The limited target specificity of CRISPR-Cas nucleases poses a challenge with respect to their application in research and therapy. Here, we present a simple and original strategy to enhance the specificity of CRISPR-Cas9 genome editing by coupling Cas9 to artificial inhibitory domains. Applying a combination of mathematical modeling and experiments, we first determined how CRISPR-Cas9 activity profiles relate to Cas9 specificity. We then used artificially weakened anti-CRISPR (Acr) proteins either coexpressed with or directly fused to Cas9 to fine-tune its activity toward selected levels, thereby achieving an effective kinetic insulation of ON- and OFF-target editing events. We demonstrate highly specific genome editing in mammalian cells using diverse single-guide RNAs prone to potent OFF-targeting. Last, we show that our strategy is compatible with different modes of delivery, including transient transfection and adeno-associated viral vectors. Together, we provide a highly versatile approach to reduce CRISPR-Cas OFF-target effects via kinetic insulation.

INTRODUCTION
The emergence of CRISPR-Cas technologies (1–3) enabled detailed genomic studies and brought a targeted therapy of genetic diseases into closer reach. The fidelity of the Cas nuclease, i.e., selectivity for the single-guide RNA (sgRNA)–matching genomic target locus as compared to OFF-target sites exhibiting partial complementarity to the RNA guide, is a key parameter to be considered for CRISPR applications. While fidelity varies between Cas orthologs (4–7) and strongly depends on the sgRNA design (8–10), target locus sequence (5, 6, 11), and mode of delivery (12), generalizable strategies to reduce OFF-target editing are highly desired. Previous studies used directed evolution or structure-guided protein engineering to identify point mutations in the Cas enzyme that reduce OFF-target editing (13–19). Complementary efforts devised rules for the design of target-specific sgRNAs (8–10). While generally very powerful, these strategies demand users to use specific CRISPR-Cas components and are often limited to a single Cas9 ortholog.

Anti-CRISPR (Acr) proteins, i.e., naturally occurring CRISPR-Cas inhibitors (20, 21), offer flexible strategies to regulate CRISPR-Cas activity and thereby to enhance the specificity of genome perturbations. Among these is AcrIIA4, a potent inhibitor of the Streptococcus pyogenes (Spy)Cas9 (22), which binds Cas9-sgRNA complexes with subnanomolar affinity (23) and impairs DNA targeting as well as nuclease function (24). Recent data show that administering AcrIIA4 shortly after SpyCas9-sgRNA delivery can reduce OFF-target editing (25), likely due to the slower editing kinetics at OFF-target sites as compared to perfect targets (26, 27). This strategy, however, has essential limitations. Sequential delivery is highly sensitive with respect to the delivery efficacy. At 50% transfection rate, for instance, only half of the cells that received Cas9/gRNA in the first transfection will also receive the Acr in the second transfection. In the other half, Cas9 would, undesirably, remain active, resulting in off-target effects. Moreover, the requirement of two separate, precisely timed delivery steps (one for Cas9-sgRNA and one for the Acr) is difficult to implement in a number of relevant application settings, e.g., in a therapeutic scenario.

To address these limitations, we here used a combination of mathematical modeling and experiments to determine Cas9 activity profiles that strongly favor ON-target over OFF-target editing events. We first show that artificially attenuated anti-CRISPR proteins either coexpressed with or fused to Cas9 can be used to fine-tune Cas9 activity to selected levels, thereby strongly increasing target specificity. Then, by calibrating our mathematical model with the obtained experimental data and additional time-lapse measurements, we quantitatively characterized ON- and OFF-target editing efficiencies depending on the target locus properties and time integral of Cas9 activity. Our original method facilitates the in silico prediction and subsequent implementation of Cas9 activity profiles that effectively insulate ON- and OFF-target editing events.

RESULTS
Fine-tuning Cas9 achieves kinetic insulation of ON- and OFF-target editing events
To investigate the possibility of insulating ON- and OFF-target editing events by fine-tuning Cas9 activity, we first devised a simple mathematical model consisting of coupled ordinary differential equations (ODEs). Our model captures the major molecular steps underlying Cas9-mediated editing in cells, namely, (i) transient expression of sgRNA and Cas9 after transfection, (ii) formation of Cas9-sgRNA complexes, (iii) binding of the complex to a genomic target locus, and (iv) target locus cleavage (Fig. 1A and table S1, see Materials and Methods for details). In this simple model, the level of Cas9-mediated target cleavage mainly depends on two parameters: the time integral of active Cas9-sgRNA complexes present in a cell and the affinity of the Cas9-sgRNA complex to a given genomic locus. High affinity of Cas9-sgRNA to a given locus, as would be
expected for perfect target sites, would thereby result in a high percentage of gene-edited cells (Fig. 1B). In contrast, lower affinities, as would be expected for OFF-target loci, require larger time integrals of active Cas9-sgRNA complexes (Fig. 1B). Notably, at sufficiently large time frames, the editing of ON-target and OFF-target loci will both reach saturation. Our model qualitatively predicts that by fine-tuning the amount of (active) Cas9-sgRNA complex to specific levels, editing at “high-affinity” loci (ON-targets) can be insulated from editing at “low-affinity” loci (OFF-targets) (Fig. 1B).

