ACS Synthetic Biology
Cite This: ACS Synth. Biol. 2019, 8, 1583−1589
Letter
pubs.acs.org/synthbio

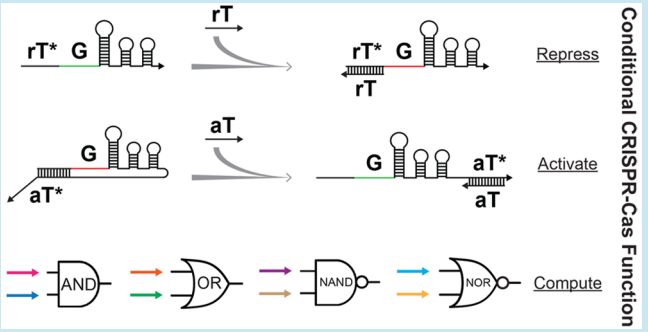
Programmable CRISPR-Cas Repression, Activation, and Computation with Sequence-Independent Targets and Triggers

Mike Jin,[∥,†,‡] Nicolas Garreau de Loubresse,[∥,†,‡] Youngeun Kim,[†,‡] Jongmin Kim,[*,†,‡,§] and Peng Yin[*,†,‡]

[†]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts 02115, United States
[‡]Department of Systems Biology, Harvard University, Boston, Massachusetts 02115, United States
[§]Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang, Gyeongbuk 37673, Republic of Korea

Supporting Information

ABSTRACT: The programmability of CRISPR-derived Cas9 as a sequence-specific DNA-targeting protein has made it a powerful tool for genomic manipulation in biological research and translational applications. Cas9 activity can be programmably engineered to respond to nucleic acids, but these efforts have focused primarily on single-input control of Cas9, and until recently, they were limited by sequence dependence between parts of the guide RNA and the sequence to be detected. Here, we not only design and present DNA- and RNA-sensing conditional guide RNA (cgRNA) that have no such sequence constraints, but also demonstrate a complete set of logical computations using these designs on DNA and RNA sequence inputs, including AND, OR, NAND, and NOR. The development of sequence-independent nucleic acid-sensing CRISPR-Cas9 systems with multi-input logic computation capabilities could lead to improved genome engineering and regulation as well as the construction of synthetic circuits with broader functionality.



KEYWORDS: *CRISPR-Cas9, guide RNA engineering, conditional guide RNA, Cas9 control, molecular computing*

Since its advent, CRISPR (clustered regularly interspaced short palindromic repeats) has shown great versatility in analyzing, controlling, and editing genomes.[1] The most common CRISPR-Cas (CRISPR-associated) system studied and used in applications consists of the *S. pyogenes* Cas9 protein, which uses two RNA species (crRNA and tracrRNA) to guide its endonuclease activity. Conveniently, crRNA and tracrRNA can be linked together and expressed as a single guide RNA (gRNA) species, and in either case the same "guide" region of the crRNA determines the complementary DNA sequence that it targets.[2] Due to its simplicity and programmability as a DNA-targeting protein, CRISPR-Cas9 has been applied successfully to epigenetic regulation, live-cell imaging, gene drives, cancer immunotherapy, and many others.[3−6]

Given the broad utility of Cas9 in applications, spatiotemporal control is important for its specificity. In addition to enabling the specification of a particular setting and timing for its on-target activity, having a conditionally controllable Cas9 would also limit its off-target activity, a side-effect which increases with exposure duration.[7] Toward this end, several methods for post-translational control of Cas9 have been developed, including photoactivation and chemical activation by small molecules.[8−11] However, these methods require engineering a new Cas9 variant for sensing and responding to each distinct stimulus.

Engineering guide RNA species for conditional control of Cas9 activity presents an attractive alternative to engineering Cas9 protein itself, and many have demonstrated the success and utility of switchable or conditional gRNA (cgRNA), a term recently proposed.[12−22] These efforts were aided by the fact that predictable structures and programmable interactions of nucleic acids lend themselves to rational design approaches to achieve desired functionality.[23,24] gRNA has proven relatively amenable to modification: for example, incorporation of RNA aptamers into gRNA enables the modified gRNA to target fluorescent proteins or transcriptional modulators to specific DNA loci and control Cas9 activity *via* ligand binding.[12,13,23,25,26] One strategy for designing cgRNA that can sense and respond to other nucleic acids introduces antisense protectors which bind to the guide region of a gRNA and repress Cas9 activity until it is released by endonuclease or photocleavage.[14,15] This strategy of using repressing complementary strands for cgRNA design can be taken further by making use of toehold-mediated strand displacement: attach-

Received: March 31, 2019
Published: July 10, 2019

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583−1589

ing a toehold domain to the antisense protector allows it to be easily removed by an activating single-stranded DNA or RNA trigger, and the protector can also be linked to the gRNA to form a single switchable cgRNA species.[16,17,20,22] cgRNA has been shown to sense endogenous RNA species in living cells as well.[18,19,21,22] This presents the exciting prospect of detecting endogenous biological signals (*e.g.*, mRNA, noncoding RNAs) with information about cell identity and state. Being able to sense specific RNA signatures could potentially limit CRISPR activity to only the desired biological contexts (*e.g.*, cell types, cell states) and minimize undesired off-target effects outside of these contexts. Indeed, much success has been demonstrated in forward-engineering of programmable RNA-based regulators that control transcription or translation *in vivo* and perform complex logic computations.[27−29]

