapeutic applications7,8. Studies of off-target effects have shown that DNA binding is far more promiscuous than RNA cleavage9–11, yet the molecular cues that govern strand scission have not been elucidated. Here we show that the conformational state of the HNH nuclease domain directly controls DNA cleavage activity. Using intramolecular Förster resonance energy transfer experiments to detect relative orientations of the Cas9 catalytic domains when associated with on- and off-target DNA, we find that DNA cleavage efficiencies scale with the extent to which the HNH domain samples an activated conformation. We furthermore uncover a surprising mode of allosteric communication that ensures concerted firing of both Cas9 nuclease domains. Our results highlight a proofreading mechanism beyond initial protospacer adjacent motif (PAM) recognition12 and RNA–DNA base-pairing1 that serves as a final specificity checkpoint before DNA double-strand break formation.

Cas9 is a large, multi-domain protein that undergoes RNA-induced conformational changes to reach a DNA-binding–competent state13. Crystal structures of apo13, single-guide RNA (sgRNA)-bound14, and sgRNA/DNA-bound15,16 Cas9 from Streptococcus pyogenes (Fig. 1a, b) have revealed distinct conformational states of the protein but failed to explain its DNA cleavage mechanism, because in each structure the HNH domain active site is positioned at least 30 Å away from the DNA cleavage site15,16. Furthermore, available structures could not explain why DNA cleavage is precluded at stably bound off-target sites with incomplete RNA-DNA complementarity. We hypothesized that functionally important HNH conformational dynamics could influence the cleavage specificity of the Cas9–guide RNA enzyme complex. To test this possibility, we developed a Förster resonance energy transfer (FRET)-based approach to investigate Cas9 structural changes in response to binding sgRNA and DNA ligands.

We generated a FRET construct to monitor Cas9 structural rearrangements upon sgRNA binding13 (Fig. 1b). Starting with a cysteine-free Cas9 variant, we introduced cysteine residues at positions D435 and E945 near the hinge region and labelled these residues with Cy3- and Cy5-maleimide dyes, generating Cas9hinge. Control labelling reactions with cysteine-free Cas9 confirmed the conjugation specificity, and doubly labelled Cas9 was fully functional for DNA cleavage (Extended Data Fig. 1a–c). Measurements from available structures revealed an expected distance change of ~60 Å upon sgRNA and DNA binding (Extended Data Table 1), and indeed, when Cy3 of sgRNA-bound Cas9hinge was excited at 530 nm, we observed a substantial decrease in energy transfer compared with apo-Cas9hinge, as evidenced by a relative increase in donor (Cy3) fluorescence relative to acceptor (Cy5) fluorescence (Fig. 1c). The observed change scaled with the molar ratio of sgRNA to Cas9, a mixture of donor-only and acceptor-only labelled Cas9hinge showed no evidence of energy transfer, and an sgRNA specific to Neisseria meningitidis Cas9 (ref. 17), which significantly impairs S. pyogenes Cas9 binding (data not shown), elicited a negligible change (Extended Data Fig. 2a–c). We conclude that the change in fluorescence intensities resulted from an sgRNA-induced, intramolecular conformational change in Cas9hinge.

Cas9hinge exhibited an ~70% decrease in energy transfer upon sgRNA binding as determined by (ratio)A, whereby the acceptor fluorescence intensity via energy transfer is normalized to that via direct excitation18,19 (Methods and Extended Data Fig. 2d). Target DNA binding induced little further change in FRET (Fig. 1c, d), consistent with available structural data (Extended Data Table 1). To identify the molecular

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1Department of Chemistry, University of California, Berkeley, Berkeley, California 94720, USA. 2Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA. 3Howard Hughes Medical Institute, University of California, Berkeley, California 94720, USA. 4Innovative Genomics Initiative, University of California, Berkeley, California 94720, USA. 5Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA. †Present address: Department of Bioengineering, Stanford University, Stanford, California 94305, USA.
determinants that trigger conformational rearrangement of Cas9, we tested truncated variants of the sgRNA (Extended Data Table 2) and found that the 20-nucleotide target recognition sequence has a critical role in controlling the Cas9 conformational state (Fig. 1d). An sgRNA lacking the entire guide segment (Δ guide1–20) generated a (ratio)A value indistinguishable from apo–Cas9 hinge while being more than 95% bound under our experimental conditions14, whereas sgRNAs containing part of the 20-nucleotide guide segment partly restored the change in (ratio)A. sgRNA variants lacking one or both hairpins at the 3′ end (Δ hairpins1–2) also generated intermediate (ratio)A values (Fig. 1d) while retaining sub-nanomolar binding affinity to Cas9 (ref. 20), and similar data were obtained with catalytically dead (D10A/H840A) dCas9 hinge (Extended Data Fig. 2e). We conclude that motifs at both ends of the sgRNA are required to stabilize a closed state of Cas9, but that in the case of Δ hairpins1–2, a fully closed state is not required for rapid cleavage kinetics20. We propose that intermediate (ratio)A changes reflect stable sgRNA–Cas9 hinge complexes interconverting between open and closed conformers.

