High-Performance Allosteric Conditional Guide RNAs for Mammalian Cell-Selective Regulation of CRISPR/Cas

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ABSTRACT: The activity of a conditional guide RNA (cgRNA) is dependent on the presence or absence of an RNA trigger, enabling cell-selective regulation of CRISPR/Cas function. cgRNAs are programmable at two levels, with the target-binding sequence controlling the target of Cas activity (edit, silence, or induce a gene of choice) and the trigger-binding sequence controlling the scope of Cas activity (subset of cells expressing the trigger RNA). Allosteric cgRNA mechanisms enable independent design of the target and trigger sequences, providing the flexibility to select the regulatory target and scope independently. Building on prior advances in dynamic RNA nanotechnology that demonstrated the cgRNA concept, here we set the goal of engineering high-performance allosteric cgRNA mechanisms for the mammalian setting, pursuing both ON → OFF logic (conditional inactivation by an RNA trigger) and OFF → ON logic (conditional activation by an RNA trigger). For each mechanism, libraries of orthogonal cgRNA/trigger pairs were designed using NUPACK. In HEK 293T cells expressing cgRNAs, triggers, and inducing dCas9: (1) a library of four ON → OFF “terminator switch” cgRNAs exhibit a median fold-change of ≈50×, a median fractional dynamic range of ≈20%, and a median crosstalk modulus of ≈9%; (2) a library of three OFF → ON “split-terminator switch” cgRNAs exhibit a median fold-change of ≈150×, a median fractional dynamic range of ≈50%, and a median crosstalk modulus of ≈4%. Further, we demonstrate that xrrNA elements that protect viral RNAs from degradation by exoribonucleases can dramatically enhance the performance of RNA synthetic biology. The high-performance allosteric cgRNAs demonstrated here for ON → OFF and OFF → ON logic in mammalian cells provide a foundation for pursuing applications of programmable cell-selective regulation.

KEYWORDS: Allosteric cgRNAs, Small conditional RNAs, Dynamic RNA nanotechnology, RNA degradation, Molecular programming, Synthetic biology

INTRODUCTION

Programmable guide RNAs (gRNAs) play a central role in the CRISPR revolution sweeping biology and medicine by directing the function of Cas protein effectors to a target gene of choice (Figure 1a), providing a versatile programmable platform for engineering diverse modes of synthetic regulation in organisms ranging from bacteria to humans. Wildtype Cas9 and Cas12a enable genome editing,5–7 while mutated catalytically dead Cas9 (dCas9) variants enable gene silencing or induction.5,7 Hence, gRNA-mediated CRISPR/Cas combines the rich functional vocabulary of different Cas effectors (edit, silence, induce) and the programmability of the gRNA. To target a new gene of choice, all that is needed is to change the sequence of the gRNA.

However, the fact that gRNAs are constitutively active is a significant limitation, making it challenging to confine gRNA activity to a desired location and time within an organism. Strategies for achieving temporal control include modulation of gRNA activity using antisense RNAs or small-molecule induction of gRNAs or Cas9.11–13 Spatiotemporal control can be achieved in photoaccessile tissues using light to uncage gRNAs,12,13 cleave antisense DNAs,14 or regulate Cas9.15 Alternatively, Cas9 can be regulated using tissue-specific promoters16,17 or microRNAs.18 Cas9 tolerates, to varying degrees, a variety of modifications to the standard gRNA structure (Figure 1b),19–21 enabling introduction of auxiliary domains to provide hooks for regulation by small molecules,22–24 protein-bound RNAs,25 nucleases,26 or nuclease-recruiting DNA or miRNAs.26,27 For generality, it is desirable to control gRNA regulatory scope in a manner that is both conditional and programmable, and for simplicity, to leverage dynamic RNA nanotechnology without relying on the functionality of additional pathways.