One desired goal for conditional Cas9 control is to be able to not only transduce the simple presence or absence of an environmental signal into ON/OFF behavior, but also perform computations on these sensory inputs. In order to realize this potential fully through cgRNA, two conditions need to be achieved. First, cgRNA should be able to sense multiple triggers and regulate its activity in response to different combinations of trigger conditions. Second, there should not be any sequence constraints on the guide sequence or the trigger(s): the trigger sequence(s) should be independent of both the guide sequence and the gRNA scaffold sequence. Earlier cgRNA designs had partial to significant sequence dependency with the guide sequence, the gRNA scaffold, or other endonuclease recognition sequence, and therefore cannot be programmed to sense arbitrary sequences given a desired DNA target.[14,16−19] This limitation has been overcome recently by newer designs for repressible and activatable sequence-independent cgRNA,[20,21] and multi-input RNA AND logic for conditional activation of Cas12a has been shown.[22] However, a unified design for both sequence-independent agRNA and rgRNA requiring no additional biological components as well as a complete demonstration of sequence-independent cgRNA computational capabilities have both yet to be achieved. A system that could programmably implement "sense sequences A and B, bind Cas9 to sequence C", where A, B, and C are independent sequences, would make conditionally active Cas9 a more effective and biologically context-specific tool in synthetic biology applications.

Here, we present sequence-independent designs for computation-capable cgRNA in both activatable (agRNA) and repressible (rgRNA) forms that regulate Cas9 activity by sensing single-stranded DNA or RNA oligonucleotide triggers and are programmable for arbitrary sequence combinations of guide and trigger. These cgRNAs are based on a unified novel design principle and demonstrate implementation of four different types of Boolean logic computations on input trigger species.

We designed agRNA and rgRNA based on our empirical observation that the formation of a double-stranded structure 5′ upstream of the guide sequence **G** achieved substantial inhibition of Cas9 activity (Figure 1). Since this structure did not have to overlap with **G** in order to achieve inhibition, this allowed for full sequence independence between **G** and any trigger **rT** or **aT** involved in the formation or separation of this structure in the cgRNA designs. rgRNA is formed by making a single-stranded 5′ extension **a*** having arbitrary sequence on the gRNA, directly adjacent to but not overlapping the guide

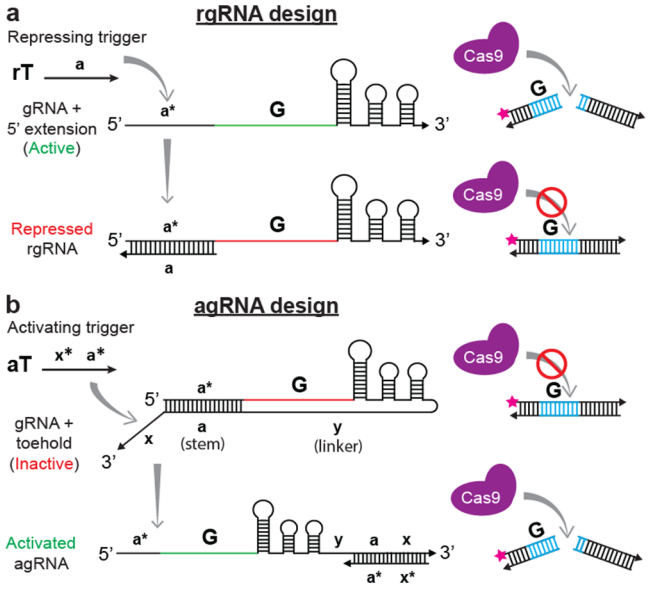


Figure 1. (a) Design of rgRNA: a double-stranded structure upstream of the guide sequence **G** (20 nt) represses Cas9 activity. Adding an extra binding domain **a*** (typically 16 nt, but longer for logic gate implementation; see Figure 3) allows Cas9 to remain active while letting **a*** act as a sensor for repressing trigger **rT** = **a**, whose binding represses Cas9 activity as measured by *in vitro* cleavage of a fluorophore-labeled dsDNA target containing sequence **G**. (b) Design of agRNA: covalently linking (linker **y**, 10 nt) the trigger-bound structure of the rgRNA as a double-stranded stem results in an agRNA after adding a toehold binding domain **x** (14 nt). This enables activation by the appropriate cognate trigger **aT** = **x*** **a***, again measured by *in vitro* Cas9 activity as in (a).

sequence **G** (Figure 1a). This extension does not substantially reduce activity. rgRNA is repressed by binding a complementary trigger **rT** with sequence **a** to the extension **a***, forming the inhibitory double-stranded structure. agRNA is formed by linking the 3′ end of gRNA to the repressor in this double-stranded stem structure using a flexible linker to alleviate potential conformational constraints, creating a single RNA species which remains repressed. Adding a toehold domain **x** to the repressor **a** allows activation of the agRNA by binding an activating trigger **aT** which is complementary to both **x** and **a**, displacing the inhibitory double-stranded structure next to **G** (Figure 1b).

