A kinetic model predicts SpCas9 activity, improves off-target classification, and reveals the physical basis of targeting fidelity

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The S. pyogenes (Sp) Cas9 endonuclease is an important gene-editing tool. SpCas9 is directed to target sites based on complementarity to a complexed single-guide RNA (sgRNA). However, SpCas9-sgRNA also binds and cleaves genomic off-targets with only partial complementarity. To date, we lack the ability to predict cleavage and binding activity quantitatively, and rely on binary classification schemes to identify strong off-targets. We report a quantitative kinetic model that captures the SpCas9-mediated strand-replacement reaction in free-energy terms. The model predicts binding and cleavage activity as a function of time, target, and experimental conditions. Trained and validated on high-throughput bulk-biochemical data, our model predicts the intermediate R-loop state recently observed in single-molecule experiments, as well as the associated conversion rates. Finally, we show that our quantitative activity predictor can be reduced to a binary off-target classifier that outperforms the established state-of-the-art. Our approach is extensible, and can characterize any CRISPR-Cas nuclease – benchmarking natural and future high-fidelity variants against SpCas9; elucidating determinants of CRISPR fidelity; and revealing pathways to increased specificity and efficiency in engineered systems.

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CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats—CRISPR-associated protein 9) has become a ubiquitous tool in the biological sciences, with applications ranging from live-cell imaging and gene knockdown/overexpression to genetic engineering and gene therapy. Streptococcus pyogenes (Sp) Cas9 can be programmed with a ~100 nucleotide (nt) single-guide RNA (sgRNA) to target DNAs based on the level of complementarity to a 20 nt segment of the sgRNA. Wildtype SpCas9 (henceforth Cas9) induces site-specific double-stranded breaks and the catalytically dead Cas9 (dCas9) mutant allows for binding without cleavage. Apart from complimentary on-targets, Cas9-sgRNA also binds and cleaves non-complementary off-targets. Off-target cleavage risks deleterious genomic alterations, which has so far impeded the widespread implementation of the CRISPR toolkit in human therapeutics.

Strong off-target sites are identified in silico by a growing set of tools. These tools use bioinformatics, machine learning, or heuristic approaches to rank genomic sites based on distinctive off-target activity scores. Though such models can identify strong off-targets, they are not quantitative and cannot assess activity on the many lesser off-targets; nor can they predict how activity changes with exposure time and enzyme concentration—even though these parameters are frequently exploited to limit off-target activity in cells.

To implement quantitative activity prediction, Cas9 targeting must be modelled in physical terms. Existing physical models assume binding equilibration before cleavage, and it remains unclear what predictive power such approaches can ultimately deliver in this non-equilibrium system. To account for the nonequilibrium nature of the targeting reaction, we construct a mechanistic model that captures binding and cleavage kinetics in integral terms. To gain insights into general mechanisms, we train and validate our model on high-throughput datasets that capture both binding and cleavage in bulk experiments. Though we restrict our training to off-targets with two or less mismatches, we accurately predict the activities on all more highly mismatched off-targets in the same datasets, as well as those reported in two independent high-throughput datasets.

To reveal the physical basis of Cas9 fidelity on genomic scales, we extract the free-energy landscapes that control PAM binding, strand-replacement, and cleavage on any target. Our characterization of Cas9 supports the notion that observed differences in binding and cleavage activities stem from a relatively long-lived DNA-bound RNA-DNA hybrid (R-loop) intermediate. This R-loop intermediate was recently observed directly in single-molecule experiments. Though we restrict our training to off-targets with two or less mismatches, we accurately predict the activities on all more highly mismatched off-targets in the same datasets, as well as those reported in two independent high-throughput datasets.

To calculate how (d)Cas9 activity evolves in time under various conditions, we also sought to compare our approach to existing binary off-target classifiers that identify strong off-targets. To this end, we reduce our quantitative activity predictor to a binary off-target classifier that outperforms the leading tools used today.