To achieve these goals and exert programmable control over the scope of gRNA activity, conditional guide RNAs (cgRNAs) change conformation in response to an RNA trigger X, conditionally directing the function of Cas to a target gene Y
Unlike a standard gRNA, a cgRNA is programmable at two levels, with the trigger-binding sequence controlling the scope of cgRNA activity and the target-binding sequence determining the subject of Cas activity. Hybridizing to the trigger changes the cgRNA conformation to perform sequence transduction between X and Y and shape transduction between active/inactive states. For maximum flexibility, this signal transduction should be allosteric so that the sequence of the target gene Y places no restriction on the sequence of the RNA trigger X, enabling independent control over the regulatory scope (using X) and the regulatory target (using Y). cgRNA mechanisms can be engineered to implement either ON → OFF logic (conditional inactivation by trigger X) or OFF → ON logic (conditional activation by trigger X) in concert with Cas variants that either edit, silence, or induce the target Y (Figure 2a), suggesting opportunities for diverse modes of tissue-selective spatiotemporal control over regulation (Figure 2b).

In *Escherichia coli*, functional cgRNAs for OFF → ON logic have been demonstrated in conjunction with dCas9 or dCas12 using a nonallosteric “toehold switch” mechanism that imposes sequence dependence between X and Y. Functional cgRNAs for ON → OFF logic have also been demonstrated in *E. coli* (in conjunction with dCas9) using allostERIC “splinted switch” and “terminator switch” mechanisms that maintain sequence independence between X and Y. The allosteric “terminator switch” mechanism was successfully ported into HEK 293T cells, providing a proof-of-principle for cgRNA regulation in the mammalian setting. Subsequently, the nonallosteric “toehold switch” mechanism was also successfully ported to HeLa cells.

In order to develop cgRNAs into a versatile platform for conditional regulation, here we focus on engineering high-performance allosteric cgRNAs in the mammalian setting. Toward this end, we measure performance using three metrics: (1) Fold-change: maximize the ON → OFF or OFF → ON conditional response ratio with/without the cognate RNA trigger (optimal value is ∞). (2) Fractional dynamic range: maximize the difference between conditional ON and OFF states as a fraction of the unconditional regulatory dynamic range of CRISPR/Cas using standard gRNAs (optimal value is 100%). (3) Crosstalk: minimize sequence (and metabolic) interactions between cgRNAs and noncognate triggers including the transcriptome (optimal value is 0%).
As a basis for comparison, consider the “terminator switch” cgRNA mechanism of Figure 4a that is conditionally inactivated by RNA trigger X (ON → OFF logic). In HEK 293T cells expressing inducing dCas9 as the protein effector, we previously observed for a library of three orthogonal cgRNA/trigger pairs a median ON → OFF conditional response of ≈4-fold, median fractional dynamic range of ≈15%, and median crosstalk of ≈30%. There is significant room for improvement in all three metrics and much higher levels of performance will be a prerequisite for cgRNAs to become useful for applications. Here, we sought to improve performance for allosteric ON → OFF logic in mammalian cells. Moreover, in contrast to previous OFF → ON cgRNAs that are nonallosteric and do not provide sequence independence between trigger X and target Y, we wondered whether protecting triggers with a 5′ xrRNA (Figure 4b), the cgRNA would have a strong ON state with activity equivalent to a standard gRNA (ideal ON state) and a clean OFF state with negligible activity equivalent to a no-target gRNA lacking the target-binding region (ideal OFF state). As expected, a strong conditional response with a nearly ideal OFF state is observed using a trigger with a 5′ xrRNA (Dengue 4; right). Single-cell fluorescence intensities via flow cytometry. Transfection of plasmids expressing inducing dCas9-VPR, Phi-YFP target gene, and either standard gRNA + no-trigger control (ideal ON state), cgRNA + no-trigger control (OFF state), no-target gRNA that lacks target-binding region + no-trigger control (ideal OFF state).