We next focused on the HNH nuclease domain. Since existing crystal structures exhibit inactive HNH domain conformations15,16, we built a model for the putative activated state by docking a homologous HNH–dsDNA crystal structure21 onto the sgRNA/DNA-bound Cas9 structure (Extended Data Fig. 3a–d). We selected two pairs of positions (S355–S867 and S867–N1054) whose inter-residue distances, according to our model, would change substantially upon target DNA binding (Fig. 2a, Extended Data Fig. 3e and Extended Data Table 1). Cas9 labelled with Cy3 and Cy5 at these sites (Cas9 HNH-1 and Cas9 HNH-2) retained nearly wild-type DNA cleavage activity (Extended Data Fig. 1c).

We observed a substantial FRET increase for catalytically inactive dCas9 HNH-1 upon target DNA binding relative to sgRNA alone (Fig. 2b), and control experiments with non-target DNA or off-target DNA substrates containing either PAM or seed mutations failed to generate this change (Fig. 2b and Extended Data Table 2). We next monitored FRET with off-target DNA substrates containing mutations distal from the PAM, which retain high-affinity Cas9 binding12,22. Remarkably, the observed (ratio)A values decreased as the number of mismatches increased (Fig. 2c), and these changes were not attributable to decreasing occupancy of the sgRNA/DNA-bound complex: direct binding assays indicate at least 89% of the dCas9 HNH-1 population should be bound to all tested DNA substrates, and increasing the concentration of dsDNA had no discernible effect on (ratio)A (Extended Data Fig. 4a, b). Our results show that the HNH domain samples a conformational equilibrium with on-target DNA that is distinct from partly matching off-target DNA, and suggest that the high FRET state corresponds to an active HNH conformation at the cleavage site.

We suspected that altered conformational states of the HNH domain could explain which off-target DNA substrates are cleaved by Cas9. Substrates with at least 4 base-pair (bp) mismatches that elicit a low (ratio)A value were cleaved slowly, if at all (Fig. 2d and Extended Data Fig. 4c), as observed previously22,23. This indicates that the inability to access the high FRET state associated with an activated HNH conformation precludes cleavage. Interestingly, substrates with only 1–3 bp mismatches at the distal end of the target sequence were cleaved at near wild-type rates despite having diminished (ratio)A values relative to the on-target. This suggests that rapidly interconverting conformational states, one of which is the activated state, may still enable rapid cleavage. Truncated sgRNAs with shorter regions of target complementarity that exhibit enhanced fidelity in genome editing experiments24 may similarly facilitate efficient on-target cleavage without stabilizing an activated HNH conformation. Single-molecule experiments will be necessary to reveal these putative dynamics, which are unavoidable averaged in our ensemble measurements.

We observed a similar pattern of (ratio)A changes using catalytically active Cas9 HNH-1 and the opposite trend of (ratio)A changes was observed with Cas9 HNH-2, a construct designed to undergo a high-to-low FRET efficiency transition upon on-target DNA binding (Fig. 2e and Extended Data Figs 3e and 4d). These data suggest that positioning of the HNH domain is largely unaffected by actual strand scission, but instead reflects a conformational equilibrium that is particularly sensitive to RNA–DNA heteroduplex formation at the distal end of the target. These observations emphasize the importance of RNA–DNA complementarity throughout the target region, rather than only the seed sequence closest to the PAM, in controlling Cas9 cleavage specificity.

The HNH and RuvC nucleases domains cleave target and non-target strands 3 bp upstream of the PAM, respectively3,25. For partly unwound off-target substrates with mismatches >10 bp further upstream, target strand cleavage is precluded by conformational control of the HNH domain. However, the mechanism by which RuvC domain-catalysed non-target strand cleavage is avoided remains unknown.
Figure 3 | RuvC nuclease activity is allosterically controlled by HNH conformational changes. a, Tested DNA substrates, with on-target (1) at top. Matched and mismatched positions of DNA target strand sequences relative to the sgRNA are coloured red and black, respectively, with the PAM in yellow. Some substrates contain internal mismatches between the two DNA strands; dashed lines indicate additional flanking sequences. Schematic at bottom right depicts identical non-target strand substrates presented to the RuvC nuclease domain in substrates 5 and 7. b, Non-target (black) and target (red) strand cleavage time courses for the indicated DNA substrates using wild-type Cas9. Exponential fits are shown as solid lines. c, (Ratio)A data for Cas9 HNH-1. Error bars in b–e, s.d.; n = 3.