We hypothesized that anti-CRISPR proteins such as AcrIIA4 could provide a simple means to fine-tune Cas9 activity to selected levels and, therefore, to test our model prediction. We thus cotransfected...
human embryonic kidney (HEK) 293T cells with vectors encoding AcrIIA4, Cas9, and a previously reported sgRNA targeting the human AAVS1 locus, which exhibits particularly strong off-target editing at two additional loci (Fig. 1C) (28). During transfection, we used relatively low AcrIIA4 vector doses (~3- to 130-fold excess of Cas9 and sgRNA vector), as ON-target editing would be completely blocked at higher Acr doses (25, 29). Seventy-two hours after transfection, we measured the frequency of insertions and deletions (InDels) at the AAVS1 locus and relevant off-target loci using T7 endonuclease assay and tracking of indels by decomposition (TIDE) sequencing. In line with the model prediction, we observed a potent reduction in off-target editing but only mild reduction in ON-target editing at selected Acr vector doses (Fig. 1, D and E, and fig. S1). To test whether this effect was independent of the specific cellular context and compatible with different modes of delivery, we packaged the different components of our system (Cas9, sgRNA, and AcrIIA4) into adeno-associated virus (AAV) vectors (Fig. 1C), which are prime vector candidates for gene therapy applications (30). For our experiments, we chose AAV serotype-2 (AAV2) that is able to efficiently transduce various cell lines (31). We coinfectected HEK 293T, HeLa (cervix carcinoma), and Huh-7 (hepatocellular carcinoma) cells with AAV2 particles encoding (i) Cas9, (ii) an sgRNA targeting the AAVS1 locus, or (iii) a previously reported AcrIIA4 variant (29) and measured InDel frequencies at the ON- and OFF-target loci. Again, at low Acr vector doses, we observed potent ON-target editing, while OFF-target editing was effectively suppressed (fig. S2).

Next, we investigated whether the identical strategy could also improve specificity when editing a different target site, namely, hemoglobin subunit beta (HBB). While separation of ON- and OFF-target editing was rather difficult when applying transient transfection for delivery of Cas9, AcrIIA4, and the HBB-targeting RNA guide (fig. S3), AAV-mediated delivery selectively limited off-target editing (Fig. 1F and fig. S1). This notable difference between delivery methods is likely due to the more homogeneous expression of constructs from AAV vectors as compared to the rather heterogeneous expression expected after transient transfection. This, in turn, allows a more precise fine-tuning of Cas9 activity in individual cells and thereby a more efficient kinetic insulation of ON- and OFF-target editing events.

**Cas-Acr fusions improve genome editing fidelity**

While coexpression of Acrs as shown above offers a highly flexible strategy to improve Cas9 specificity without the necessity of altering Cas9 or the sgRNA itself, this approach is rather sensitive with respect to the Acr dose. Moreover, the amounts of Cas9, sgRNA, and Acr and thus the ratio of the Cas9-sgRNA complex to Acr will vary between individual cells after co-delivery into a population of cells. Thus, there will be a considerable fraction of cells, in which inhibition is either too weak to reduce off-target editing or too strong to allow ON-target editing, both of which is undesired.

We hypothesized that a more robust fine-tuning of Cas9 activity could be achieved by covalently linking Acrs to Cas9 via genetic fusion, thereby eliminating the problem of varying Acr to Cas9-sgRNA complex ratios. In this configuration, which in the following we refer to as Cas-Acr, every Cas9 molecule carries its own inhibitory domain and therefore can exist in an active or an inactive state. At equilibrium, the fractions of molecules populating the active or inactive states depend on the strength of the fused Acr, i.e., by modulating the Acr strength, Cas9 activity can be fine-tuned to desired levels (Fig. 2A). As one might expect, fusing wild-type AcrIIA4 to Cas9 blocks Cas9 activity (almost) entirely (Fig. 2, B to G, Cas9 wt samples). Thus, to enable the envisaged fine-tuning, we used a previously reported set of AcrIIA4 domain insertion mutants (fig. S4) (29) as well as AcrIIA4 point mutants (32), which display various Cas9 inhibition potencies when coexpressed with Cas9. We then fused these attenuated AcrIIA4 variants to the Cas9 C terminus via long (40 residue) glycine-serine linkers and prescreened the resulting 10 Cas-Acr variants using the previously used AAVS1 and HBB locus targeting sgRNAs. As hoped, a number of Cas-Acr variants not only showed ON-target editing efficiencies comparable to wild-type Cas9 but also displayed strongly reduced off-target editing (fig. S5). We then selected three Cas-Acr variants (Ins. 5, N39A, and D14A/G38A in fig. S3) that showed different levels of specificity gain and compared their ON- and OFF-target editing frequencies with that of wild-type Cas9 using five different sgRNAs. Genome editing specificity was strongly improved for all Cas-Acr variants as we observed in T7 endonuclease experiments (Fig. 2, B to G, and fig. S6) and further confirmed by targeted amplicon sequencing (fig. S7). In several cases, e.g., for HEK, RUNX, and EMX, this improvement came at the cost of reduced ON-target editing (Fig. 2, B to G, and figs. S6 and S7). Using a Cas-Acr fusion based on a particularly weak inhibitor (AcrIIA4 mutant D14A/Y15A) not only improved ON-target editing for these sgRNAs but also markedly increased off-target editing (Fig. 3 and fig. 8G, Cas-Acr samples). This suggests an intrinsic trade-off between the gain in specificity and reduction in ON-target editing that can be achieved, which is dependent on the used sgRNA.