RESULTS AND DISCUSSION

In considering avenues to increase cgRNA performance, we wondered whether endogenous RNA degradation pathways might be undermining current performance. To protect against RNA degradation, flaviviruses use exoribonuclease-resistant RNA (xrRNA) motifs, that form a mechanical block to halt diverse 5′ exoribonucleases (Figure 3a). xRNAs have previously been appended to mRNAs to study decay intermediates. Here, we explore whether xRNAs can enhance the performance of RNA synthetic biology.

As a starting point, we tried expressing xrRNA motifs (e.g., Figure 3a) from five viruses at the 5′ end of an RNA trigger used to regulate the terminator switch cgRNA of Figure 4a. The best of the xrRNAs dramatically improved trigger performance, yielding a cleaner OFF state with lower fluorescence (Figure 3b). Figure 3c compares cgRNA/trigger performance for a standard trigger (left) and for a trigger with a 5′ xrRNA from Dengue 4 (right). Using the xrRNA trigger, the OFF state distribution is dramatically improved, achieving nearly the ideal OFF state corresponding to a no-target gRNA lacking the target binding region. Based on this dramatic performance enhancement for one cgRNA/trigger pair, we next wondered whether protecting triggers with a 5′ xrRNA might boost performance across the board for our library of previously published terminator switch cgRNAs.

The terminator switch cgRNA mechanism of Figure 4a is conditionally inactivated by RNA trigger X (ON → OFF logic). Compared to a standard gRNA (Figure 1b), the cgRNA has a modified terminator region with an extended loop and rationally designed sequence domains “d-e-f.” Hybridization of the RNA trigger X (protected by an xrRNA at the 5′ end) to these modified domains is intended to form a structure incompatible with cgRNA mediation of dCas9 function. This cgRNA mechanism is allosteric—the trigger down-regulates cgRNA:dCas9 function not by sequestering the target-binding region (orange in Figure 4a), but by hybridizing to the distal trigger-binding region (blue). Hence, the sequences of the RNA trigger X and the regulatory target Y are fully independent.

In HEK 293T cells expressing the cgRNA, inducing dCas9-VPR as the protein effector, and a fluorescent protein reporter (Phi-YFP) as the target gene Y, we expect a decrease in fluorescence upon expression of the RNA trigger X (conditional logic: “if not X then Y”; Figure 4b). An ideal cgRNA would have a strong ON state with activity equivalent to a standard gRNA (ideal ON state) and a clean OFF state with negligible activity equivalent to a no-target gRNA lacking the target-binding region (ideal OFF state). As expected, a strong conditional response with a nearly ideal OFF state is observed using a trigger with a 5′ xrRNA (Figure 4c).

