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# Conditional Recruitment to a DNA-Bound CRISPR−Cas Complex Using a Colocalization-Dependent Protein Switch

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Cite This: https://dx.doi.org/10.1021/acssynbio.0c00012 Read Online

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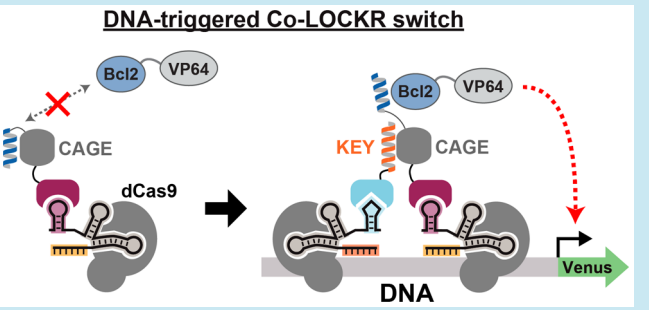
**ABSTRACT:** To spatially control biochemical functions at specific sites within a genome, we have engineered a synthetic switch that activates when bound to its DNA target site. The system uses two CRISPR−Cas complexes to colocalize components of a *de novo*-designed protein switch (Co-LOCKR) to adjacent sites in the genome. Colocalization triggers a conformational change in the switch from an inactive closed state to an active open state with an exposed functional peptide. We prototype the system in yeast and demonstrate that DNA binding triggers activation of the switch, recruitment of a transcription factor, and expression of a downstream reporter gene. This DNA-triggered Co-LOCKR switch provides a platform to engineer sophisticated functions that should only be executed at a specific target site within the genome, with potential applications in a wide range of synthetic systems including epigenetic regulation, imaging, and genetic logic circuits.




**KEYWORDS:** *CRISPR−Cas, Co-LOCKR, protein switch, genetic circuit*



Catalytically inactive CRISPR−Cas complexes, which bind but do not cleave DNA, can programmably recruit functional proteins to specific genomic target sites with applications for transcriptional control, epigenetic regulation, and imaging.[1,2] One outstanding challenge for the field is that these effector proteins can produce nonspecific background effects. For example, epigenetic modifiers can mark off-target sites in the genome,[3] while unbound imaging probes can produce background signal that obscures the specific target site of interest.[4] Similarly, efforts to engineer long-range DNA loops are challenging in part because the desired interaction between two DNA-bound CRISPR−Cas complexes competes with the free, unbound CRISPR−Cas complexes in the cell.[5] In each of these cases, the problem is that the effector protein remains functional even when it is not bound at a specific DNA target site. As a first step to address this general problem, we have developed a conditional system in which an effector protein can be activated only when the CRISPR−Cas complex is bound to its DNA target.

To engineer a conditional, DNA-triggered effector protein, we envisioned making the activity of the effector protein dependent on colocalization of two CRISPR−Cas complexes. Ideally, assembly of the functional effector would occur only when the two complexes are brought into proximity at adjacent genomic target sites. To achieve this behavior, we turned to a recently developed system called colocalization-dependent latching orthogonal cage-key (Co-LOCKR),[6] a designed protein switch that activates only when its two components, the cage and key, are colocalized. The cage conformationally regulates a protein interaction module (the "latch" peptide) that becomes activated only when key binding displaces the latch and exposes the interaction module (Figure 1).[7,8] By appropriate tuning of the cage, latch, and key affinities, the conformational switch occurs only when the key and cage are colocalized to the same subcellular location and in close physical proximity.[6]

To couple the conformational switch to DNA binding, we can use two orthogonal CRISPR−Cas complexes to recruit a Co-LOCKR cage and key to adjacent genomic target sites. When both the cage and key are colocalized on DNA, the Co-LOCKR switches to the active state and exposes the protein interaction module. When the cage-tethered CRISPR−Cas complex is not bound to DNA, the cage adopts the inactive state (Figure 1). Thus, provided that the cage−key interaction is too weak to form without colocalization, the system should effectively function as a sensor for DNA binding: only CRISPR−Cas complexes that are bound to DNA and appropriately positioned will switch to the active state. An additional feature of this approach is that off-target binding events should not activate the switch, as any off-target sites for the cage or key CRISPR−Cas