cgRNA designs were verified *in vitro*, with Cas9 activity quantified using a denaturing polyacrylamide gel shift assay based on the amount of fluorophore-labeled dsDNA cleaved. The results showed that for rgRNA, the single-stranded 5′ extension **rT*** itself resulted in only a slight decrease in DNA cleavage in the ON state (Figure 2a, blue columns compared to violet columns with same guide), with 65−75% of DNA cleaved by the various unrepressed rgRNAs. Upon binding a repressing trigger **rT**, however, rgRNA showed an 8- to 15-fold decrease in DNA cleavage (Figure 2a), to only 4−9% of DNA cleaved. Likewise, agRNA by itself, with its domain **aT*** bound to its single-stranded 5′ extension to form the inhibitory double-stranded structure, did not result in substantial DNA cleavage: the agRNAs alone showed 6−8% DNA cleavage for G1 and 8−21% for G5 (Figure 2b). Introduction of activating trigger **aT** resulted in a substantial increase in DNA cleavage to 55−70%, levels similar to positive control (Figure 2b, orange columns compared to violet columns with same guide). This

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583−1589

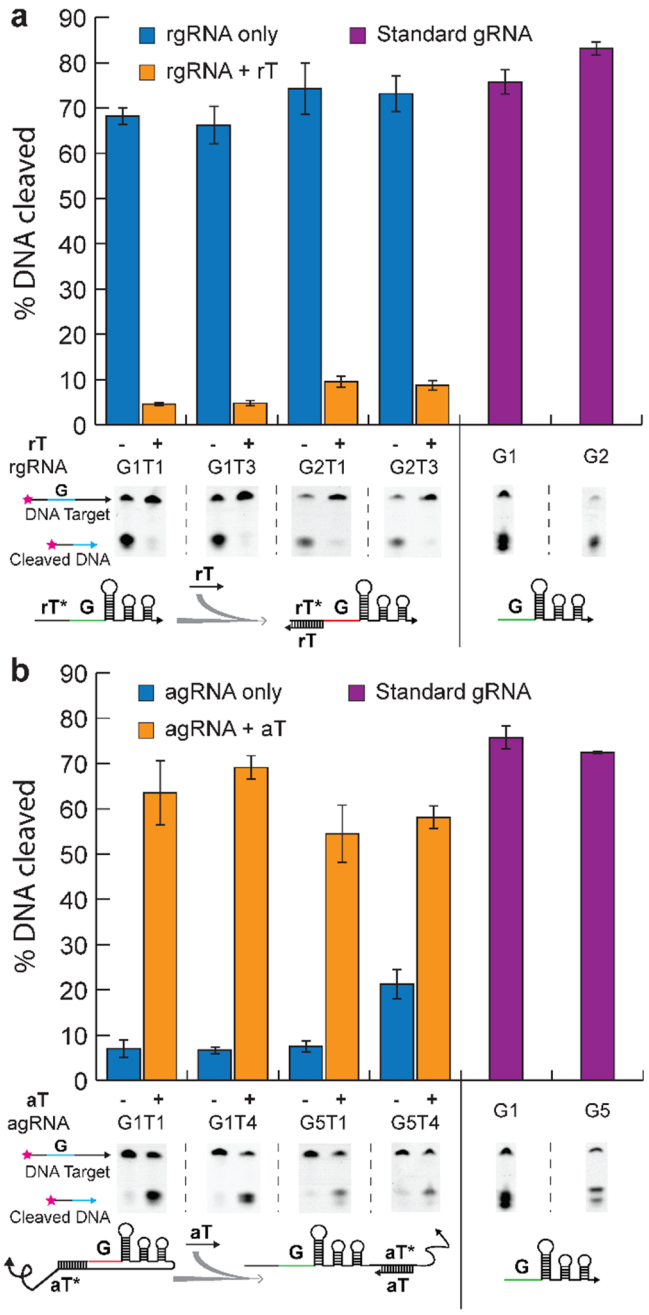


Figure 2. Demonstration of programmability and sequence independence for (a) rgRNA and (b) agRNA, each showing Cas9 activity with and without trigger, quantified by percentage of dsDNA target cleaved, for all combinations of two guide sequences with two trigger sequences. cgRNA domain lengths are as described in Figure 1, and a tail was added to agRNA to enhance performance (see main text for details). Denaturing polyacrylamide gel images below each graph show cleavage of 10 nM dsDNA target by 125 nM Cas9 and 250 nM cgRNA in the presence of 1 μM repressing or activating trigger (**rT** or **aT**) after 1 h incubation at 37 °C. See Methods and Table S2.1, Table S3 in Supporting Information for additional details.

constitutes a 9- to 10-fold increase for agRNAs with guide G1, and 3- to 7-fold for G5.