Results

The kinetic model. In Fig. 1a we show the reaction pathway that underpins the Cas9 targeting reaction on every target. The reaction starts with Cas9-sgRNA ribonucleoprotein complex exiting the solution state to specifically bind to a 3nt protospacer adjacent motif (PAM) DNA sequence—canonically 5’-NGG-3’—via protein-DNA interactions. Binding to the PAM sequence (state 0) opens the DNA double helix, and allows the first base of the target sequence to hybridize with the sgRNA, forming the first R-loop state (state 1). The DNA double helix further denatures as the RNA-DNA hybrid is extended in the guide-target strand-replacement reaction (state 2–20). The hybrid grows and shrinks in single-nucleotide steps, until it is either reversed and Cas9 dissociates, or it reaches completion at 20 base pairs (bp) in state 20. If the full hybrid is formed, Cas9 can use its HNH and RuvC nuclease domains to cleave both DNA strands.

If we know the conversion rates in Fig. 1a for a particular guide and target, the reaction scheme can be solved to calculate the binding and cleavage probabilities at any time (Methods). Fully parameterizing the model over all guide and target sequences requires the estimation of ~10^20 rates. To render parameter estimation tractable, we make four mechanistic-model assumptions:

1. Mismatch positions are more important than mismatch types (e.g. G-G vs. G-A). This can be directly inferred from data, and we treat all 12 mismatch types equally.
2. Mismatch energies are determined by local interactions. The energetic cost of multiple mismatches is taken to be equal to the sum of the energetic costs of the individual mismatches.
3. dCas9 differs from Cas9 only in that dsDNA bond-cleavage catalysis is completely suppressed (k_cat = 0); all other rates are taken to be identical.
4. All selectivity is governed by the hybrid-bond-reversal rates. Hybrid-bond-formation rates are treated as equal, independent of complementarity and location.

These assumptions reduce the total number of microscopic parameters to 44 (see Methods): the concentration dependent rate of PAM binding from solution (k_on) and the associated free-energy gain (F_{0}); a single internal forward bond-formation rate (k_f); 20 free energies dictating R-loop progression at the on-target (F_{1}, …, F_{20}); 20 free-energy penalties for mismatches at different R-loop positions (\delta_{e_{1}}, …, \delta_{e_{20}}); and the rate at which the final cleavage reaction is catalyzed for Cas9 (k_cat). Once model parameters are estimated, all possible off-target free energies can be directly calculated using assumptions 1–4 above. In Fig. 1b we illustrate the calculation taking us from the on-target (pink) to the off-target (blue) free-energy landscape with mismatches entering the hybrid at the 3rd and 15th bp. How to translate between free energies and rates is detailed in Methods.

Base-pairing interactions, protein-DNA interactions, and induced conformational changes contribute to the stability of the Cas9- sgRNA-DNA complex. To account for the varying nature of these interactions, we allow for varying gains and losses in the on-target free-energy landscape as the hybrid is extended. These variable gains and losses allow for the formation of metastable states on the on-target, and constitutes an essential extension of our previous fixed-gain model for RNA-guided nuclease kinetics, as well as of models describing DNA displacement reactions occurring in solution.

Training on binding and cleavage for moderately mismatched targets. We seek to reveal general properties of SpCas9 DNA targeting on genomic scales. To this end, we train and validate our model on data from two highly reproducible bulk-biochemical experiments performed on a large library of moderately to highly mismatched off-targets. The first set (NucleoSEQ) has 97% correlation between replicated experiments, and estimates the effective cleavage rates (k_{clv}^{eff}) for a library of off-targets exposed to Cas9-sgRNA for 16 hours. The second set (CHAMP) has 94% correlation between replicated experiments, and reports on the effective association constant (K_{A}^{eff}) over the same library and guide, but this time exposed to dCas9-sgRNA.
for 10 min. In Methods we detail how the experiments are modeled.