We hypothesized that this activity would be sensitive to HNH domain conformational changes. We first separately measured HNH and RuvC domain cleavage rates for a panel of partly mismatched substrates and found that both strands were consistently cleaved in synchrony (Fig. 3a, b and Extended Data Fig. 5a, b). We next used shorter DNA substrates with or without internal mismatches, such that Cas9-mediated DNA unwinding up to the site of an sgRNA–DNA mismatch would theoretically present identical substrates to the RuvC domain active site (Fig. 3a). After separately measuring non-target strand cleavage kinetics and Cas9 HNH-1 FRET, we observed a tight correlation between RuvC domain cleavage activity and the presence of an activated HNH conformational state (Fig. 3c and Extended Data Fig. 5c–e). This finding provides strong evidence that HNH conformational dynamics exert allosteric control over the RuvC nuclease domain. Furthermore, the RuvC domain could still effectively cleave the non-target strand of a bubbled substrate that induced an activated HNH conformation, but whose target strand could not be cleaved by the HNH domain because of mismatches in the seed (Fig. 3d, e). Together, these data argue that HNH conformational changes, but not HNH nuclease function, trigger RuvC domain nuclease activity.

We wondered how Cas9 achieves this functional coupling. The HNH domain is inserted between RuvC domain motifs II and III, but linkers connecting both domains are consistently dispersed in available crystal structures and there are relatively few inter-domain contacts.13,15,16 (Extended Data Fig. 6a). We purified an HNH deletion construct, ΔHNH–Cas9 (Extended Data Fig. 6a–c), that retained nearly wild-type DNA binding activity while being defective in non-target strand cleavage by the RuvC domain (Fig. 4a, b and Extended Data Fig. 6d). Thus, the HNH domain is required for RuvC nuclease domain activation but is dispensable for RNA-guided DNA targeting.

Finally, we sought to identify the basis of allostery between the HNH and RuvC domains. We hypothesized that two α-helices connecting the HNH and RuvC III motifs (residues S909–N940), previously shown to adopt an extended conformation and proposed to assist the HNH domain in approaching the cleavage site,13 were instead acting as a signal transducer (Extended Data Fig. 7a). We introduced a series of proline residues to specifically disrupt this α-helix and found that target strand cleavage kinetics by the HNH domain were minimally affected (Fig. 4c–e and Extended Data Fig. 7b, c). In stark contrast, RuvC domain nuclease activity was almost completely blocked with an E923P/T924P–Cas9 mutant, and this effect could be reversed with the corresponding alanine mutations (Fig. 4d, e and Extended Data Fig. 7c). The finding that this effect was not confined to highly conserved residues supports the idea that disruption of the helix-forming propensity of this region, and not specific point mutations, disabled the RuvC domain. We conclude that an intact extended α-helix acts as an allosteric switch to communicate the HNH conformational change to the RuvC domain and activate it for cleavage. Understanding the precise mechanism of activation will probably require additional structures of Cas9 in a pre-cleavage state, with the intact non-target strand substrate bound in the RuvC active site.