agG1T1 (*i.e.*, **ag**RNA with **G**uide sequence **1**, **T**rigger sequence **1**, where each trigger sequence number denotes the same sequence used within agRNA designs or within rgRNA designs, whereas guide sequence numbers denote the same sequence across all cgRNA designs; Table S2.1) also showed some sensitivity to single-nucleotide polymorphisms (SNPs) in the trigger sequence, with even 1 SNP present in the trigger sequence resulting in a 40% reduction in target dsDNA cleavage compared to correct trigger sequence (Figure S1). For noncognate triggers with higher sequence dissimilarity, erroneous activation levels were negligible, as shown by cross-reactivity testing (Figure S2). rgRNA did not show a similar decrease in repression for repressing triggers with SNPs (data not shown), as a repressing trigger with a few SNPs still contains plenty of correctly pairing bases to bind strongly. SNP detection can in principle be optimized using molecular competition: for example, a toehold probe setup could be used to further improve SNP detection sensitivity.[30]

Investigation of the parameter space surrounding the agRNA design using agG1T1 revealed some trends for optimizing domain lengths for the best ON/OFF activity ratio. First, we observed that the addition of a 15 nt ssRNA extension at the 3′ end of the agG1T1 toehold domain improved its ON/OFF ratio by both reducing the "leakage" Cas9 activity in the OFF state (without activating trigger **aT**) and increasing activity in the ON state (with trigger present) (Figure S3). In fact, the same 15 nt sequence added to agRNAs with different guide/trigger sequences appeared to uniformly improve ON/OFF ratios in a similar fashion by either reducing leakage or increasing ON activity (Figure S4).

Next, variation of 3 different sections of agG1T1 gave an apparent optimal set of domain lengths: stem length of 16 nt, linker length of 10 nt, and spacer length of 0 nt between guide and stem (Figure S5). Stem lengths shorter than 16 nt appeared to increase activity in the OFF state, presumably due to decreased binding energy of the stem. On the other hand, increasing the stem to 20 nt eliminated activity in the ON state (Figure S5a). For the other two domain variations, activity in the OFF state was relatively unaffected, whereas a 10 nt linker between gRNA scaffold and stem produced the highest activity in the ON state (Figure S5b), and any additional distance between the guide sequence and the double-stranded stem decreased activity in the ON state (Figure S5c). Given a long spacer between the guide and stem, the local configuration of agRNA in the OFF state may resemble the canonical gRNA with a single-stranded 5′ extension.

In case some sequence dependency of guide and trigger is allowed, the double-stranded structure can partially overlap the guide sequence in agRNA (Figure S6a). This agrees with previous works demonstrating that direct binding of complementary strands to the guide sequence of gRNAs inhibits Cas9 activity.[15,18] For a sequence-dependent design tested which placed the 16 bp double-stranded stem directly overlapping the guide sequence, an ON/OFF activity ratio of around 20 was observed. This ratio exceeded that of any of the sequence-independent agRNAs tested (Figure S6b). The single-stranded extensions on the 5′ end of gRNAs still appeared to slightly inhibit Cas9 activity: the ON state of a sequence-dependent agRNA (*i.e.*, with no 5′ extension on the guide sequence) showed 76% DNA cleavage (Figure S6), on par with 76% cleavage observed with canonical gRNA (Figure 2), whereas with 16 nt and 26 nt single-stranded 5′ extensions, the cleavage levels dropped to around 65% (Figure 2a, rgG1T1) and 60% (Figure 3e, 3f, "0 0" columns), respectively.

Both agRNA and rgRNA can sense multiple trigger species to evaluate logic expressions based on the presence of other nucleic acid sequences (DNA, Figure S7; RNA, Figure 3), for example, detecting two trigger inputs that both must be

