EXTENDED DATA FIGURE LEGENDS

Extended Data Figure 1 | Activity assays of reagents used in single-molecule experiments. a, Cleavage assays were conducted using radiolabeled 55-base-pair (bp) DNA substrates that contained the six λ-DNA sequences targeted in **Fig. 1d**. Each DNA substrate (~1 nM) was incubated with 100 nM Cas9:RNA complex reconstituted using the corresponding guide RNA, and reaction products were resolved by 10% denaturing polyacrylamide gel electrophoresis (PAGE). Reactions contained 3x-FLAG-tagged Cas9 (where indicated) or untagged, wild-type Cas9. * denotes further trimming of the non-target strand. **b,** Cleavage assay of λ-DNA under conditions identical to those used in single-molecule experiments. Full-length λ-DNA (25 ng μl^{-1}) was incubated with 10 nM Cas9:RNA reconstituted using the λ6 guide RNA, and reaction products were resolved by agarose gel electrophoresis. Successful cleavage is expected to generate DNA products that are 42,051 and 6,451 bp in length. When present, imaging components included anti-FLAG antibody-coated quantum dots, YOYO1, BSA, glucose, and glucose oxidase/catalase.

Extended Data Figure 2 | Binding histograms and Gaussian fits for λ-DNA target binding, and analysis of off-target binding. a, Binding distributions for dCas9 programmed with λ1-λ6 guide RNAs were measured as described in the Materials and Methods, and the data for each individual experiment was then bootstrapped and fit with a Gaussian curve. Shown in number of base pairs is the mean, μ, and standard deviation, σ, obtained from each fit, as well as the expected location of each target site in λ-DNA. **b**, Distribution of Cas9:RNA binding events for λ2 crRNA (N = 2,330, top) and spacer 2 crRNA (N = 2,190, bottom). The density of PAM sites throughout

the λ-DNA substrate is shown in red. **c**, Survival probabilities for non-target binding events with λ2 and spacer 2 crRNA. Data were collected at 25 mM KCl.

Extended Data Figure 3 | DNA binding by apo-dCas9 and dCas9:RNA. a, Electrophoretic mobility gel shift assay (left) with radiolabeled 55-bp target DNA and increasing concentrations of dCas9:RNA, using a 10X excess of crRNA:tracrRNA over dCas9. The quantified data (right) were fit with a standard binding isotherm (solid line), and data from three such experiments yielded a equilibrium dissociation constant (K_d) of 0.49 ± 0.21 nM. **b,** Results for apo-dCas9 shown as in (**a**). Data from three independent experiments yielded a K_d of 26 ± 15 nM. **c,** crRNA:tracrRNA duplex and heparin dissociate apo-dCas9 bound to non-specific DNA, but not dCas9:RNA complexes bound to target DNA. 55-bp DNA substrates were pre-incubated with the indicated reagent for 15 minutes at 37 °C, at which point non-targeting crRNA:tracrRNA duplex (10–1000 nM) or heparin (0.01–100 μg mL^{-1}) was added. Reactions were incubated an additional 15 minutes at 37 °C and then resolved by 5% native PAGE. Reactions at the far right show that apo-dCas9 pre-bound to target DNA can be dissociated by complementary crRNA:tracrRNA and re-bind the same DNA in complex with RNA. Note the distinct mobilities of DNA in complex with apo-dCas9 versus DNA in complex with dCas9:RNA.

Extended Data Figure 4 | Target DNA cleavage products remain bound to Cas9:RNA. a,b, DNA substrates 72 nucleotides (nt) in length were radiolabeled at either their 5' or 3' ends and annealed to an unlabeled complementary strand, where indicated (top). The non-target strand contains the PAM (yellow box), whereas the target strand contains the sequence complementary to crRNA (red). Each DNA substrate (~1 nM) was incubated with 100 nM Cas9:RNA complex for

30 minutes at room temperature, using nuclease-inactive D10A/H840A Cas9 (d), both nickase mutants (D10A, n1; H840A, n2), and wild-type (WT). Half the reaction volume was quenched in formamide gel-loading buffer containing 25 mM EDTA and analyzed by 10% denaturing PAGE to verify the expected cleavage pattern of each sample (**a**). The other half of each reaction was analyzed by 5% native PAGE to determine whether the radiolabeled DNA fragment remained bound to Cas9:RNA (**b**). Aside from an apparent reduced affinity for the single-stranded target strand after cleavage, WT Cas9:RNA shows an affinity for all four possible radiolabeled DNA products that is indistinguishable from the affinity of dCas9:RNA for uncleaved DNA substrates. Note that the order of samples in (**a**) and (**b**) is identical. The additional band present for double-stranded DNA substrates in (**a**) results from incomplete denaturation and partial migration of intact duplex into the gel (*).