Figure 4 | Mechanism of communication between the HNH and RuvC nuclease domains to achieve concerted DNA cleavage. a, Target DNA binding assay with dCas9 and ΔHNH–Cas9, resolved by native polyacrylamide gel electrophoresis (PAGE) (top); for gel source data, see Supplementary Fig. 1. Quantified data are below; binding fits are shown as solid lines. b, Target DNA cleavage assay with dCas9, wild-type (WT) Cas9, and ΔHNH–Cas9, resolved by denaturing PAGE. S, substrate; NT, cleaved non-target strand; T, cleaved target strand. c, Magnified view of the sgRNA/DNA-bound Cas9 structure (top) highlights two α-helices connecting the HNH domain carboxy (C) terminus and RuvC III amino (N) terminus. Bottom shows sequence alignment of this region, and residues mutated to proline or alanine are indicated (arrows). d, Target DNA cleavage assay with the indicated Cas9 variants, resolved by denaturing PAGE. e, Target (red) and non-target (black) strand cleavage time courses with the indicated Cas9 variants (for WT-Cas9 data, see Fig. 3b). Exponential fits are shown as solid lines. Error bars in a and e, s.d.; n = 5 and 3, respectively. f, Model for conformational control of target cleavage by CRISPR–Cas9.
Our data support a model in which the Cas9 endonuclease uses multiple levels of regulation to ensure accurate target DNA cleavage (Fig. 4f and Supplementary Video 1). After identification of potential targets via PAM binding and directional DNA unwinding dependent on sgRNA–DNA complementarity, recognition of on-target DNA drives a conformational change in the HNH nuclease domain that enables productive engagement with the scissile phosphate. Importantly, this same structural transition triggers RuVc domain catalytic activity, ensuring concerted cleavage of both DNA strands. Partially conformational off-target DNA sequences may stably bind Cas9, but by failing to drive HNH conformational changes, avoid cleavage. The recent crystal structure of sgRNA/DNA-bound Cas9 from *Staphylococcus aureus* (~17% sequence identity with *S. pyogenes* Cas9) also exhibits an inactive HNH conformation, suggesting that conformational control of the HNH domain is a general feature of all Cas9 enzymes. Furthermore, this proofreading mechanism is strikingly similar to the R-loop locking mechanism used by the RNA-guided targeting complex (Cascade) from type I CRISPR-Cas systems, in which RNA–DNA heteroduplex formation at the PAM-distal end of the target exerts allosteric control over Cascade conformational rearrangements near the PAM-proximal end that are required for subsequent target cleavage 22,27. Beyond providing fundamental insights into the mechanism of DNA interrogation by Cas9, our findings have important implications for the use of Cas9 as a genome engineering technology. For example, our data can explain why little cleavage occurs at off-target DNA sequences identified in chromatin immunoprecipitation followed by sequencing (ChiP-seq) experiments 9–11, and suggest that DNA nicking by the native Cas9 enzyme is disfavoured in cells owing to concerted cutting by the HNH and RuVc nuclease domains. Finally, our findings demonstrate an exciting opportunity to use protein conformational changes that report on target DNA recognition for fluorescence-based readout of DNA binding in cells.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. van der Oost, J., Westra, E. R., Jackson, R. N. & Wiedenheft, B. Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nature Rev. Microbiol.* **12**, 479–492 (2014).
2. Barrangou, R. & Marraffini, L. A. CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. *Mol. Cell.* **54**, 234–244 (2014).
3. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
4. Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262–1278 (2014).
5. Doudna, J. A. & Charpentier, E. Genome editing: The new frontier of genome engineering technology. *Nature Biotechnol.* **32**, 570–576 (2014).
6. Sternberg, S. H. & Doudna, J. A. Expanding the biologist's toolkit with CRISPR-Cas9. *Mol. Cell.* **58**, 568–574 (2015).
7. Wu, X., Kriz, A. J. & Sharp, P. A. Target specificity of the CRISPR-Cas9 system. *Quart. Biol.* **2**, 59–70 (2014).
8. Gori, J. L. et al. Delivery and specificity of CRISPR-Cas9 genome editing technologies for human gene therapy. *Hum. Gene Ther.* **26**, 443–451 (2015).
9. Wu, X. et al. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nature Biotechnol.* **32**, 670–676 (2014).
10. Tsai, S. Q. et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nuclease. *Nature Biotechnol.* **33**, 187–197 (2015).
11. Ran, F. A. et al. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* **520**, 186–191 (2015).
12. Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62–67 (2014).
13. Jinek, M. et al. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* **343**, 1247997 (2014).
14. Jiang, F., Zhou, K., Ma, L., Gressel, S. & Doudna, J. A. A Cas9–guide RNA complex reorganized for target DNA recognition. *Science* **348**, 1477–1481 (2015).
15. Nishimasu, H. et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949 (2014).
16. Anders, C., Niewoehner, D., Duerst, A. & Jinek, M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* **513**, 569–573 (2014).
17. Hou, Z. et al. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc. Natl Acad. Sci. USA* **110**, 15644–15649 (2013).
18. Majumdar, Z. K., Hickerson, R., Noller, H. F. & Clegg, R. M. Measurements of internal distance changes of the 3OS ribosome using FRET with multiple donor-acceptor pairs: quantitative spectroscopic methods. *J. Mol. Biol.* **351**, 1123–1145 (2005).
19. Clegg, R. M. Fluorescence resonance energy transfer and nucleic acids. *Methods Enzymol.* **211**, 353–388 (1992).
20. Wright, A. V. et al. Rational design of a split-Cas9 enzyme complex. *Proc. Natl Acad. Sci. USA* **112**, 2984–2989 (2015).
21. Bierثmpfel, C., Yang, W. & Suck, D. Crystal structure of T4 endonuclease VII resolving a Holliday junction. *Nature* **449**, 616–620 (2007).
22. Szczelkun, M. D. et al. Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. *Proc. Natl Acad. Sci. USA* **111**, 9798–9803 (2014).
23. Cencic, R. et al. Protospecler adjacent motif (PAM)-distal sequences engage CRISPR Cas9 DNA target cleavage. *PLoS One* **9**, e109213 (2014).
24. Fu, Y., Sander, J. D., Reyton, D., Casico, V. M. & Joung, J. K. Improving CRISPR-Cas nucleosome specificity using truncated guide RNAs. *Nature Biotechnol.* **32**, 279–284 (2014).
25. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl Acad. Sci. USA* **109**, E2579–E2586 (2012).
26. Nishimasu, H. et al. Crystal structure of *Staphylococcus aureus* Cas9. *Cell* **162**, 1113–1126 (2015).
27. Ruvkauksis, M. et al. Directional R-loop formation by the CRISPR-Cas surveillance complex cascade provides efficient off-target site rejection. *Cell Reports* **10**, 1534–1543 (2015).
28. Briner, A. E. et al. Guide RNA functional modules direct Cas9 activity and orthogonality. *Mol. Cell* **56**, 333–339 (2014).
29. Robert, X. & Gouet, P. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res.* **42**, W320–W324 (2014).