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583−1589

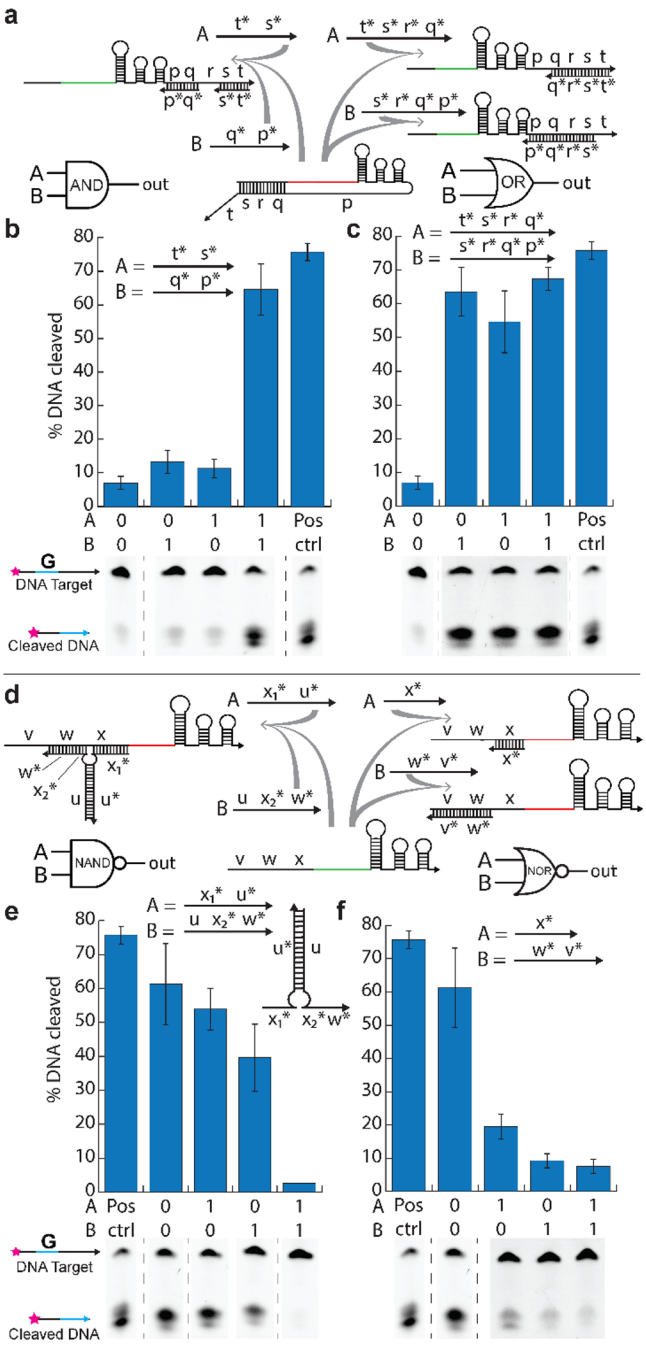


Figure 3. Logic gate implementation using RNA triggers on cgRNA designs. (a–c) Implementation of 2-input AND (both inputs must be present for activation) and 2-input OR (either input present results in activation) with agG1T1. Domain lengths **p** = 10 nt, **q** = 4 nt, **r** = 8 nt, **s** = 4 nt, **t** = 14 nt. (d–f) Implementation of 2-input NAND (both inputs must be present for repression) and 2-input NOR (either input present results in repression) with rgG1T1e. Domain lengths **u** = 21 nt, **v** = 10 nt, **w** = 6 nt, **x** = 10 nt, $\mathbf{x}_1$ = 8 nt, $\mathbf{x}_2$ = 2 nt. Denaturing polyacrylamide gel images below each graph show cleavage of 10 nM dsDNA target by 125 nM Cas9 and 250 nM cgRNA (or standard gRNA as positive control) in the presence of 1 μM of each trigger after 1 h incubation at 37 °C. See Figure S8 for uncropped gel image for (e). See also Methods and Table S2.1, Table S8 in Supporting Information for additional details.

present to evaluate as TRUE, consequently demonstrating a logical AND expression. All of the logic gate designs presented here used trigger sequences that had no sequence dependence on either the gRNA scaffold or the guide sequence.

agRNA can implement 2-input AND with two triggers, which individually only open up part of the double-stranded stem, so that both triggers must be present to open the entire stem (Figure 3a). This was validated by PAGE assay, where both triggers with agRNA produces Cas9 activity comparable to standard gRNA positive control (Figure 3b). Similarly, 2-input OR can be implemented by extending both triggers such that any single trigger can open the entire stem starting from separate toeholds on the agRNA (Figure 3a). PAGE results showed that either trigger produced Cas9 activity comparable to positive control (Figure 3c).

rgRNA implements 2-input NAND using two triggers where the binding domain of any single trigger is too short for stable binding with the rgRNA; self-assembly of the two triggers forms a trigger complex that can stay bound to the 5′ gRNA extension (Figure 3d). Two-input NOR was implemented using nonoverlapping triggers such that each can stably bind to distinct parts of the 5′ extension (Figure 3d). Again, this was validated by PAGE assay for both NAND and NOR (Figure 3e, 3f). Note that, for NAND and NOR logic, in order to provide more space for trigger binding, the 5′ end of the rgRNA was extended by an additional 10 nt for a total of 26 nt (Table S2.1). Interestingly, the 2-input NOR design demonstrated that a double-stranded structure even 10 nt upstream of the rgRNA's guide sequence can effectively repress Cas9 activity (Figure 3f, S7).

These design principles for logic gate implementation are both generalizable and constitute only part of a rich design space. The self-assembling trigger design for 2-input NAND can be generalized to greater numbers of triggers, as 3-input NAND was implemented similarly with 3 self-assembling triggers (Figure S9). Moreover, the programmable structure of nucleic acids provides ample possibility for alternate logic gate implementations: for instance, the AND gate implementation could utilize a self-assembling trigger design much as the 2- and 3-input NAND implementations, as this design with up to 4 self-assembling strands has been successfully used to activate riboswitches using strand displacement with 4-input AND logic.[29] On the basis of previous literature, the kinetics of strand displacement from a trigger complex should still be much faster than Cas9 kinetics, and therefore kinetic considerations are not of primary significance in determining cgRNA switching performance with multiple trigger inputs.[31,32] Beyond the two-input logic gates presented here, we can also implement other logic such as NIMPLY using trigger sequestration (Figure 4) and in principle extend to any multi-input logic by layering gates.[33]