We estimate the model parameters by minimizing the total experimental-error weighted residue between prediction and experiment for off-targets (see Methods) with no more than two mismatches in the NucleaSeq (Fig. 2a–c) and CHAMP (Fig. 2d–f) experiments. The rates and association constants from different types of mismatches are averaged (see Methods and Supplementary Data 1), and the optimal solution is sought with a Simulated Annealing algorithm59 (see Methods).

The two training sets differ significantly (Fig. 2, and Supplementary Fig. 1a). Our model still reproduces effective cleavage rates (Fig. 2a–c) and effective association constants (Fig. 2d–f) with a Pearson correlation of 93% and 98% respectively, and quantitatively captures the difference between binding and cleavage activity. The time and concentration dependence of (d)Cas9 activity can be explored through a dashboard we provide (see Code Availability).

Validation on highly mismatched targets and independent data sets. Apart from the data we use for training (two or less mismatches), the NucleaSeq15 and CHAMP1531 sequence libraries also includes block-mismatched targets with more than two mismatches. In Fig. 3a, b we show that we quantitatively predict effective association constants on these highly mismatched targets at a correlation of 98%. Our method also successfully separates out the single dominating off-target present among highly mismatched targets in the NucleaSeq experiments (Supplementary Fig. 1b), resulting in a perfect correlation.

To further validate our model, we test against two data sets from HTS-FLIP experiments reported in the literature11. The first independent validation set records the association rate relative to the on-target, estimated over 1500 seconds of exposure to dCas9-sgRNA at 1 nM concentration (Fig. 3c–e). The second independent validation set records the dissociation rate relative to the on-target, estimated over 1500 seconds following 12 hours of exposure to a saturating dCas9-sgRNA concentration (Fig. 3f–h). Our model quantitatively captures the relative association rates for all reported targets with 82% correlation (Fig. 3e). For the relative dissociation rates, the correlation is more modest at 46% (Fig. 3h), and the quantitative agreement is lost in some regions (Fig. 3f–h). We still seem to capture the general trends on moderately mismatched targets (Fig. 3f, g), though our model will never give binding/dissociation rates above/below that of the on-target, as is reported for some highly mismatched targets (Fig. 3e, h).

Physical characterization of SpCas9 and the intermediate R-loop state. As our model parameters carry physical meaning, estimating them from data amounts to characterizing the system in physical terms. For Cas9, it has been experimentally shown that R-loop progression is controlled by an intermediate metastable state on the on-target42. We expect this intermediate state to show up as a local minimum in our estimated on-target free-energy landscape. The free energy of any metastable state will have a strong influence on the observed dynamics, and we expect such energies to be well constrained by the data. We expect barriers between metastable states to be harder to resolve, as the details of barrier regions matter less for the observable dynamics.

We here report 33 near-equivalent optimization runs that all resulted in a residue that fell within 15% of the best solution found (see Supplementary Video 1). In Fig. 4a we plot the resulting on-target free-energy landscapes, with the optimal solution highlighted in pink. As expected, we see metastable states in the on-target free-energy landscape. With Cas9 in solution or PAM-bound, we have a well-defined free-energy minimum where the R-loop is closed (C). The on-target free energy (Fig. 4a) increases substantially when forming the first hybrid bp in state 1, and remains relatively high and poorly constrained up to and including state 8. The energy of state 9–12 are well constrained, and among them we find a second local minimum. We identify these states as belonging to an intermediate (I) R-loop state. For hybrids of length 13 to 19 bp we again see an ill-constrained barrier, ending when we enter a well-constrained local minimum of a fully formed hybrid at state 20. This last minima defines the open (O) R-loop.

Mismatch penalties are all around 5k_BT (Fig. 4b), but show reproducible variation along the hybrid. Comparing Fig. 2a, d with Fig. 4b, it is clear that variations in mismatch penalties in the first 8 states correlate strongly with the measured effective cleavage rate/dissociation constant on targets with a single seed mismatch at the corresponding hybrid position. It is not clear if these variations are due to varying interactions with the protein,
or reflects the fact that the possible mismatch types vary with position. In Fig. 4c we show the remaining rates needed to predict Cas9 cleavage activity at any target, time, and Cas9-sgRNA concentration (see Methods).