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Cas9 and nucleic acid preparation. S. pyogenes Cas9 was cloned into a custom pET-based expression vector encoding an N-terminal His6-tag followed by maltose-binding protein (MBP) and a TEV protease cleavage site. Point mutations were introduced using site-directed mutagenesis or around-the-horn PCR and verified by DNA sequencing. dCas9 refers to catalytically inactive (dead) Cas9 containing D10A and H840A mutations. ΔHNH-Cas9 contained a deletion of residues T769–K918 and replacement with a GGSGSGS linker. The HNH domain for add-back experiments (Extended Data Fig. 6d) encoded residues N776–G907. All Cas9 variants were purified as described11 and confirmed using 5–10% denaturing PAGE.

DNA substrates (Extended Data Table 2) were prepared from commercially synthesized oligonucleotides (Integrated DNA Technologies). DNA duplexes without internal mismatches were prepared and purified by native PAGE as described11. DNA duplexes containing internal mismatches or overlaps were prepared by mixing a 5× molar excess of one strand with its complementary strand in hybridization buffer (20 mM Tris-Cl pH 7.5, 100 mM KCl, 5 mM MgCl2), heating at 95°C for 1–2 min, and slow-cooling on the benchtop. For FRET experiments, the non-target strand was in excess over the target strand; for biochemical cleavage experiments, the non-radio-labelled strand was in excess over the radiolabelled strand.

Preparation of dye-labelled Cas9. Labelling reactions were conducted in Cas9 gel filtration buffer (20 mM Tris-Cl pH 7.5, 200 mM KCl, 5% glycerol, 1 mM TCEP) and contained 10 μM Cas9 and 200 μM Cy3- and Cy5-maleimide (GE Healthcare). Dyes were initially dissolved in anhydrous DMSO before being mixed with Cas9, and the final DMSO concentration did not exceed 5%. Reactions were incubated in the dark for 2 h at room temperature (−22°C) followed by incubation overnight at 4°C. Reactions were quenched by adding 10 mM DTT, and labelled Cas9 was separated from free dye by size-exclusion chromatography on a Superdex 200 10/300 column. Samples were then concentrated, snap frozen in liquid nitrogen, and stored at −80°C. Control labelling reactions contained either cysteine-free Cas9 or only one of the two dyes.

FRET experiments. All fluorescence measurements were conducted at room temperature in reaction buffer (20 mM Tris-Cl pH 7.5, 100 mM KCl 5 mM MgCl2, 5% glycerol, 1 mM DTT), supplemented with 50 μg ml−1 heparin to reduce non-specific DNA binding12. Reactions (60 μl) with Cas9 (C805/D435C/C574S/E945C-Cas9 labelled with Cy3/Cy5) and dCas9 (Cy3/Cy5) and additional nuclease-inactivating D10A/H840A mutations) contained either 50 nM or 100 nM Cas9, and, when present, a 10× molar excess of sgRNA and target DNA, respectively. Reactions (60 μl) with Cas9 (C805/S335C/C574S/S867C-Cas9 labelled with Cy3/Cy5) and dCas9 (C805/S335C/C574S/S867C-N1054C-Cas9 labelled with Cy3/Cy5) contained 50 nM Cas9, and, when present, 200 nM sgRNA and DNA unless otherwise indicated.

We observed substantial aggregation of apo-Cas9 upon 10 min incubation at 37°C, as indicated by apparent intermolecular FRET with a single-cysteine Cas9 (C805/C574S/S867C) that had been labelled with a mixture of Cy3- and Cy5-maleimide (data not shown). This aggregation could be completely avoided by incubating reactions for 10 min at room temperature instead, centrifuging reactions for 1 min in the dark for 2 h at room temperature (~22°C) followed by incubation overnight at 4°C. Reactions were quenched by adding 10 mM DTT, and labelled Cas9 was separated from free dye by size-exclusion chromatography on a Superdex 200 10/300 column. Samples were then concentrated, snap frozen in liquid nitrogen, and stored at −80°C. Control labelling reactions contained either cysteine-free Cas9 or only one of the two dyes.