In addition to demonstrating programmability across a variety of sequences, rgRNA and agRNA are compatible with both DNA and RNA triggers. DNA and RNA versions of activating trigger aT1 both successfully activate agG1T1 (Figure S10), and implementation of four logic gates AND, OR, NAND, and NOR was achieved using DNA (Figure S7) and RNA (Figure 3) triggers.

rgRNA can also be used for more specialized applications involving conditional Cas9 activation: including a toehold on the repressing trigger enables derepression of the switch using an activating strand **T1Rp*** that is the reverse complement of the extended repressing trigger **T1Rp** (Figure 4). This mode of activation can be advantageous where thresholding is required: with an excess of repressing trigger present, derepressing

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583–1589

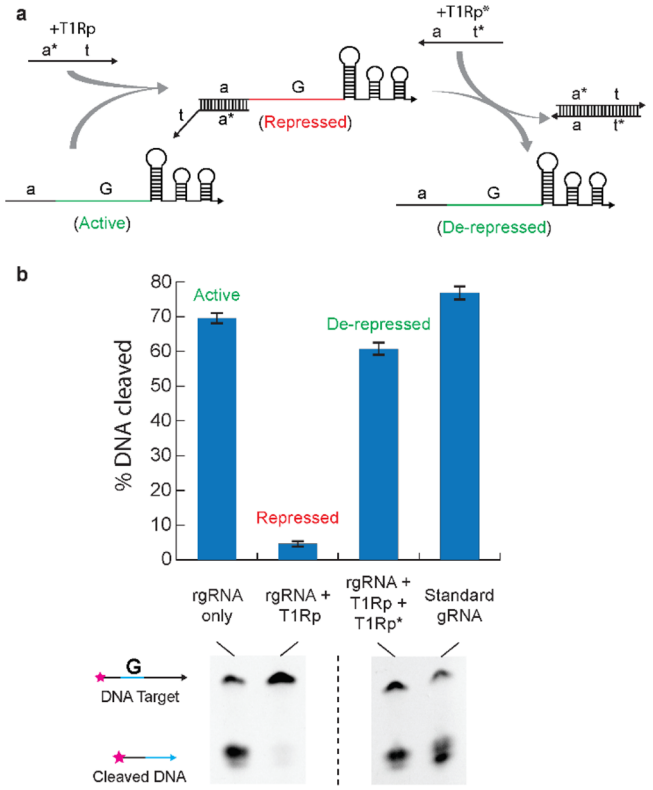


Figure 4. (a) Repression and derepression of rgRNA. Repressor **T1Rp** is first introduced, having binding domain **a*** (16 nt) and toehold **t** (14 nt), at a threshold concentration. The derepressing trigger **T1Rp***, at sufficient concentration, can then reactivate the repressed rgRNA by binding and removing **T1Rp**. (b) Denaturing polyacrylamide gel showing cleavage of 10 nM dsDNA target by 125 nM Cas9 and 250 nM rgG1T1 or standard gRNA as positive control. 500 nM of initial repressing trigger **T1Rp** was overcome by 1000 nM derepressing trigger **T1Rp*** added halfway (30 min) through 1 h incubation at 37 °C. See Figure S11 for complete PAGE results and Methods, Table S2.1, and Table S6 in Supporting Information for additional details.

triggers cannot achieve significant activation of Cas9 activity until it titrates out the repressing triggers (Figure S11).

Finally, agRNA likewise demonstrated conditional activity with dCas9, "dead" Cas9 with no nuclease activity but retaining its sequence-specific DNA binding activity.[34,35] *In vitro* activation of dCas9 was demonstrated using agG1T1LS (long stem) using a gel shift assay (Figure S12). At present, it is not clear whether dCas9 does not stably bind to the OFF state cgRNA, or the dCas9-cgRNA complex cannot stay bound to its DNA target. The applicability of switchable cgRNA to dCas9 makes cgRNA equally relevant to the plethora of applications involving dCas9 in addition to those involving standard Cas9 including genetic or epigenetic regulation and synthetic circuits. When combined with dCas9-based transcription regulators, cgRNA could enable synthetic biologists to implement modules with RNA input and RNA output in a single step.

In summary, we have demonstrated programmable, computation-capable cgRNA that can conditionally activate (agRNA) or repress (rgRNA) the activity of Cas9 by performing logic gate functions on nucleic acid triggers that have no sequence dependency on the guide and scaffold domains of gRNA. Moreover, we have shown the novel empirical result that the formation of a double-stranded structure adjacent to the guide sequence is sufficient for *in vitro* repression of Cas9 activity. Our work utilizes this fact as a unifying design principle and represents the first demonstration of both activation and repression of cgRNA within the same system. It is also the first example of an activatable sequence-independent cgRNA which does not rely on any other cellular machinery: recent works showed either sequence-independent cgRNA repression[20] or sequence-independent cgRNA activation requiring additional cellular endonucleases.[21] Toward the goal of programmable Cas9 with complex sensing and computational capabilities, the sequence independence of the sensed triggers from the gRNA target and scaffold sequences represents a critical feature, but more importantly, the demonstration of multi-input logical computations represents a key step forward in this space.