Next, we compared side-by-side our Cas-Acr variants to the latest generation of high-fidelity Cas9s, namely, HypaCas9 (19), xCas9 (15), and Sniper-Cas9 (14). We found that the high-fidelity Cas9 benchmarks showed a very diverse behavior when combined with different sgRNAs (Fig. 3 and fig. S8). Sniper-Cas9, for example, was potent and specific when using the HBB sgRNA but showed strong off-target editing when combined with the AAVS1 and RUNX sgRNAs. HypaCas9 and xCas9, on the other hand, performed particularly well in combination with the EMX sgRNA. HypaCas9, however, showed diminished ON-target editing for HBB and RUNX, and xCas9 showed particularly prominent off-target editing for AAVS1. The Cas-Acr fusions achieved highest specificity on AAVS1 but were outperformed by at least one of the benchmarks on the other three loci. Together, these results suggest that (i) kinetic insulation can be very powerful on specific loci (e.g., AAVS1) and (ii) even the latest generation of high-fidelity Cas9 variants still show strong off-target editing for some sgRNAs, an issue that might be addressable, at least in part, via kinetic insulation (see Discussion).

**A quantitative model of Cas-Acr action explains locus-dependent specificity gains**

To quantitatively characterize the relation between ON-target editing efficiency and specificity in detail in the context of our Cas-Acr fusion approach, we extended our initial model by including the inhibitory states of Cas-Acr variants (Fig. 4A). In particular, the model explains the transient expression of sgRNA, Cas9 or Cas-Acr mRNA, and Cas9 or Cas-Acr protein in transfected cells. Cas9 and active as well as inactive Cas-Acr proteins can be reversibly bound to sgRNA molecules. Cas-Acr is reversibly inactivated by the fused Acr. Thereby, the model effectively describes how the ratio between activation and inactivation rates of Cas-Acr variants is related to the
time integral of active complexes of Cas-Acr:sgRNA (see Materials and Methods and tables S1, S6, and S7 for details).

The model was calibrated with experimental data on editing frequencies after transient expression of Cas9, Cas-Acr containing unmodified AcrIIA4, as well as Cas-Acr variants “Ins. 5,” “N39A,” and “D14A/G38A” (Fig. 4B and fig. S9; see fig. S10 for the complete set of model fits and data and Materials and Methods for details).

Moreover, the model was calibrated with time-lapse measurements of Cas9–green fluorescent protein (GFP) and mCherry-AcrIIA4 expression recorded by life-cell fluorescence microscopy (fig. S9). For parameter estimation, the model was fitted to time-resolved ON- and OFF-target editing efficiencies as observed for the AAVS1-targeting sgRNA in combination with different Cas-Acr variants as well as wild-type Cas9 (fig. S9). The model was calibrated with experimental data on editing frequencies after transient expression of Cas9, Cas-Acr containing unmodified AcrIIA4, as well as Cas-Acr variants “Ins. 5,” “N39A,” and “D14A/G38A” (Fig. 4B and fig. S9; see fig. S10 for the complete set of model fits and data and Materials and Methods for details).
further fitted to ON- and OFF-target efficiencies measured for the EMX1, RUNX1, and HEK locus when using wild-type Cas9 as well as ON-target editing frequencies measured for the different Cas-Acr variants. Corresponding OFF-target editing data for the Cas-Acr variants were used for model validation. Simulations using the calibrated model predicted the experimentally measured OFF-target rates in the Cas-Acr validation dataset with high precision (Fig. 4C and fig. S10).
**Fig. 4. A mathematical model of Cas-Acr action explains improved specificity and informs experimental planning.**