Extended Data Figure 5 | Cas9:RNA acts as a single-turnover enzyme. a, Agarose gel electrophoresis (1%, TBE buffer) was used to assess cleavage of plasmid DNA containing a λ2 target sequence as a function of Cas9:RNA concentration. DNA (25 nM) was incubated with the indicated concentration of Cas9:RNA, and aliquots were removed at each time point and quenched with gel loading buffer containing 25 mM EDTA. The gel was stained with ethidium bromide, and the quantified data is presented in **Fig. 2b**. **b,** Similar turnover experiments were conducted with 25 nM radiolabeled λ2 oligoduplex substrates and increasing concentrations of Cas9:RNA. Cleavage data were visualized by phosphorimaging, and * denotes further trimming of the non-target strand. **c,** Turnover experiments with 25 nM Cas9:RNA were repeated at 37 °C and with a 10X excess of crRNA:tracrRNA over Cas9. Neither condition significantly stimulates turnover. **d,** Quantified data from experiments in (**b**) and (**c**) show that each reaction reaches its maximum

yield after ~1 minute and does not increase with further incubation time, demonstrating that Cas9:RNA exhibits single-turnover activity. Note that the observed requirement for a slight stoichiometric excess of Cas9:RNA over DNA to reach reaction completion is likely a result of our enzyme preparations not being 100% active. While modest turnover (2.5-fold) was observed at a single enzyme:substrate stoichiometry in Jinek *et al.* (*Science* **337**, 816-821 [2012]), our results clearly demonstrate that the reaction yield remains proportional to the molar ratio between Cas9:RNA and DNA across a range of concentrations.

Extended Data Figure 6 | Analysis of competition cleavage assays. a, Representative cleavage assays as a function of competitor DNA concentration, using a competitor containing 12 PAM sites. Radiolabeled λ1 target DNA (1 nM) was incubated with 10 nM Cas9:RNA and increasing concentrations of the competitor, and reaction products at each time point were resolved by 10% denaturing PAGE. Cleavage data were visualized by phosphorimaging, and * denotes further trimming of the non-target strand. **b,** Shown are the conditional survival probabilities for the radiolabeled target DNA at each concentration of 12-PAM competitor. **c,** Shown is the change in survival probability of the target DNA, $\Delta P_s(t)$, as a function of 12-PAM competitor concentration. The area under each curve represents the amount of time that Cas9:RNA spent on the competitor DNA during the reaction. **d,** Competition data with a panel of substrates that have no complementarity to the guide RNA and variable numbers of PAMs, and a perfect target sequence with single base-pair mutation in the PAM. The data are presented similarly to **Fig. 3c**, but the time bound to competitor is shown for all five concentrations of competitor tested.

Extended Data Figure 7 | PAM sites in non-target DNA are bound specifically by dCas9:RNA. a, None of the competitors from **Fig. 3c** can be cleaved, including one that bears full complementarity to the crRNA but contains a single base-pair mutation in the PAM. Radiolabeled competitor DNAs and target DNA (1 nM) were incubated with 100 nM WT Cas9:RNA for the indicated time, and reaction products were assessed by 10% denaturing PAGE. * denotes further trimming of the non-target strand. **b,** PAM-rich competitor DNAs interfere with target DNA binding by dCas9:RNA. The same radiolabeled 55-bp target DNA from **Fig. 3b,c** was pre-mixed with increasing concentrations of the indicated competitor DNA and then incubated with 10 nM dCas9:RNA for 60 minutes at 37 °C. Binding reactions were resolved by 5% native PAGE. **c,** dCas9:RNA has increased affinity for non-target DNA containing multiple PAM sequences. The indicated radiolabeled DNA substrates (~0.02 nM) were incubated with increasing concentrations of dCas9:RNA for 60 minutes at 37 °C, and reactions were resolved by 5% native PAGE. The observed well-shifting at high concentrations may result from multiple dCas9:RNA molecules binding the same DNA substrate.