The achievement and refinement of a fully sequence-independent cgRNA capable of logical computations could provide substantial utility in further applications. Due to the low amount of cross-talk between agRNAs, this scheme could additionally be multiplexed to allow conditional targeting of Cas9 to multiple loci by multiple distinct RNA triggers and may be extended to arbitrary DNA- or RNA-guided endonuclease systems. As the system requires only Cas9, cgRNA, and the input RNAs without any additional proteins or ligands, sequence-independent RNA-sensing cgRNAs could prove useful for implementing complex gene circuits. These circuits based on similar design principles may work in biological contexts as well, since the inhibitory double-stranded structure relies only on Watson−Crick base pairing. Indeed, sequence-constrained and sequence-independent cgRNAs have already been described in bacterial and mammalian cells,[14,18,21] and concentration-thresholded activation of Cas9/dCas9 with information-computing capabilities may be useful for a wide variety of biological applications, potentially enabling genome editing, genome regulation, or transcription regulation conditional on cellular RNA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00141.

Detailed methods, DNA and RNA sequences used, and supplemental figures (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: jongmin.kim@postech.ac.kr.
*E-mail: py@hms.harvard.edu.

ORCID

Mike Jin: 0000-0001-7237-6697
Youngeun Kim: 0000-0002-4020-939X
Jongmin Kim: 0000-0002-2713-1006
Peng Yin: 0000-0002-2769-6357

Author Contributions

‖M.J. and N.G.d.L. contributed equally.

Notes

The authors declare the following competing financial interest(s): Peng Yin is cofounder of Ultivue Inc. and NuProbe Global.

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583−1589

■ ACKNOWLEDGMENTS

This work was supported by Office of Naval Research grants N000141612410 and N000141310593; National Institutes of Health grants 1R01EB01865901 and 1DP1GM13305201; and National Science Foundation grant CCF1317291 to P.Y.; M.J. was supported by the National Science Foundation Graduate Research Fellowship Program (DGE1745303). N.G.d.L. was supported by the Human Frontier Science Program (HFSP Long-Term Fellowship LT 000522/2015) and the Philippe Foundation. J.K. acknowledges a Wyss Institute Director's Cross-Platform Fellowship. A provisional patent has been filed based on this work. P.Y. is cofounder of Ultivue Inc. and NuProbe Global.

■ REFERENCES

(1) Wang, F., and Qi, L. S. (2016) Applications of CRISPR Genome Engineering in Cell Biology. *Trends Cell Biol. 26*, 875−888.

(2) Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science 337*, 816−821.

(3) Liu, X. S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. A., and Jaenisch, R. (2016) Editing DNA Methylation in the Mammalian Genome. *Cell 167*, 233−247.

(4) Chen, B., Gilbert, L. A., Cimini, B. A., Schnitzbauer, J., Zhang, W., Li, G. W., Park, J., Blackburn, E. H., Weissman, J. S., Qi, L. S., and Huang, B. (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell 155*, 1479−1491.

(5) Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M., Baker, D., Marois, E., Russell, S., Burt, A., Windbichler, N., Crisanti, A., and Nolan, T. (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nat. Biotechnol. 34*, 78−83.

(6) Su, S., Hu, B., Shao, J., Shen, B., Du, J., Du, Y., Zhou, J., Yu, L., Zhang, L., Chen, F., Sha, H., Cheng, L., Meng, F., Zou, Z., Huang, X., and Liu, B. (2016) CRISPR-Cas9 mediated efficient PD-1 disruption on human primary T cells from cancer patients. *Sci. Rep. 6*, 20070.

(7) Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., and Zhang, F. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol. 31*, 827−832.

(8) Polstein, L. R., and Gersbach, C. A. (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. *Nat. Chem. Biol. 11*, 198−200.

(9) Zetsche, B., Volz, S. E., and Zhang, F. (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat. Biotechnol. 33*, 139−142.

(10) Liu, K. I., Ramli, M. N., Woo, C. W., Wang, Y., Zhao, T., Zhang, X., Yim, G. R., Chong, B. Y., Gowher, A., Chua, M. Z., Jung, J., Lee, J. H., and Tan, M. H. (2016) A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing. *Nat. Chem. Biol. 12*, 980−987.

(11) Nihongaki, Y., Kawano, F., Nakajima, T., and Sato, M. (2015) Photoactivatable CRISPR-Cas9 for optogenetic genome editing. *Nat. Biotechnol. 33*, 755−760.

(12) Liu, Y., Zhan, Y., Chen, Z., He, A., Li, J., Wu, H., Liu, L., Zhuang, C., Lin, J., Guo, X., Zhang, Q., Huang, W., and Cai, Z. (2016) Directing cellular information flow *via* CRISPR signal conductors. *Nat. Methods 13*, 938−944.

(13) Tang, W., Hu, J. H., and Liu, D. R. (2017) Aptazyme-embedded guide RNAs enable ligand-responsive genome editing and transcriptional activation. *Nat. Commun. 8*, 15939.

(14) Ferry, Q. R., Lyutova, R., and Fulga, T. A. (2017) Rational design of inducible CRISPR guide RNAs for de novo assembly of transcriptional programs. *Nat. Commun. 8*, 14633.