(A) Overview of the mathematical model of gene editing with Cas-Acr constructs. The model accounts for turnover of plasmids, sgRNA, Cas-Acr mRNA, and protein; transition between the active and inhibited states (Cas-Acrinh); sgRNA binding (Cas-Acr:sgRNA, Cas-Acrinh:sgRNA); association with a target gene; and gene editing. (B) Exemplary model fits to time-resolved T7 endonuclease assay measurements using the AAVS1-targeting sgRNA and either wild-type Cas9 or the Cas-Acr variant Ins. 5 (see fig. S10 for the full set of fits). (C) ON- and OFF-target editing efficiencies for sgRNAs targeting the AAVS1, EMX1, RUNX1, or HEK locus are shown together with model simulations of editing efficiencies for either wild-type Cas9 or the indicated Cas-Acr variants. The model was calibrated with ON- and OFF-target editing efficiencies for AAVS1 and ON-target editing efficiencies for EMX1, RUNX1, and HEK. OFF-target editing measurements for EMX1, RUNX1, and HEK were used for model validation. (D) and (E) Kinetic insulation of ON- and OFF-target editing by Cas-Acr variants. (D) Data points are shown together with inhibitor strengths as estimated by model fitting. Kinetic insulation is achieved for inhibitor strengths that fall between sigmoidal curves for ON- and OFF-target editing. (E) The calibrated model can be used to predict the ratio between ON- and OFF-target editing efficiencies resulting from Cas-Acr variants. (F) Model simulations of the ratio between ON- and OFF-target editing efficiencies relative to ON-target editing efficiency illustrate the trade-off between Cas9 fidelity and ON-target editing efficiency. Cas-Acr variants can be selected on the basis of highest tolerated OFF-target editing efficiencies.
Last, the calibrated model was used to quantitatively dissect the kinetic insulation of ON- and OFF-target editing events by Cas-Acr. Simulated editing efficiencies at ON- and OFF-target sites followed sigmoidal curves dependent on the strength of the Cas9-fused inhibitor (Fig. 4, D and E, and fig. S11). Notably, inhibitor strengths of the Cas-Acr variants D14A/G38A, Ins. 5, and N39A are all in the regions of the maximal slope of the ON-target editing efficiency curves but below the region of the maximal slopes of the OFF-target editing efficiency curves for the studied sgRNAs, explaining why these variants are well suited for insulation of ON- and OFF-target editing events. According to parameter estimates, transition rates to the inhibited state of the Cas-Acr variants carrying AcrIIA4 mutants were decreased by factors between 16 and 19 relative to the Cas-Acr variant containing wild-type AcrIIA4, explaining why these variants still enable potent ON-target editing (table S7). To further characterize the capability of tuning the activity of Cas9, we simulated the ratio between ON- and OFF-target editing efficiencies (Fig. 4, E and F). The dependence of this ratio on the strength of the Cas9-fused inhibitor was reflected by sigmoidal curves for each of the edited genes (Fig. 4E). Notably, inhibitor strengths of the variants Ins. 5, N39A, and D14A/G38A were in the upper-to-medium part of the sigmoidal curves (Fig. 4E). Together, these data show that the Cas-Acr variants hit the desired “sweet spot” in the Cas9 activity profile characterized by efficient insulation of ON- and OFF-target events (Fig. 4F).

DISCUSSION

The specificity of Cas nucleases is a critical parameter to consider when applying the CRISPR technology in both basic research and gene therapy. Using a combination of mathematical modeling and wet-lab experiments, we here showed that Cas9 activity can be fine-tuned to selected levels by either coexpression of or covalent fusion to (attenuated) anti-CRISPR proteins, thereby selectively limiting off-target editing. From a mechanistic point of view, our approach harnesses a simple principle derived from enzyme kinetics, i.e., we increase the competition between optimal and suboptimal substrates (ON- and OFF-target sites, respectively) by reducing the pool of active enzyme (Cas9) to desired levels. The editing kinetics at ON- and OFF-target loci is dependent on physical constraints (e.g., affinity of Cas9-sgRNA to respective loci), which are specific to each sgRNA and target locus. The greater the difference is between editing kinetics at ON- and OFF-target sites, the easier it is to kinetically insulate ON- and OFF-target editing events. This explains why, for AAVS1, OFF-target editing can be reduced without affecting ON-target editing, while for other loci such as EMX and HEK, reduction in OFF-target editing will—to some extent—also affect the ON-target editing efficiency. Even so, in the latter cases, the reduction in OFF-target editing is greater than that in ON-target editing, i.e., overall, there is a gain in specificity.

While fine-tuning of Cas9 activity could, in principle, also be achieved by either reducing the Cas9 expression level or the time in which Cas9 is active, both of these strategies are more difficult to put into practice, at least when delivering genetic constructs encoding the different components. Reducing the expression level would typically require reducing vector doses, which might unintendedly reduce transfection/transduction rates and also increase heterogeneity, both of which is highly undesired. Confining the time in which Cas9 is active either requires external control, e.g., via chemicals (33, 34) or light (29, 35), or depends on synthetic circuits mediating a negative feedback, which might be more difficult to tweak toward desired levels of Cas9 activity (36). In contrast, our Cas-Acr approach is simple and compatible with different modes of delivery and functions robustly for various loci and in different cell lines.