**R-loop dynamics captures single-molecule experiments.** The recent direct observation of the R-loop dynamics between metastable states allows us to further test our model against quantitative single-molecule data. To this end, we define a coarse-grained model (Fig. 5a) and calculate the effective rates between metastable states from our microscopic free-energy landscapes (see Methods). In Supplementary Fig. 2 we show that predictions based on our coarse-grained model replicate those of the microscopic model.

Using effective rates between metastable states, we can rationalize the broad strokes of Cas9 fidelity by considering a few important examples. For on-targets (Fig. 5b), the transition between the PAM bound state and the intermediate R-loop state is reversible ($k_{\text{IP}} \approx k_{\text{OP}}$) (Fig. 5c). Complexes that enter the intermediate state typically also enter the fully opened state ($k_{\text{IP}} \ll k_{\text{IP}}$). The transition from intermediate to open R-loop configuration is irreversible ($k_{\text{IO}} \gg k_{\text{OI}}$), and entering the open configuration guarantees cleavage ($k_{\text{IO}} \ll k_{\text{cat}}$). Taken together, the on-target reaction is essentially unidirectional toward cleavage, once the intermediate state is entered. The transition into the intermediate R-loop state is rate-limiting ($k_{\text{IP}} \ll k_{\text{IP}} \ll k_{\text{cat}}$) for cleavage.

Mismatches between the target DNA and the sgRNA have differential effects on R-loop propagation depending on position. A PAM-proximal mismatch (position 1–8) (Fig. 5d) strongly suppresses the rate of transition from a closed to intermediate R-loop state (Fig. 5e). In contrast, a PAM-distal mismatch (position 12–17) (Fig. 5f) limits the effective rate of cleavage by reducing the intermediate to open transition rate (Fig. 5g), and allowing for re-closure of the R-loop before entering the open state ($k_{\text{IO}} \approx k_{\text{IP}}$).

These observations are in agreement with the experimental observation, and in Fig. 5c, e we use purple triangles to indicate measured rates when available at zero torque. We quantitatively
predict the conversion rates out of the intermediate R-loop state. The model also captures the position of the on-target intermediate state as being around hybrid length 9–12. Our model does not capture the rate of the open to intermediate transition, and future work will have to determine if this is due to a difference in experimental conditions or because our choice of training data is ill-suited to determine the free energies past the intermediate state.

Our model predicts rates on all off-targets, and so extends and refines the long-established rule of thumb that off-target rejection in the PAM proximal seed requires only one mismatch, while off-target rejection outside the seed region requires multiple mismatches. In particular, our model quantifies the intermediate activity resulting from PAM distal mismatch, and so enables prediction of activity titration.

**R-loop dynamics resembles conformational dynamics.** Next, we wondered what structural properties of Cas9 give rise to the free-energy landscape of Fig. 4a. A comparison between DNA-bound and unbound Cas9-sgRNA structures have revealed that Cas9 repositions its HNH and RuvC nuclease domains to catalyze cleavage and unbound Cas9-sgRNA complexes have been identified as a third intermediate conformer. The relative position and occupancy of the HNH states is affected by R-loop mismatches, and Ivanov et al. suggest that the intermediate R-loop state is linked to the intermediate structural state seen in FRET experiments. To test this hypothesis, we mimicked the experiments of Dagdas et al., and considered the time evolution of the occupancy of our metastable R-loop states for two target sequences (Fig. 6). The HNH-domain completes its conformational change within seconds after Cas9-sgRNA binds to on-target DNA, and our model demonstrates a similar behavior for R-loop progression (Fig. 6a). The intermediate structural state is visited only transiently, as is the intermediate R-loop state in our model (Fig. 6a). Compared to the on-target, PAM-distal mismatches maintain the entry rate into the intermediate structural state, while increasing the time spent in this state; again in close agreement with our findings for the intermediate and open metastable R-loop states in the presence of a PAM distal mismatch (Fig. 6b). Taken together, our model supports the notion that the intermediate R-loop state is linked to the intermediate structural state seen in FRET experiments.