**Received:** January 9, 2020




https://dx.doi.org/10.1021/acssynbio.0c00012
ACS Synth. Biol. XXXX, XXX, XXX−XXX

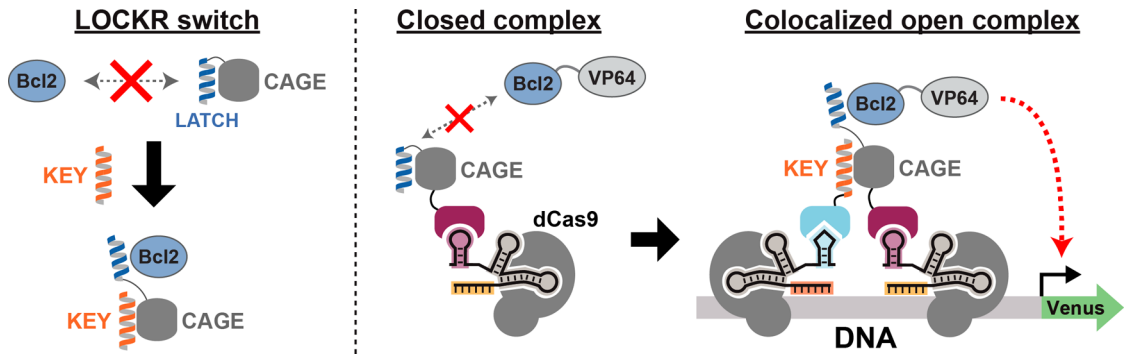



**Figure 1.** Colocalized CRISPR−Cas complexes can sense DNA binding. The LOCKR switch is a designed protein that can switch between two conformational states.[7] Colocalization of the key and cage with orthogonal CRISPR−Cas complexes to adjacent genomic target sites on DNA releases the latch, which allows recruitment of the Bcl2−VP64 transcriptional activator. CRISPR−Cas complexes are specified for either cage or key recruitment using orthogonal 3′ RNA hairpins that recruit RNA binding proteins fused to the cage or key, respectively.