In addition, our calibrated model of gene editing by Cas-Acr variants can inform the optimization of future gene editing experiments. If ON- and OFF-targeting efficiencies resulting from gene editing with wild-type Cas9 are known, the model can be used to predict ON- and OFF-targeting efficiencies for Cas-Acr variants to select an optimal construct. Furthermore, our model can be used to predict the maximally achievable increase in the specificity for different genes and sgRNAs. Thereby, our model can assist the implementation of the kinetic insulation principle developed in this work.

ON- and OFF-target editing would be expected to follow specific kinetic trajectories for practically any Cas enzyme, as the basic physical properties that contribute to the different kinetics (e.g., locus-specific affinity of the Cas-sgRNA complex) apply to any CRISPR nuclease. We thus speculate that the principle of kinetic insulation applied in this work should be easily transferrable to other CRISPR-Cas systems. Moreover, the ongoing rapid discovery and characterization of anti-CRISPR proteins improve our ability to fine-tune the activity of Cas enzymes in time and space (29, 37).

Last, we note that the strategies developed in this work, in particular the Cas-Acr fusion variants, are not meant to replace existing high-fidelity CRISPR-Cas variants, the latter of which provide a great resource to the community. On the contrary, we believe that in the future, a combination of several approaches, such as engineered Cas enzymes, improved sgRNAs and, additionally, kinetic control, e.g., via attenuated Acr variants as shown here, will likely be applied to maximize efficiency and specificity in CRISPR genome editing. Together, our Cas-Acr constructs in combination with the calibrated mathematical model represent a powerful resource to enhance the precision of CRISPR-Cas genome perturbations in living cells by fine-tuning Cas9 activity.

MATERIALS AND METHODS

Plasmids
All vectors were created by classical restriction enzyme cloning. Oligonucleotides as well as synthetic, double-stranded DNA fragments (gBlocks) were obtained from Integrated DNA Technologies. A list of all constructs used or created in this study is shown in table S3, and annotated vector sequences (GenBank files) are provided in data file S1. Vectors expressing Cas9, Cas9 fused to GFP (Cas9-GFP), wild-type AcrIIA4, different AcrIIA4-LOV2 hybrids, or a U6 promoter–driven sgRNA bearing the improved F+E scaffold (38) have been previously reported by us (29, 37) (see Addgene no. 113033-113039). The mCherry-AcrIIA4 vector was created by fusing an mCherry coding sequence to the N terminus of wild-type AcrIIA4 using overlap extension polymerase chain reaction (PCR). A construct expressing Cas9 fused to wild-type AcrIIA4 via a 40-residue glycine-serine (GS) linker was created by cloning a synthetic DNA fragment encoding the GS linker–AcrIIA4 fragment into vector CMV–SpyCas9 (Addgene no. 103033) via Eco RI/Hind III. The AcrIIA4 fragment in the resulting construct was subsequently replaced by PCR fragments encoding AcrIIA4-LOV2 fusions or AcrIIA4 point mutants via Bam HI/Hind III. To generate the AcrIIA4-LOV2 PCR fragments, our previously reported AcrIIA4-LOV2 vectors were used as template (29). The AcrIIA4 point mutants were created by first amplifying a vector encoding

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wild-type AcrIIA4 (Addgene no. 113037) with 5′-phosphorylated primers introducing the point mutation(s). The resulting vectors were then used as template to generate PCR fragments encoding AcrIIA4 mutants. sgRNA expression vectors were created by inserting binding complementary sequences into vector pAAV-RSV–GFP–U6–sgRNA scaffold (Addgene no. 113039) by oligo cloning via BbsI.

BPK4410, a human expression plasmid for SpCas9 Cluster 1 (HypaCas9), was a gift from J. Doudna and K. Joung (Addgene plasmid no. 101178; http://n2t.net/addgene:101178; RRID:Addgene_101178). xCas9 3.7 was a gift from D. Liu (Addgene plasmid no. 108379; http://n2t.net/addgene:108379; RRID:Addgene_108379). p3S-Snper-Cas9 was a gift from J. Lee (Addgene plasmid no. 113912; http://n2t.net/addgene:113912; RRID:Addgene_113912). In all cloning procedures, PCRs were performed using Q5 Hot Start High-Fidelity DNA Polymerase [New England Biolabs (NEB)] followed by agarose gel electrophoresis to analyze PCR products. Bands of the expected size were cut out from the gel, and the DNA was purified by using the QIAquick Gel Extraction Kit (Qiagen). Restriction digests and ligations were performed with corresponding enzymes from NEB by following the manufacturer’s protocols. Following ligation, plasmids were transformed into chemically competent Top10 cells, and plasmids were extracted and purified using the QIAamp DNA Mini or Plasmid Plus Midi Kit (all from Qiagen).