Figure 3. Enhancing RNA trigger performance via protection with 5′ xrRNAs. (a) Secondary structure of a representative xrRNA motif. Gray shading denotes duplex regions; darker bases base pair to each other to form a pseudoknot. Arrowhead denotes 3′ end. (b) Screening for an enhanced cgRNA OFF state using triggers with a 5′ xrRNA (xrRNA motifs from five different viruses: Murray Valley encephalitis (MVE), West Nile virus (WNV), Zika, Dengue (Dengue 4), and Yellow Fever (Yf)). Raw fluorescence depicting ON → OFF conditional response to cognate trigger (standard trigger or trigger with 5′ xrRNA) in HEK 293 T cells. (c) cgRNA trigger performance: weak OFF state using standard trigger (left); nearly ideal OFF state using trigger with 5′ xrRNA (Dengue 4; right). Single-cell fluorescence intensities via flow cytometry. Transfection of plasmids expressing inducing dCas9-VPR, Phi-YFP target gene, and either standard gRNA + no-trigger control (ideal ON state), cgRNA + no-trigger control (OFF state), no-target gRNA that lacks target-binding region + no-trigger control (ideal OFF state).
Figure 4. Allosteric ON → OFF terminator switch cgRNAs with inducing dCas9 in mammalian cells. (a) cgRNA mechanism: the constitutively active cgRNA is inactivated by hybridization of RNA trigger X. Rational design of cgRNA terminator region (domains “d-e-f” : 6 nt linker, 4 nt stem, 30 nt loop) and complementary trigger region (domains “P-e-d”). (b) Conditional logic: “if not X then Y”. (c) Expression of RNA trigger X (xrRNA + 40 nt unstructured + hU6 terminator) toggles the cgRNA from ON → OFF, leading to a decrease in fluorescence. Single-cell fluorescence intensities via flow cytometry. Transfection of plasmids expressing inducing dCas9-VPR, Phi-YFP target gene Y, and either standard gRNA + no-trigger control (ideal ON state), cgRNA + no-trigger control (ON state), cgRNA + RNA trigger X (OFF state), or no-target gRNA that lacks target-binding region + no-trigger control (ideal OFF state). (d-g) Programmable conditional regulation using four orthogonal cgRNAs (Q, R, S, T). (d) Raw fluorescence depicting ON → OFF conditional response to cognate trigger. (e) Fold change = ON/ OFF. (f) Fractional dynamic range = (ON − OFF)/(ON + OFF) where ON corresponds to signal for cgRNA + trigger p for p ∈ [X₀, X₁, X₂, X₃]. Bar graphs depict mean ± estimated standard error of the mean (with uncertainty propagation) calculated based on the mean single-cell fluorescence over 1545–3970 cells for each of N = 3 replicate wells. dynamic range, note that cgRNA T has the strongest ON state but is the hardest to turn OFF, resulting in the lowest fold-change but the highest fractional dynamic range. The median crosstalk between noncognate/trigger pairs is ≅4%, and the median crosstalk modulus is ≅9%. Negative crosstalk values correspond to a situation where the ON state is stronger with a noncognate trigger than with the no-trigger control (presumably a form of metabolic rather than sequence crosstalk). For terminator switch cgRNAs, the use of triggers with a S’ xrRNA enables high-performance cell-selective regulation that opens the door to pursuit of applications for ON → OFF logic.

Previous demonstrations of OFF → ON logic using cgRNAs have employed a “toehold switch” mechanism that is nonallosteric, preventing independent selection of trigger X and regulatory target Y. With OFF → ON logic, the constitutive state is OFF so it is especially critical to achieve a clean OFF state to prevent spurious signal accumulation prior to expression of the trigger X. In our experience, it is challenging to completely (yet reversibly) inactivate a cgRNA via intramolecular interactions. Hence, we reasoned that to generate a constitutively clear OFF state it could be beneficial to use a “split cgRNA” concept in which the cgRNA is expressed in two (or more) pieces whose assembly into a functional cgRNA is mediated by the trigger. For programmability, the trigger must interact with a portion of the cgRNA whose sequence can be designed, and for allosterity the designable sequence must also be independent of the target Y. Our terminator switch mechanism for ON → OFF logic (Figure 4a) demonstrates that the stem of the S’ terminator hairpin is designable, and truncation studies of a standard gRNA suggested that the stem of this hairpin is necessary for function (Figure S26). Hence, as a promising route to implementing allosteric OFF → ON logic with a clean OFF state, we decided to test a “split-terminator switch” mechanism in which the S’ terminator hairpin is replaced by a terminator duplex between the cgRNA and the trigger.