DNA cleavage experiments presented in Figs 2d and 4b, d and Extended Data Figs 1c and 6d used 5′-[32P]DNA duplex substrates radiolabelled on both strands; all other cleavage experiments used DNA duplex substrates with a single 5′-[32P]radiolabelled strand that had been annealed to a 5× molar excess of unlabelled complementary strand. Cas9 and sgRNA were pre-incubated at 37°C for 10 min in reaction buffer before adding DNA. Cleavage reactions were performed at room temperature and contained 1 nM DNA and 100 nM Cas9–sgRNA complex. Aliquots were removed at various time points and quenched by mixing with an equal volume of formamide gel loading buffer supplemented with 50 mM EDTA.

Cleavage products were resolved by 10% denaturing PAGE and visualized by phosphorimaging (GE Healthcare). Cleavage reactions were performed at room temperature and contained 1 nM DNA and 100 nM Cas9–sgRNA complex. Aliquots were removed at various time points and quenched by mixing with an equal volume of formamide gel loading buffer supplemented with 50 mM EDTA.

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Extended Data Figure 1 | Biochemical preparation and DNA cleavage activity of dye-labelled Cas9. a, Size-exclusion chromatograms of Cy3/Cy5-labelling reactions with cysteine-free Cas9 (C80S/C574S) or the two double-cysteine Cas9 variants used to generate Cas9hinge and Cas9HNH-1. Reactions contained 10 μM Cas9 and 200 μM Cy3- and Cy5-maleimide, and were separated on a Superdex 200 10/300 column (GE Healthcare). Cysteine-free Cas9 was unreactive. b, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of unlabelled and dye-labelled Cas9 variants. The gel was scanned for Cy3 and Cy5 fluorescence (right) before being stained with Coomassie blue (left). For gel source data, see Supplementary Fig. 1. c, Representative radiolabelled DNA cleavage assay with wild-type (WT) Cas9 and doubly labelled Cas9 variants used in this study, resolved by denaturing PAGE (left); quantified data and exponential fits are shown on the right. S, substrate; NT, cleaved non-target strand; T, cleaved target strand. Error bars, s.d.; n = 3.
Extended Data Figure 2 | Fluorescence control experiments with Cas9<sub>hinge</sub> and dCas9<sub>hinge</sub>, and representative analysis of fluorescence emission spectra to calculate (ratio)<sub>A</sub>. a, Fluorescence emission spectra of 50 nM Cas9<sub>hinge</sub> in the presence of increasing concentrations of full-length sgRNA. Protein and sgRNA concentrations were calculated under non-denaturing conditions using theoretical extinction coefficients. b, Fluorescence emission spectra of (1) Cy3-labelled Cas9<sub>hinge</sub>, (2) Cy5-labelled Cas9<sub>hinge</sub>, and (3) an equal mixture of Cy3-Cas9<sub>hinge</sub> and Cy5-Cas9<sub>hinge</sub> upon excitation at 530 nm. The minor fluorescence peak for Cy5 in the mixed sample results from residual absorbance of Cy5-Cas9<sub>hinge</sub> at 530 nm and not from intermolecular FRET (compare spectra 3 with 4, which is a sum of spectra 1 and 2). c, Fluorescence emission spectra of Cas9<sub>hinge</sub> in the presence of sgRNA substrates specific to S. pyogenes (Spy) or N. meningitidis (Nme) Cas9. d, Determination of the (ratio)<sub>A</sub> parameter, which is proportional to FRET efficiency. Shown for apo-Cas9<sub>hinge</sub> are (1) an emission spectrum of Cy3/Cy5-Cas9<sub>hinge</sub> upon excitation of the donor at 530 nm; (2) an emission spectrum of donor only Cy3-Cas9<sub>hinge</sub> upon excitation of the donor at 530 nm, normalized to 1; (3) the extracted fluorescence of the acceptor via energy transfer, obtained by subtracting 2 from 1; and (4) an emission spectrum of Cy3/Cy5-Cas9<sub>hinge</sub> upon direct excitation of the acceptor at 630 nm. (Ratio)<sub>A</sub> is calculated by dividing the integrated intensity (650–800 nm) of 3 by the integrated intensity of 4. e, (Ratio)<sub>A</sub> data for dCas9<sub>hinge</sub> in the presence of the same sgRNA substrates tested with nuclease-active Cas9<sub>hinge</sub> in Fig. 1e. Error bars, s.d.; n = 3.
Extended Data Figure 3 | Modelling of the HNH domain docked at the cleavage site, and design of the Cas9HNH-2 FRET construct. a, The scissile phosphate and flanking nucleotides of a DNA substrate co-crystallized with the phage T4 endonuclease VII (endo VII; PDB 2QNC; left) were aligned with the scissile phosphate and flanking nucleotides of the DNA target strand in the sgRNA/DNA-bound Cas9 crystal structure (PDB 4UN3; middle). Structural alignment of the Cas9 HNH domain with endonuclease VII (middle) results in a model of how the Cas9 HNH domain docks at the cleavage site (right). Catalytic residues are labelled, target strands are shown in red and pink, and a magnesium ion is depicted as a blue sphere. b, Conservation rendering of the sgRNA/DNA-bound Cas9 crystal structure, generated using ConSurf, shows that the most highly conserved patches of the HNH domain, including the active site, are solvent-exposed in the observed conformation. The HNH domain is omitted from the view on the left for clarity. c, Magnified view of the HNH domain in its observed conformation (left) and the model for the docked state (right), coloured as in b. The DNA target strand fits snugly in a groove on the HNH domain in the model, with the most highly conserved patches located in the immediate vicinity of the scissile phosphate. DNA and sgRNA are coloured red and orange, respectively. d, The conformational flexibility of the HNH domain in available Cas9 crystal structures is revealed by structural alignment of the nuclease lobe (RuvC and PI domains) from two sgRNA/DNA-bound structures (PDB accession numbers 4UN3 and 4O08) and the sgRNA-bound structure (PDB 4ZT0). The modelled docked state from a is shown. e, Design of Cas9HNH-2 FRET construct. Measured distances between ~N1054 and S867 in the sgRNA/DNA-bound Cas9 structure and a model of the HNH domain docked at the cleavage site are indicated. Putative conformational changes of the HNH domain are shown with a black arrow.
Extended Data Figure 4 | Evidence that variable \((\text{ratio})_A\) values for dCas9\(_{\text{HNH-1}}\) reflect distinct conformational states/dynamics, and FRET data for Cas9\(_{\text{HNH-2}}\). a, DNA binding assay with dCas9 and either on-target DNA or off-target DNAs containing 2, 4, or 8-bp mismatches at the PAM-distal end. Binding fits are shown as solid lines and yield equilibrium dissociation constants (\(K_d\)) of 0.80, 6.7, 19, and 20 nM, respectively. Given these values, 99%, 96%, 89%, and 89% of dCas9 should be bound to DNA under the conditions used for FRET experiments in Fig. 2c (50 nM dCas9\(_{\text{HNH-1}}\), 200 nM DNA). b, \((\text{ratio})_A\) data for 50 nM dCas9\(_{\text{HNH-1}}\) in the presence of 1 \(\mu\)M sgRNA and either 200 nM, 400 nM, or 1 \(\mu\)M off-target DNAs containing 2- or 4-bp mismatches. Data for sgRNA only and on-target DNA are shown for comparison. c, DNA cleavage time courses for the indicated DNA substrates using wild-type Cas9. Exponential fits are shown as solid lines, and extracted rate constants are shown in Fig. 2d. d, Fluorescence emission spectra of Cas9\(_{\text{HNH-2}}\) in the presence of the indicated substrates. The inset shows \((\text{ratio})_A\) values; mut, mutation. Error bars in a and b–d, s.d.; \(n = 3–5\) and 3, respectively.
Extended Data Figure 5 | Additional experimental support for dependence of RuvC nuclease activity on HNH conformational changes. 