**Cell culture**

Before use, all cell lines were authenticated and tested negative for mycoplasma contamination via a commercial service (Multiplexion, Heidelberg). Cells were maintained at 5% CO2 and at 37°C in a humidified incubator and passed every 2 to 4 days, i.e., when reaching 70 to 90% confluency. HEK 293T and HeLa cells were cultivated in 1× Dulbecco’s modified Eagle’s medium supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) (all Thermo Fisher Scientific), and 10% (v/v) fetal calf serum (Biochrom AG). The Huh-7 medium was additionally supplemented with 1 mM nonessential amino acids (Thermo Fisher Scientific).

**AAV lysate production**

Low-passage HEK 293T cells were used for the production of AAV-containing cell lysates. Cells were seeded into six-well plates (CytoOne) at a density of 350,000 cells per well. The following day, cells were triple-transfected with (i) an AAV helper plasmid carrying AAV2 rep and cap genes, (ii) an adenoviral plasmid providing helper functions for AAV production, and (iii) the AAV vector plasmid using 1.33 μg of each construct and 8 μl of TurboFect Transfection Reagent (Thermo Fisher Scientific) per well. The AAV vector plasmid encoded either (i) a U6 promoter–driven sgRNA targeting the AAVS1 locus as well as an RSV promoter–driven GFP (used as transfection reporter), (ii) Cas9, or (iii) the AcrIIA4 variant (LOV2–AcrIIA4 Insertion 3 in table S2; Addgene no. 113036). Three days after transfection, cells were collected in 300 μl of phosphate-buffered saline (PBS) and subjected to five freeze-thaw cycles by alternating between snap-freezing in liquid nitrogen and 37°C in a water bath. Centrifugation at 18,000 g was applied for 10 min to remove cell debris, and the supernatant containing AAV particles was stored at 4°C until use.

**T7 endonuclease assay, TIDE sequencing, and targeted amplicon sequencing**

Table S4 shows the genomic ON-target/OFF-target sites relevant to this study. For transfection-based T7 assays, HEK 293T cells were seeded at a density of 12,500 cells per well and a culture volume of 100 μl per well into 96-well plates (Eppendorf). The following day, cells were transfected with jetPRIME (Polyplus-transfection) using 0.3 μl of jetPrime reagent per well and as detailed in the following. For experiments shown in Fig. 1E and figs. S2 and S3, cells were cotransfected with (i) 66 ng of Cas9 expression construct, (ii) 66 ng of sgRNA constructs, and (iii) different doses of Acr construct as indicated in the figures. To keep the total amount of DNA transfection constant between all samples, DNA was topped up to 200 ng per well using an irrelevant vector. For experiments shown in Fig. 2 and figs. S4, S5, S6, and S7C, cells were cotransfected with (i) 66 ng of Cas9 or Cas-Acr vector, (ii) 66 ng of sgRNA expression construct, and (iii) 66 ng of an irrelevant stuffer DNA.

For transduction-based T7 assays, HEK 293T cells were seeded at a density of 3500 cells per well, and HeLa and Huh-7 cells were seeded at a density of 3000 cells per well into 96-well plates. The following day, cells were cotransduced with 33 μl of Cas9 AAV lysate, 33 μl of sgRNA AAV lysate, and the indicated volume of AcrIIA4 AAV lysate. The transduction volume was always topped up with PBS to a total volume of 100 μl. The transduction was repeated 24 hours after the first transduction. Seventy-two hours after transfection or after (first) transduction, the medium was aspirated and cells were lysed using DirectPCR lysis reagent (VIAGEN Biotech) supplemented with proteinase K (Sigma-Aldrich).

For T7 assays and TIDE sequencing, the genomic target locus and relevant off-target loci were PCR-amplified with primers flanking the corresponding ON-target/OFF-target sites (table S5) using Q5 Hot Start High-Fidelity DNA Polymerase (NEB). For TIDE sequencing analysis, PCR amplicons were purified from 1% agarose gels using the QIAquick Gel Extraction Kit (Qiagen) followed by Sanger sequencing (Eurofins, Germany). Percentages of modified sequences were quantified using the TIDE web tool (https://tide.deskgem.com/). For T7 assays, five microliters of PCR amplicon was diluted 1:4 in buffer 2 (NEB), and then heated up to 95°C and slowly cooled down to room temperature to allow heteroduplex formation using nexus GSX1 Mastercycler (Eppendorf) and the following temperature steps: 95°C/5 min, 95° to 85°C at −2°C per second, 85° to 25°C at −0.1°C per second. Then, 0.5 μl of T7 endonuclease (NEB) was added; samples were mixed and incubated for 15 min at 37°C. Next, gel loading dye (NEB) supplemented with 1% GelRed (Biotium) was added and samples were then loaded onto 2% tris-borate-EDTA agarose gels. Voltage (100 V) was applied for 40 min to resolve DNA fragments. The Gel IX20 system equipped with a 2.8-megapixel/14-bit scientific-grade charge-coupled device camera (INTAS) was used for gel documentation. To calculate the InDel percentages from the gel images, T7 bands were quantified using the ImageJ (http://imagej.nih.gov/ij/) gel analysis tool. Peak areas were measured, and percentages of insertions and deletions [InDel (%)] were calculated using the formula indel (%) = 100 × (1 − (1 − fraction cleaved) ½), whereas the fraction cleaved = (cleavage product bands)/ (cleavage product bands + PCR input band). Full-length T7 assay gel images are shown in fig. S12.