Figure 5a displays a “split-terminator switch” mechanism that is conditionally activated by RNA trigger X (OFF → ON logic). Compared to a standard gRNA (Figure 1b), the cgRNA is incomplete, containing only one-half of the stem region of the S’ terminator hairpin (sequence domain “d”). Hybridization of the RNA trigger X to domain “d” is intended to form a terminator duplex that restores cgRNA function. This mechanism is allosteric because the trigger and cgRNA interact via a terminator duplex (blue in Figure 5a) distal to the target-binding region (orange). The resulting full sequence independence between RNA trigger X and target gene Y provides the flexibility for X to control regulatory scope independent of the choice of Y. Initially, we expected to use a long terminator duplex to achieve stable hybridization with a strong regulatory effect, but preliminary studies with duplexes of 40, 30, 20, 10, 8, 6, or 4 bp revealed relatively modest performance differences, with the best performance achieved using the shortest terminator duplex of only 4 bp (Section S6.6). This short duplex length, which matches the stem length of the original terminator hairpin, suggests that dCas9 may assist in stabilizing formation of the terminator duplex in the cgRNA:trigger:dCas9 complex.
trigger X (Figure 5c). Notably, the OFF state approaches the ideal OFF state of a no-target gRNA lacking the target-binding region. The raw data for a library of three orthogonal split-terminator switch cgRNAs (M, N, O) (Figure 5d) reveals that all three cgRNAs have notably clean OFF states in the absence of trigger and generate strong ON states in response to expression of the corresponding cognate trigger. Across the three cgRNA/trigger pairs, we observe a median fold-change of $\approx 150\times$ (Figure 5e), a median fractional dynamic range of $\approx 50\%$ (Figure 5f), and median crosstalk of $\approx 4\%$ (Figure 5g). With this high level of performance in mammalian cells, split-terminator switch cgRNAs likewise open the door to pursuit of applications for OFF $\rightarrow$ ON logic.

For both the ON $\rightarrow$ OFF terminator switch mechanism and the OFF $\rightarrow$ ON split-terminator switch mechanism, sequence design was performed using the NUPACK reaction pathway designer.\textsuperscript{40,41} Sequence design was formulated as a multistate optimization problem using target test tubes to represent reactant and product states of cgRNA/trigger hybridization, as well as to model crosstalk between orthogonal cgRNAs (Figure 6).\textsuperscript{41} Sequence design was performed subject to complementarity constraints inherent to the reaction pathway (Figure 4a; domain “d” complementary to “d*”, etc.), as well as to biological sequence constraints imposed by the regulatory target Y, the protein effector (dCas9), and the hU6 terminator; see the constraint shading in Figure 6. Sequences were optimized by reducing the ensemble defect quantifying the average fraction of incorrectly paired nucleotides over the multibupe ensemble,\textsuperscript{41-43} implementing both a positive design paradigm (explicitly design for on-pathway elementary steps) and a negative design paradigm (explicitly design against off-pathway crosstalk).\textsuperscript{41} The discovery that xrRNA viral protection elements can dramatically enhance the performance of RNA synthetic
biology seems likely to have important consequences beyond the scope of cgRNAs. In our exploratory studies, we found the utility of xrRNAs to be highly context dependent. For the ON \(\rightarrow\) OFF terminator switch mechanism (Figure 4), xrRNAs from several viruses dramatically enhanced performance when expressed S' of the trigger, while a number of other xrRNAs expressed in the same location significantly impeded performance (Figure S21). From among the best-performing xrRNAs, we selected the Dengue 4 xrRNA motif for further study.\(^6\) We tried placing it S' of a cgRNA or a standard gRNA, but the xrRNA interfered with cgRNA/gRNA function, substantially reducing the strength of target induction in both cases (Figures S22). For a library of four orthogonal terminator switch cgRNAs (Q, R, S, T), the improvement in fold change using triggers with a S' xrRNA was striking, and the gains in fractional dynamic range were also substantial (Figure S24). Surprisingly, for the OFF \(\rightarrow\) ON split-terminator switch mechanism (Figure 5), triggers performed slightly worse with a S' xrRNA than without (Figure S30). This difference in the efficacy of xrRNA triggers for the two cgRNA mechanisms may derive from the fact that for ON \(\rightarrow\) OFF logic the trigger is expected to function in the absence of dCas9 (Figure S14), while for the OFF \(\rightarrow\) ON logic the trigger is expected to function in complex with dCas9 (Figure S15), potentially leading to differential degrees of protection from the protein effector.