**Kinetic modelling improves genome-wide off-target prediction.** Current methods for identifying strong off-targets rank genomic sequences according to various measures of activity. They do not quantitatively predict biochemically measurable parameters, nor do they normally capture changes in conditions or activity over time. Our approach overcomes these limitations, and we do not suggest that these benefits should be abandoned in order to construct a binary off-target classifier. Still, to strengthen the case for including the full non-equilibrium nature of the problem in any Cas9 modelling, we reduce our quantitative kinetic model to a binary classifier (Supplementary Data 1). The quoted correlation coefficients are Pearson-correlation coefficients, and correlation plots are displayed with log-scales to show the quantitative agreement also for weak targets. The dashed lines in the correlation plots correspond to perfect quantitative prediction.
(CFD) score\textsuperscript{12}, since it is a much-used tool for off-target classification.

To compare our model against the three selected off-target classifiers, we choose to rank all genomic sites based on cleavage activity in the low enzyme-concentration limit (see Methods). We make our comparison over all canonical PAM sites in the human genome. True positive off-targets are collected from sequencing-based cleavage experiments that used industry-standard sgRNAs and reported multiple off-target cleavage sites\textsuperscript{35–38,40,41,62} (Supplementary Table 1). We tested how well our kinetic model’s ranking of activity compares to that of the CFD score\textsuperscript{12}, CRISPRoff\textsuperscript{28}, and uCRISPR\textsuperscript{24}. For each sgRNA, we separately tested the models by using the union (sites found in any experiment) and intersection (sites found in every experiment) sets of the reported off-target sites as true positives. We perform precision-recall (PR) analysis (Supplementary Fig. 3) rather than DNA cleavage.
ately mismatched targets, we extract the physical parameters of both time and complexity model strongly suggest that the path to increased predictive power and therapeutic relevance runs through bottom-up modelling of RNA-guided nucleases in kinetic terms.

Taken together, we have shown that our mechanistic and kinetic model gives biophysical insight and quantitative predictive power far beyond the training sets. This predictive power is only expected to increase when including sequence features and allowing for alternative PAM sequences in future modelling efforts. SpCas9 is only one of many RNA-guided nucleases with biotechnological applications, and other CRISPR associated nucleases (such as Cas12a, Cas13 and Cas14) offer a diversification genome-engineering toolkit. These nucleases can all be characterized with our approach, and it will be especially interesting to compare the free-energy landscape of our SpCas9 benchmark to that of engineered and natural (e.g. N. meningitides Cas9) high-fidelity Cas9 variants.

Methods
Modelling of the (d)Cas9 targeting reaction. We consider a single DNA target sequence with a PAM, in contact with (d)Cas9-sgRNA in solution at fixed concentration (Fig. 1a). (d)Cas9-sgRNA binding to the PAM site is assumed to be first order, $$k_{in} = k_{in}^0[\text{Cas9} - \text{sgRNA}]$$

where [Cas9-sgRNA] is the concentration of active complexes relative to some reference concentration (we use 1 nM). Binding is followed by a Cas9-mediated strand exchange reaction between sgRNA and the DNA. Once a 20 bp hybrid is formed, Cas9 can cleave the DNA, while dCas9 cannot. We model the targeting recognition as a stochastic hopping process along a sequence of states: target unbound (n = 0), PAM bound (n = 0), and strand exchange (n = 1, 2, ..., 20). We use the column vector $P(t) = (P_{un}(t), ... , P_{20}(t))^T$ to represent the probabilities of being in the various states at time t. The evolution of probabilities is captured by the Master Equation

$$\dot{P}(t) = K \cdot P(t),$$

where K is a tri-diagonal rate matrix. Letting $k_{n}^b$ be the forward (n → n + 1) transition rate, $k_{n}^b$ to be the backward (n → n - 1) transition rate (Fig. 1a), and defining $k_{-1}^b = 0,$
we can give the elements of $K$ as

$$K_{mn} = \begin{cases} 
  k_{m,n}^{\text{off}} & m = n - 1 \\
  -k_{m,n}^{\text{on}} - k_{n,m}^{\text{on}} & m = n \\
  k_{n,m}^{\text{on}} & m = n + 1 \\
  0 & |n - m| \geq 2.
\end{cases}$$