For targeted amplicon sequencing, a first-step PCR was performed by PCR amplifying the genomic ON-target/OFF-target loci with primers carrying 5′ Illumina Nextera sequencing adapters (forward: 5′-TC-GTCCGACCCTAGGTGTAATAAGAGACAG-[locus-specific sequence]-3′; reverse: 5′-GTCCTGTGGGATCTGAGATGTTATAAGAGACAG-[locus-specific sequence]-3′) (table S5). The second-step PCR for introducing barcodes, sequencing on an Illumina MiSeq platform, and 3′ Illumina Nextera sequencing adapters (forward: 5′-AGCTTCGACCTGAGATGTTATAAGAGACAG-[locus-specific sequence]-3′; reverse: 5′-GTCCTGTGGGATCTGAGATGTTATAAGAGACAG-[locus-specific sequence]-3′) (table S5).
machine and downstream bioinformatics for quality control, and calling of CRISPR-induced Indels was performed via the CRISPR-Cas9 commercial sequencing service (Microsynth) using their in-house pipelines.

**Fluorescence microscopy and image analysis**

Cells were seeded into eight-well Glass Bottom μ-Slides (ibidi) at a density of 9000 cells per well for HeLa and 10,000 cells per well for HEK 293T and a volume of 300 μl of medium per well. The following day, cells were cotransfected with (i) 25 ng of Cas9-GFP, 25 ng of sgRNA AAVS1 construct, and 25 ng of stuffer DNA (pBluescript) or (ii) 25 ng of Cas9-GFP, 25 ng of mCherry-AcrIIA4, and 25 ng of sgRNA AAVS1 construct using 0.2 μl of jetPrime per well. Imaging was performed at 12, 18, 24, 48, and 72 hours after transfection using a Leica SP8 confocal laser scanning microscope equipped with automated CO₂ and temperature control; an ultraviolet, argon, and solid-state laser; as well as an HCX PL APO 40× oil objective (numerical aperture = 0.7). The identical imaging settings were applied to all samples as detailed in the following. GFP fluorescence was recorded using the 488-nm laser line for excitation, and the detection wavelength was set to 493 to 578 nm. mCherry fluorescence was recorded using the 552-nm laser line for excitation, and the detection wavelength was set to 578 to 789 nm. Laser power was 0.25%, and gain was set to 800 V. For each field of view, a 40-µm Z-stack (40 slices) was recorded, and five fields of view were recorded per sample and time point. A single-plane bright-field image was recorded in parallel. A previously reported HeLa reference cell line expressing known GFP and mCherry molecules per cell (39) was subjected to the identical imaging conditions.

For image analysis, cells were manually segmented using the freehand selection tool in ImageJ using the bright-field channel, and the area of each cell was measured. The segments were then applied to measure mean fluorescence in z-projections of the GFP and mCherry stacks. The number of fluorescent molecules per cell was then calculated using the following formula

\[ \text{FM(sample)} = \frac{A\text{(sample)} \cdot I\text{(sample)}}{A\text{(ref)} \cdot I\text{(ref)}} \cdot \text{FM(ref)} \]

whereby FM(sample) and FM(ref) represent the number of fluorescent molecules per cell, A(sample) and A(ref) represent the cell area, and I(sample) and I(ref) represent the fluorescence intensity, after background subtraction, in a particular cell in the sample cell or reference (ref) cell line, respectively.

**Mathematical modeling and parameter estimation**

To quantitatively describe gene editing dynamics by Cas9 or Cas-Acr variants, an ODE model was developed. The model describes the transient expression of sgRNAs, Cas9 or Cas-Acr mRNAs, and Cas9 or Cas-Acr proteins, binding of sgRNAs to Cas9 or Cas-Acr variants, activation and inhibition of Cas-Acr variants, as well as gene editing by active complexes of Cas9 or Cas-Acr variants and sgRNAs. A model without Cas-Acr species was used for initial simulations (table S1). This model consisted of nine equations containing a total of 11 parameters. Three types of models were defined for simultaneous model fitting to experimental data: (i) a model describing turnover of plasmids, mRNAs, and proteins, consisting of 7 equations; (ii) a Cas9 model consisting of 39 equations; and (iii) Cas-Acr models containing 47 equations (table S6). A total of 32 parameters were estimated by model fitting to 75 data points.