The present work significantly advances the performance of cgRNAs in the mammalian setting. xrRNA motifs that protect viral RNAs from degradation by diverse exoribonucleases are demonstrated to have significant context-dependent utility for RNA synthetic biology. Libraries of orthogonal cgRNA/trigger pairs achieve a median fold-change response of \(\approx 500\) for ON \(\rightarrow\) OFF logic and \(\approx 150\) for OFF \(\rightarrow\) ON logic, a level of performance suitable for pursuit of applications. There is still room to improve fractional dynamic range by roughly a factor of 5 for ON \(\rightarrow\) OFF logic and roughly a factor of 2 for OFF \(\rightarrow\) ON logic. Median crosstalk between noncognate cgRNA/trigger pairs, capturing both sequence and metabolic effects, is \(\approx 9\%\) in magnitude for ON \(\rightarrow\) OFF logic and \(\approx 4\%\) for OFF \(\rightarrow\) ON logic.

High-performance cgRNAs open the possibility of restricting synthetic regulation to a desired cell type, tissue, or organ without engineering the organism. This could be achieved by selecting an endogenous RNA trigger X with the desired spatial and temporal expression profile, enabling biologists to exert spatiotemporal control over regulation. For example, conditional gene silencing would query genetic necessity and conditional gene activation would query genetic sufficiency. To shift conditional regulation to a different tissue or developmental stage, the cgRNA would be reprogrammed to recognize a different input X with the desired spatial and temporal expression profile. Figure 2b illustrates the range of cell-selective spatiotemporal control that is conceptually achievable in embryos or bacterial mixtures by combining the conditionality of cgRNA logic (ON \(\rightarrow\) OFF and OFF \(\rightarrow\) ON) and the functionality of Cas variants (edit, silence, induce). The same molecular logic has attractive therapeutic potential, with X as a programmable disease marker and Y as an independent programmable therapeutic pathway, enabling selective treatment of diseased cells leaving healthy cells untouched (Figure 2c).

In order to realize the vision of cell-selective spatiotemporal regulatory control with cgRNAs, it is critical to develop cgRNA mechanisms that can efficiently detect endogenous RNA triggers (e.g., subsequences of full-length mRNAs), significantly increasing the difficulty of achieving a strong conditional response.\(^28,30,44\) Further, while the split-terminator switch mechanism is allosteric, enabling independent selection of trigger X and target Y, a portion of the trigger may be constrained by the sequence and/or structure of the terminator region 3' of the terminator duplex (shaded gray in Figure 5a). Such a constraint would not be limiting for synthetic biology applications (where the trigger can be rationally designed), but it poses a challenge in the context of detecting endogenous triggers. Further study of the structure/function relationship between the terminator region and Cas is warranted as insights into these interactions have facilitated critical advances in cgRNA mechanism design.

## EXPERIMENTAL SECTION

For each mechanism, orthogonal cgRNA/trigger pairs were designed using NUPACK (nupack.org).\(^40,41\) A cgRNA expression plasmid and a trigger expression plasmid were cotransfected with a plasmid expressing an inducing dCas9-VPR fusion\(^37\) and a reporter plasmid containing a gRNA binding site upstream of a minimal CMV promoter for Phi-YFP expression.\(^39,45\) The four plasmids were transiently transfected into HEK 293T cells with Lipofectamine 2000 and grown for 24 h, with end-point fluorescence measured via flow cytometry. Data analysis was performed on cells expressing high levels of both cgRNA and trigger fluorescent protein transfection controls. In contrast to our previous studies where the no-trigger control employed a plasmid lacking the hU6-promoter-trigger-poly(T) sequence,\(^21\) here our no-trigger control uses a random pool of triggers to provide a sequence-generic approximation of the metabolic load of trigger expression.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00037.

Methods, sequences, plasmids, schematics, flow cytometry replicates, and additional studies (PDF)

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Notes

The authors declare the following competing financial interest(s): Patent applications filed by the California Institute of Technology.

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