(15) Jain, P. K., Ramanan, V., Schepers, A. G., Dalvie, N. S., Panda, A., Fleming, H. E., and Bhatia, S. N. (2016) Development of Light-Activated CRISPR Using Guide RNAs with Photocleavable Protectors. *Angew. Chem., Int. Ed. 55*, 12440−12444.

(16) Jakimo, N., Chatterjee, P., and Jacobson, J. M. (2018) ssRNA/DNA-Sensors *via* Embedded Strand-Displacement Programs in CRISPR/Cas9 Guides, *bioRxiv*.

(17) Cox, K. J., Subramanian, H. K. K., Samaniego, C. C., Franco, E., and Choudhary, A. (2019) A universal method for sensitive and cell-free detection of CRISPR-associated nucleases. *Chem. Sci. 10*, 2653−2662.

(18) Siu, K. H., and Chen, W. (2019) Riboregulated toehold-gated gRNA for programmable CRISPR-Cas9 function. *Nat. Chem. Biol. 15*, 217−220.

(19) Lee, Y. J., Hoynes-O'Connor, A., Leong, M. C., and Moon, T. S. (2016) Programmable control of bacterial gene expression with the combined CRISPR and antisense RNA system. *Nucleic Acids Res. 44*, 2462−2473.

(20) Hanewich-Hollatz, M. H., Chen, Z., Hochrein, L. M., Huang, J., and Pierce, N. A. (2019) Conditional Guide RNAs: Programmable Conditional Regulation of CRISPR/Cas Function in Bacterial and Mammalian Cells *via* Dynamic RNA Nanotechnology. *ACS Cent. Sci.*, DOI: 10.1021/acscentsci.9b00340.

(21) Wang, X. W., Hu, L. F., Hao, J., Liao, L. Q., Chiu, Y. T., Shi, M., and Wang, Y. (2019) A microRNA-inducible CRISPR-Cas9 platform serves as a microRNA sensor and cell-type-specific genome regulation tool. *Nat. Cell Biol. 21*, 522−530.

(22) Oesinghaus, L., and Simmel, F. C. (2019) Switching the activity of Cas12a using guide RNA strand displacement circuits. *Nat. Commun. 10*, 2092.

(23) Briner, A. E., Donohoue, P. D., Gomaa, A. A., Selle, K., Slorach, E. M., Nye, C. H., Haurwitz, R. E., Beisel, C. L., May, A. P., and Barrangou, R. (2014) Guide RNA functional modules direct Cas9 activity and orthogonality. *Mol. Cell 56*, 333−339.

(24) Nishimasu, H., Ran, F. A., Hsu, P. D., Konermann, S., Shehata, S. I., Dohmae, N., Ishitani, R., Zhang, F., and Nureki, O. (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell 156*, 935−949.

(25) Wang, S., Su, J. H., Zhang, F., and Zhuang, X. (2016) An RNA-aptamer-based two-color CRISPR labeling system. *Sci. Rep. 6*, 26857.

(26) Zalatan, J. G., Lee, M. E., Almeida, R., Gilbert, L. A., Whitehead, E. H., La Russa, M., Tsai, J. C., Weissman, J. S., Dueber, J. E., Qi, L. S., and Lim, W. A. (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell 160*, 339−350.

(27) Green, A. A., Silver, P. A., Collins, J. J., and Yin, P. (2014) Toehold switches: de-novo-designed regulators of gene expression. *Cell 159*, 925−939.

(28) Chappell, J., Westbrook, A., Verosloff, M., and Lucks, J. B. (2017) Computational design of small transcription activating RNAs for versatile and dynamic gene regulation. *Nat. Commun. 8*, 1051.

(29) Green, A. A., Kim, J., Ma, D., Silver, P. A., Collins, J. J., and Yin, P. (2017) Complex cellular logic computation using ribocomputing devices. *Nature 548*, 117−121.

(30) Zhang, D. Y., Chen, S. X., and Yin, P. (2012) Optimizing the specificity of nucleic acid hybridization. *Nat. Chem. 4*, 208−214.

(31) Genot, A. J., Zhang, D. Y., Bath, J., and Turberfield, A. J. (2011) Remote toehold: a mechanism for flexible control of DNA hybridization kinetics. *J. Am. Chem. Soc. 133*, 2177−2182.

(32) Zhang, D. Y., and Winfree, E. (2009) Control of DNA strand displacement kinetics using toehold exchange. *J. Am. Chem. Soc. 131*, 17303−17314.

(33) Tamsir, A., Tabor, J. J., and Voigt, C. A. (2011) Robust multicellular computing using genetically encoded NOR gates and chemical 'wires'. *Nature 469*, 212−215.

(34) Mali, P., Aach, J., Stranges, P. B., Esvelt, K. M., Moosburner, M., Kosuri, S., Yang, L., and Church, G. M. (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol. 31*, 833−838.

(35) Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., and Lim, W. A. (2013) Repurposing

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583−1589

CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell 152*, 1173−1183.

DOI: 10.1021/acssynbio.9b00141
ACS Synth. Biol. 2019, 8, 1583−1589