In the following, model assumptions and steps to iteratively refine the model shall be described. The experimental dataset comprised measurements related to protein turnover and gene editing. However, several reactions in between were experimentally inaccessible. For this reason, we tried to limit the problem of parameter unidentifiability by parsimoniously defining model parameters. Taking into account that the sizes of plasmids for expressing sgRNAs, Cas9, or Cas-Acr variants were of the same order of magnitude, the same degradation rate was assumed for all plasmids. Furthermore, the model assumes the same degradation rate k_{deg,Cas9} and all Cas-Acr species (Cas-Acr, Cas-Acr_{inf, Cas-Acr:sgRNA}, and Cas-Acr_{inf, Cas-Acr:sgRNA}) independent on their activation state and sgRNA binding. Similarly, degradation of different sgRNAs was described by one parameter, k_{deg,sgRNA}. If Cas-Acr as part of complexes with sgRNA is degraded, it is assumed that sgRNA remains within the cell. Thereby, the model pertains flexibility regarding a potential sgRNA-rescuing effect of Cas9 in consistency with the observation that otherwise very short-lived sgRNA is protected from degradation after binding to Cas9 (40).

We assumed that binding of sgRNA to Cas9 or Cas-Acr variants was fast compared to other processes such as translation or gene editing. A quasi-steady state was achieved by fixing the binding parameter k_{sgRNA:Cas9} to a large value and effectively only estimating the dissociation constant K_{d,sgRNA} = k_{sgRNA:Cas9}/k_{sgRNA,Cas9}. At first, we tried to fit the model with equal K_{d,sgRNA} values for the sgRNA targeting four different genes. We realized that estimating K_{d,sgRNA} individually for sgRNAs resulted in a substantially improved model fit, indicated by a difference in the Akaike information criterion of ΔAIC = 53. This implies that affinities to Cas9 or Cas-Acr variants varied between sgRNAs.

Similarly, we first assumed the same parameter for the maximal editing efficiency for experiments in all targeted genes. In the model, this parameter served as initial value D_{on} for the fraction of unedited genes. In case that all target sites in transfected cells can be edited, this parameter equals the percentage of transfected cells expressing sgRNA and Cas9 or Cas-Acr variants. Estimating this parameter individually for the four edited genes, D_{on,i} with i = 1...4 for AAVS1, EMX1, RUNX1, and HEK, improved the model fit considerably (ΔAIC = 42).

For Cas-Acr variants, a common activation parameter per individual inactivation parameters were defined. Inhibitor strengths for Cas-Acr variants with mutated AcrIIA4 (Ins. 5, N39A, and D14A/G38A) were estimated relative to Cas-Acr wt with unmodified AcrIIA4. To this end, inhibition parameters for Cas-Acr variants were defined as a product between the common parameter k_{inh,CasAID} and parameters γ_j with j = 1...4 for Cas-Acr wt, Ins. 5, N39A, and D14A/G38A, and γ_1 ≡ 1 for Cas-Acr wt (table S6).

It was observed before that mismatches between gRNAs and target sites take influence on the unbinding rather than on the binding kinetics of Cas9:sgRNA complexes (40). For this reason, individual parameters k_{off,target,j} were estimated for the unbinding of active Cas9:sgRNA or Cas-Acr:sgRNA complexes from target sites, whereas the common binding parameter k_{on,target} was defined for all genes.

To explain differences between ON-target and OFF-target editing, factors φ_{OFF,target,j} between unbinding rates of Cas9:sgRNA or Cas-Acr:sgRNA complexes for ON- and OFF-targets were estimated separately for the four genes.

Besides the model variables D_{on}, the concentrations of plasmids for expression of gRNA ([P_{gRNA}]), coexpression of Cas9 and Acr...
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SUPPLEMENTARY MATERIALS

Model simulations were performed with custom scripts in MATLAB (The MathWorks, Natick, MA, USA). For parameter estimations, the MATLAB toolbox PottersWheel (www.potterswheel.de) was used (41). A total of 500 multistart local optimizations were conducted followed by profile likelihood estimation to determine parameter confidence intervals. Parameter estimates, parameter bounds, and parameter confidence intervals are listed in table S7. For simulating the model parts documented in table S6 using the parameter estimates listed in table S7, MATLAB files are available as supplementary data (data file S2).

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**Author contributions:** D.N. conceived the study and designed the experiments. S.A. and M.D.H. carried out experiments and performed data analysis. S.A., S.M.K., M.D.H., and D.N. interpreted data. S.M.K. performed mathematical modeling. A.H. performed initial tests on the Cas-Acr constructs. R.E. and D.N. jointly directed the work. S.M.K. and D.N. wrote the manuscript with input from all authors. **Competing interests:** S.A., S.M.K., M.D.H., A.H., and D.N. are inventors of data. S.M.K. performed mathematical modeling. A.H. performed initial tests on the Cas-Acr constructs. R.E. and D.N. jointly directed the work. S.M.K. and D.N. wrote the manuscript with input from all authors. **Data and materials availability:** Cas-Acr and sgRNA expression vectors will be made available via Addgene. For constructs created in this study, annotated vector sequences (GenBank files) are provided in data file S1. Scripts for model simulation in MATLAB are provided in data file S2. Additional data related to this paper may be requested from the authors.

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