**a**, Panel of DNA substrates tested in **b**, with on-target (1) at top. Matched and mismatched positions of DNA target strand sequences relative to the sgRNA are coloured red and black, respectively, with the PAM in yellow. Some substrates contain internal mismatches between the two DNA strands; dashed lines indicate additional flanking sequence. 

**b**, Kinetics of non-target (black) and target (red) strand cleavage for the indicated DNA substrates.

**c**, Panel of DNA substrates tested in **d** and **e**, depicted as in **a**, (Ratio)\textsubscript{A} data for Cas9\textsubscript{HNH-1} in the presence of the indicated DNA substrates.

**d**, Non-target strand cleavage kinetics of the RuvC domain for the indicated DNA substrates. Error bars in **b**, **d**, **e**, s.d.; \( n = 3 \).
Extended Data Figure 6 | Design, purification, and DNA cleavage activity of ΔHNH-Cas9. a, Domain organization of WT- and ΔHNH-Cas9 (top), showing the residues that were replaced with a GGS2 linker to generate ΔHNH-Cas9. Magnified view of connections between the HNH domain and RuvC II and III motifs in the apo (left) and sgRNA/DNA-bound (right) Cas9 crystal structures, as well as in the ΔHNH-Cas9 construct. Disordered linkers and the introduced GGS2 linker are shown as dashed lines. b, Size-exclusion chromatograms of WT- and ΔHNH-Cas9 using a Superdex 200 16/60 column (GE Healthcare). c, SDS–PAGE analysis of dCas9 (D10A/H840A), WT-Cas9, ΔHNH-Cas9, and the purified HNH domain (residues 776–907). Expected molecular masses are 159 kDa, 159 kDa, 142 kDa, and 16 kDa, respectively. For gel source data, see Supplementary Fig. 1. d, Representative radiolabelled DNA cleavage assay with WT-Cas9, ΔHNH-Cas9, ΔHNH-Cas9 in the presence of excess HNH domain, and HNH domain alone, resolved by denaturing PAGE.
Extended Data Figure 7 | Structural analysis and perturbation of the HNH–RuvC III linker. a, Molecules A (left) and B (right) of the sgRNA/DNA-bound Cas9 crystal structure (PDB 4O08). Molecule A has an ordered HNH domain and HNH–RuvC III linker, whereas these are both disordered in molecule B; the missing density for the HNH domain is replaced with the modelled docked state (right). Another prominent difference is the N-terminal region of the RuvC III motif (blue helices), which rearranges from a helix–loop–helix in molecule A into an extended helix in molecule B. Proline pairs were inserted to prevent formation of this extended helix.