The Master Equation has the formal solution

$$P(t) = \exp(Kt) \cdot P(0),$$

which can be computed numerically, given any set of rates $K$ and initial probabilities $P(0)$. The above expression, with initial probabilities and rates adjusted to experimental conditions (see below), allows us to capture the full time-dependent evolution of the targeting reaction in quantitative terms.

**Parameter reduction.** Based on the mechanistic-model assumption 1, we average the data over mismatch types (see below), and only keep track of if there is a match or a mismatch at every position. Model assumption 3 means that the model of dCas9 is the same as for Cas9, but with $k_{2}^{\text{on}} = 0$. Model assumption 4 implies that $k_{m}^{\text{on}} = k_{m}^{\text{on}} = \cdots = k_{n}^{\text{on}} = k_{1}^{\text{on}}$. To see the implications of model assumption 2, we move to a description in terms of free energies.

Denote the free energy of any state $n$ with $F_{n}$, and imagine that states $n$ and $n - 1$ are allowed to mutually equilibrate. Equilibration means that the relative occupancy is described by Boltzmann weights and that there are no net probability currents between the states

$$F_{n}^{\text{eq}} = \frac{\exp(-\beta \epsilon_{n})}{\sum_{m} \exp(-\beta \epsilon_{m})} P_{m} = \exp(-\beta \epsilon_{n}) F_{n-1}^{\text{eq}},$$

The above relationships tie rates to free-energy differences through

$$\Delta F_{n} = F_{n} - F_{n-1} = k_{q} T \ln \left( \frac{k_{m}^{\text{on}}}{k_{m}^{\text{off}}} \right).$$

Using $\eta = -1$ as the free-energy reference ($F_{-1} = 0$), the assumption that binding is first-order implies

$$F_{0} = F_{0}^{\text{eq}} = k_{q} T \ln([\text{Cas9} - \text{sgRNA}]).$$

Here $F_{0}^{\text{eq}}$ is the free energy of the PAM bound state at the reference concentration (1 nM). Mechanistic-model assumption 2 now implies that $\Delta F_{n} = 0$ only depends on if there is a mismatch at position $n$ or not, and we can write

$$\Delta F_{n} = \epsilon_{n} \text{match} + \epsilon_{n} \text{mismatch}.$$

Here $\epsilon_{n}$ is the free-energy increase when extending the hybrid from length $n - 1$ to length $n$ if the nth hybrid bp is correctly matched, and $\delta_{m}$ is the additional energy needed when the bp is incorrectly matched. We can write the backward transition rates as

$$k_{n}^{\text{off}} = \begin{cases} 
  k_{m}^{\text{off}} \exp(\frac{\epsilon_{n}}{k_{B} T}) & n = 0, \\
  \frac{k_{m}^{\text{off}}}{k_{B} T} & n = 1, \ldots, 20.
\end{cases}$$

The model is now parameterized in terms of 41 free energies ($\epsilon_{1}, \epsilon_{2}, \ldots, \epsilon_{20}, \delta_{1}, \ldots, \delta_{20}$ and three forward rates ($k_{m}^{\text{on}}, k_{1}^{\text{on}}, k_{2}^{\text{on}}$)).