Extended Data Figure 8 | Cas9:RNA binds and cleaves bubble-containing DNA substrates with mismatches to the crRNA that are otherwise discriminated against within the context of perfect duplexes. a, dCas9:RNA has weak affinity for a substrate containing a 2-bp mismatch to the crRNA (middle), whereas a substrate presenting the same mismatches within a small 2-nt bubble (right) is bound with an affinity nearly indistinguishable from a perfect target substrate (left), in agreement with data presented in **Fig. 3e**. The indicated DNA substrates were incubated with increasing concentrations of dCas9:RNA for 60 minutes at 37 °C, and reactions were resolved by 5% native PAGE. **b,** The same bubble-containing substrate in (**a**) is cleaved with

similar kinetics as a perfect substrate (compare right and left time courses), whereas a perfectly base-paired substrate with the same pattern of complementarity to the crRNA is cleaved with substantially reduced kinetics (middle). Radiolabeled DNA substrates (1 nM) were incubated with 100 nM WT Cas9:RNA for the indicated time, and reaction products were resolved by 10% denaturing PAGE. * denotes further trimming of the non-target strand.

Extended Data Figure 9 | PAM recognition activates the nuclease activity of Cas9. a, DNA substrates were prepared using the λ2 target sequence as indicated (top), where the flanking region extending beyond the PAM was 16 bp (cleavage experiments) or 26 bp (binding experiments). **b,** For cleavage experiments, substrates were prepared by annealing the radiolabeled target strand (i.e. substrate 2) to a 5X excess of cold complement, and 1 nM DNA was reacted with 50 nM Cas9:RNA at room temperature. Reaction products were resolved by 10% denaturing PAGE, and the quantified data were fit with single-exponential decays (solid lines). Results from three independent experiments yielded apparent pseudo-first order cleavage rate constants of 9.0 ± 2.0 min^{-1} (substrate 1), 0.067 ± 0.027 min^{-1} (substrate 2), 0.066 ± 0.024 min^{-1} (substrate 3), and 7.3 ± 3.2 min^{-1} (substrate 4), and are presented as values relative to substrate 1 in **Fig. 4b**. Rate constants for substrates 2 and 3 are likely overestimates, since the reactions did not approach completion and the data were best fit with amplitudes well below 1. **c,** For binding experiments, substrates were gel purified after annealing the radiolabeled target strand (i.e. substrate 2) to a 10X excess of cold complement. Binding reactions contained ~0.1 nM DNA and increasing concentrations of dCas9:RNA, and were incubated at 37 °C for one hour before being resolved by 5% native PAGE. The quantified data were fit with standard binding isotherms (solid lines). Results from three independent experiments yielded apparent K_d values of 0.27 ± 0.14 nM (substrate 1), 0.28 ± 0.12

nM (substrate 2), 0.59 ± 0.18 nM (substrate 3), and 0.21 ± 0.06 nM (substrate 4), and are presented as values relative to substrate 1 in **Fig. 4b**.

Extended Data Table 1 | RNA and DNA substrates used in this study.

* crRNA guide sequences and complementary DNA target strand sequences are shown in red. PAM sites (5'-NGG-3') are highlighted in yellow on the non-target strand when adjacent to the target sequence, except for PAM competitors in which case all PAMs are highlighted.

† The reverse complement of the T7 promoter is indicated in bold.

‡ The second nucleotide of the λ1-targeting crRNA was mutated from G to A to match the λ1 target duplex sequence.

§ The underlined base pairs were mutated relative to the wild-type λ-DNA sequence in order to remove all PAM sites from the substrate other than the PAM immediately adjacent to the target sequence. The crRNA was mutated accordingly, as were the λ1 competitor DNAs.

|| The duplex was cloned into EcoRI and BamHI sites on pUC19.

NA, not applicable.