b, Target (red) and non-target (black) strand cleavage time courses with the indicated Cas9 variant. Exponential fits are shown as solid lines.

c, Kinetics of target (red) and non-target (black) strand cleavage for the indicated Cas9 mutants. ND, cleavage not detected. Error bars in b and c, s.d.; n = 3.
## Extended Data Table 1 | Measured distances between residues labelled with FRET pairs

| Structure used                        | Inter-residue distance * |
|--------------------------------------|--------------------------|
|                                       | D435–E945    | S355–S867 | S867–N1054 |
| Apo (4CMP)                           | 21 Å         | 79 Å      | 6 Å        |
| sgRNA-bound (4ZT0)                   | 78 Å         | 81 Å      | 7 Å        |
| sgRNA/DNA-bound (4OOS mol A)         | 77 Å †       | 61 Å      | 34 Å ‡     |
| sgRNA/DNA-bound (4UN3)               | 83 Å         | 59 Å      | 28 Å §     |
| sgRNA/DNA-bound, HNH docked state || 21 Å         | 57 Å §     |

*Distances were measured between Cα atoms of the indicated residues, except where indicated, for the denoted structures (PDB accession numbers in parentheses).
†E945 is disordered in the structure; an average of measured distances to T941 and I950 is reported.
‡N1054 is disordered in the structure; an average of measured distances to T1048 and I1063 is reported.
§N1054 is disordered in the structure; an average of measured distances to I1050 and K1059 is reported.
|| The docked state for the HNH domain was generated using PDB accession numbers 4UN3 and 2QNC.
**Extended Data Table 2** | RNA and DNA substrates used in this study

| Description | Sequence * |
|-------------|------------|
| A1-targeting sgRNA | 5′-GACCAUAAAGAAGGCACTGGCCGTGAATACATTAGTTTATGACG-3′ |
| A1-targeting sgRNA, Alt site | 5′-GACCAUAAAGAAGGCACTGGCGGTGAAATCTTACATTGTTATGACG-3′ |
| A1-targeting sgRNA, Δguide1-5 | 5′-GACCAUAAAGAAGGCACTGCTGATGAAATCTTACATTGTTATGACG-3′ |
| A1-targeting sgRNA, Δguide1-10 | 5′-GACCAUAAAGAAGGCACTGCTGATGAAATCTTACATTGTTATGACG-3′ |
| A1-targeting sgRNA, Δguide1-15 | 5′-GACCAUAAAGAAGGCACTGCTGATGAAATCTTACATTGTTATGACG-3′ |
| A1-targeting sgRNA, Δguide1-20 | 5′-GACCAUAAAGAAGGCACTGCTGATGAAATCTTACATTGTTATGACG-3′ |
| A1-targeting sgRNA, Δtarget1 | 5′-GACCAUAAAGAAGGCACTGCTGATGAAATCTTACATTGTTATGACG-3′ |
| A1-off target DNA, Substrate 2, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 3, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, 1-pm mismatch | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, 2-pm mismatch | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, 3-pm mismatch | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, 4-pm mismatch | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, 6-pm mismatch | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, 8-pm mismatch | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 2, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 3, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 4, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 5, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 6, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 7, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 8, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 9, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 10, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 11, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 12, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 13, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 14, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |
| A1-off target DNA, Substrate 15, ED Fig | 5′-AAGAGGACTCCGTTGCTCAAAAAGAGAGACG-3′ |

*sgRNA guide sequences and matching DNA target strand sequences are shown in red. PAM sites (5′-NGG-3′) are highlighted in yellow on the non-target strand. Internal mismatches in select DNA substrates are denoted by misaligned text on the non-target strand.

†All sgRNA constructs contain remnants of the BamHI sequence on the 3′ end resulting from run-off in vitro transcription.

§sgRNA specific to *N. meningitidis* (*Nme*) Cas9 contains an additional 3′ extension, which does not affect activity (data not shown), for purposes unrelated to this study.

Δguide1–20 sgRNA contains an extraneous 5′-G from in vitro transcription.