**Predicting NucleaSeq cleavage rates.** To produce predictions for training and validation, we model experimental setups. To model NucleaSeq data, we use the solution to the Master Equation to calculate the expected cleaved fraction at any complementarity pattern. NucleaSeq is performed by exposing targets to saturating concentrations of Cas9-sgRNA, which we model by setting $F_{0} = -100 k_{B} T$ and taking $P_{i,0}(t) = 1, P_{i,0,20}(t) = 0$ as initial conditions. As done in the original experiment, we record the fraction of DNA that remains uncleaved ($\chi = \sum_{n=0}^{20} P_{n,0}(t)$) at the time points $t = 0, 12, 60, 180, 300, 1800, 3000, 18000, s$, and 60000 s, and fit-out a single effective rate constant $k_{m}^{\text{off}}$. There is no a priori reason for the uncleaved fraction to follow an exponential decay, but as long as we follow the experimental data-analysis protocol we can use the effective cleavage rates to train and validate our model.

**Predicting CHAMP association constants.** We model the CHAMP experiments\textsuperscript{15} by calculating the bound fraction ($\sum_{n=0}^{20} P_{n,0}(t)$) of dCas9-sgRNA after 10 min at concentrations 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM and 300 nM, starting with the probabilities $P_{i,0}(t) = 1, P_{i,0,20}(t) = 0$. We use the equilibrium binding fraction

$$P_{m}^{\text{emp}} = \frac{[\text{Cas9} - \text{sgRNA}]}{[\text{Cas9} - \text{sgRNA}]} + 1/k_{m}^{\text{off}}$$

to fit out an effective association constant $k_{m}^{\text{off}}$. Again, there is no a priori reason to believe that this non-equilibrium system will equilibrate within 10 min, but as long as we follow the experimental data-analysis protocol we can use $k_{m}^{\text{off}}$ for training and validation.

**Predicting HITS-FLIP association rates.** To predict measured association rates in the HITS-FLIP experiment\textsuperscript{14}, we assume the recorded fluorescence signal to be proportional to our calculated bound fraction of dCas9-sgRNA, when starting with the probabilities $P_{i,0}(t) = 1, P_{i,0,20}(t) = 0$. Following the experiments we use linear regression to extract an effective association rate by fitting a straight line to the bound fraction at time points 500 s, 1000 s and 1500 s.

**Predicting HITS-FLIP dissociation rates.** To predict measured dissociation rates in the HITS-FLIP experiment\textsuperscript{14}, we again compared the fluorescence signal to our calculated bound fraction of dCas9, starting with the probabilities $P_{i,0}(t) = 1, P_{i,0,20}(t) = 0$. We let the protein associate at saturating concentrations for 12 h, and record the resulting occupational probabilities. We then use these probabilities as new initial probabilities, while also letting $k_{m} = 0 ([\text{Cas9} - \text{sgRNA}] = 0)$ in $K$, before further evolving the system. This allows us to model complex dissociation in the presence of a high concentration of competitor on-targets in solution. Following the experiments, we fit an exponential decay to our predictions at time-points 500 s, 1000 s, and 1500 s.

**Averaging over mismatch types.** Our model does not account for mismatch types, and for training we need to average over all experimentally measured mismatch sequences $s$ consistent with a mismatch pattern $p$. We expect rates to be proportional to exponentional transition-state free energies, and association constants to be controlled by exponentiated binding free energies. We therefore choose to perform our mismatch-type averages over the logarithm of rates and association constants, bringing these averages close to averages of energies. For measured quantities $m = k_{m}^{\text{on}}$ or $k_{m}^{\text{off}}$, we chose a weighted mismatch-type average

$$\log_{\text{mm}} m_{p}^{s} = \sum_{\text{sequences with } p} W_{s} \log_{\text{mm}} m_{p}^{s}.$$

Here $m_{p}^{s}$ is the measured value for target sequences $s$. We take the weights to be given by

$$W_{s} = \frac{1}{\delta \log_{\text{mm}} m_{p}^{s}}.$$

Here $\delta \log_{\text{mm}} m_{p}^{s}$ is the experimental error for the logarithm of the measurement at a particular sequence $s$. This choice of weights minimizes the error-normalized square deviation on the sequence resolved data, if we have complete freedom to set the average for each mismatch pattern. Our model is more constrained then this, but with this weighing our model could—at least in principle—give the best possible approximation of the sequence resolved data. The squared error in the mismatch-type average can be calculated as

$$\delta.$$

**Cost function.** We look to simultaneously optimize our predictions of both effective cleavage rates from NucleaSeq ($k_{m}^{\text{off}}$) and effective dissociation constants from CHAMP ($k_{m}^{\text{on}}$). We combine the cost from each experiment

$$X = X_{\text{eff}} + X_{\text{on}}$$

by summing log deviations

$$X_{p} = \sum_{s} W_{p} \log_{\text{mm}} m_{p}^{s} - \log_{\text{mm}} m_{p}^{s}.$$

In the above $m_{p}$ represent the model prediction for the average measured quantity at mismatch pattern $p$. The weights $w_{p}$ are chosen so the error-weighted contribution from the on-target, the 20 singly mismatched off-targets, and the 20 - 19/2 = 199 doubly mismatched off-targets are weighted equally as groups

$$w_{p} = \frac{1}{\delta \log_{\text{mm}} m_{p}^{s}}.$$

**Simulated annealing.** The Simulated Annealing algorithm\textsuperscript{19} is commonly used for high-dimensional optimization problems. We optimize with respect to model parameters $F_{0}^{\text{eq}}, \epsilon_{1}, \ldots, \epsilon_{20}, \delta_{1}, \ldots, \delta_{20}, \log_{\text{mm}} [\text{Cas9 - sgRNA}] / \text{s}, \log_{\text{mm}} [\text{Cas9}],$ and $\log_{\text{mm}} (k_{m}^{\text{on}} / \text{s}).$ Trial moves are generated by adding a uniform noise of magnitude $\alpha$ to the present value of each model parameter. The process is initiated with a noise strength $\alpha = 0.1$. In the initiation cycle the temperature is adjusted until we have an acceptance fraction of 40–60% over 1000 trial moves, based on the Metropolis condition. After this initial
Calculating coarse-grained transition rates. First we find the intermediate state on every possible target. As the central-local minimum in free energy (Fig.4a) can be slightly displaced by mismatches on off-targets, we seek the free-energy minimum for every possible target. As the central-local minimum in free energy (Fig.4a) can be slightly displaced by mismatches on off-targets, we seek the free-energy minimum.

Constructing a binary off-target predictor. We rank all canonical PAM sites in canonical PAM sites in the human genome according to their relative cleavage rate in the low concentration limit. In this limit, the cleavage rate is given by the PAM binding rate times the probability to cleave once the PAM site is bound. As the PAM binding rate is expected to depend on the sgRNA sequence, we can rank our off-targets based on the cleavage probability once bound,

\[ P_{\text{off-target}}(s) = \frac{k_{\text{off}} e^{-\Delta G_{\text{off}}}}{k_{\text{on}} e^{-\Delta G_{\text{on}}} + k_{\text{off}} e^{-\Delta G_{\text{off}}}}. \]

Here \( p(s) \) is the mismatch pattern of sequence \( s \).

Statistics & Reproducibility. Only experimental data giving physical positive values for mismatch-averaged rates and association constants were included in the correlation analysis. See Supplementary Data 1.

Data availability. The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Mismatch averaged experimental data used for training and validation (Figs. 2 and 3), estimated microscopic parameters (Fig. 4), and genome-wide off-target classification evaluation (Fig. 7b–e), are all provided as Supplementary Data 1.

Code availability. The code enabling quantitative off-target activity prediction for any guide-target pair is available on our GitHub page [https://github.tudelft.nl/deken_group/SpatCas9_kinetic_model_dashboard]. There you will also find a small database application, allowing time resolved activity predictions given a particular sequence and enzyme concentration. A clone of the repository at publication is also permanently available at [https://doi.org/10.5281/zenodo.5790798]. The purpose made optimization code will be made available upon request.

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