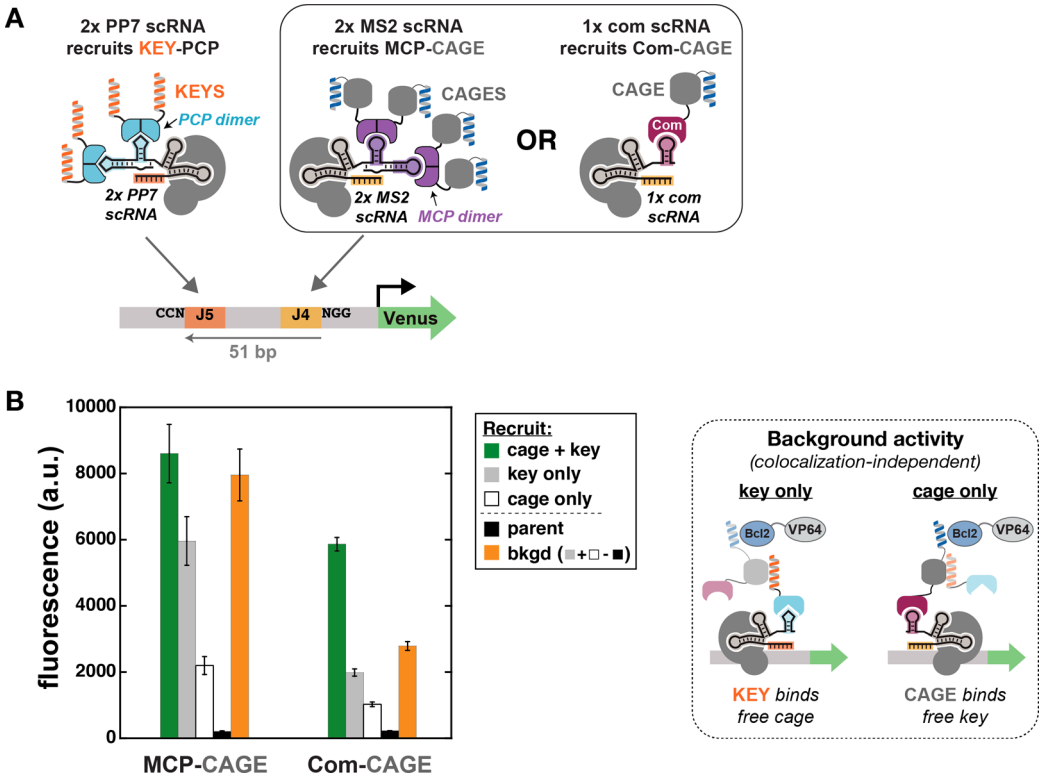



**Figure 2.** Colocalization-dependent activation of a transcriptional reporter. (A) A 2× PP7 scRNA targets the upstream site (J5) and recruits four key−PCP fusion proteins. At the downstream site (J4), a 2× MS2 scRNA recruits four MCP−cage fusion proteins. Alternatively, a 1× com scRNA recruits one Com−cage fusion protein. (B) Fluorescence reporter activity upon cage−key colocalization. For the MCP−cage, the observed "cage + key" signal appears to arise primarily from colocalization-independent opening of the Co-LOCKR switch. For the Com−cage, the observed "cage + key" signal is significantly stronger than the background colocalization-independent activation. The background is the additive sum of the "key only" and "cage only" samples (Figure S1), and the errors are propagated by adding in quadrature. Data for parents were obtained with the unmodified parent strains yKL016 and yKL014. Fluorescence values are means ± SD for at least three biological replicates.

complexes are unlikely to be in close proximity to each other. Conceptually similar approaches have been used for gene editing with paired Fok1 nucleases, nickases, and alternative DNA binding domains.[9−14]

We demonstrate here that Co-LOCKR switches can be coupled to CRISPR−Cas complexes to function as a proximity-sensitive sensor of DNA binding. We use a reporter gene assay to assess the switch function, and we systematically vary the stoichiometry, separation distance on DNA, linker lengths, expression levels, and protein interaction strengths to identify the key parameters that enable optimal colocalization-dependent switch activation on DNA. The use of tunable, designed proteins to implement a DNA-binding-dependent protein switch lays the foundation for a variety of future applications, including synthetic epigenetic modifications, imaging tools, rewiring of genome structure, and genetic logic gates, that could benefit from coupling of a biochemical function to DNA binding.

## RESULTS

To engineer a DNA-dependent Co-LOCKR switch, we need one CRISPR−Cas complex that recruits the cage protein and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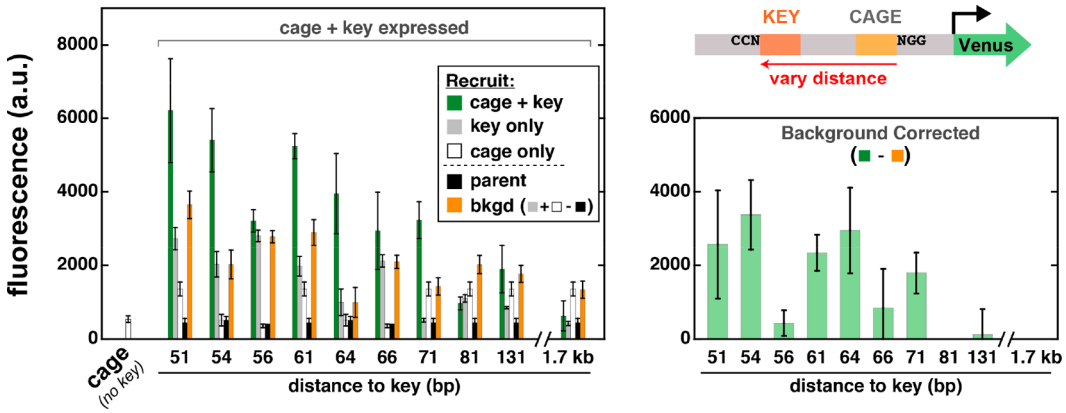


**Figure 3.** Sensitivity of the CRISPR Co-LOCKR switch (2× PP7−key and 1× com−cage) to the target site spacing. The background colocalization-independent activation is calculated from the sum of the "key only" and "cage only" samples as described in Figure S1. Subtracting the background from the observed "cage + key" activity gives the background-corrected values shown. Data for parent strains were obtained by transforming the parental strains yKL014, yRK244, and yRK245 with empty vector (pRS316). Fluorescence values are means ± SD for at least three biological replicates.

another CRISPR−Cas complex programmed to an adjacent genomic site that recruits the key protein. To recruit different proteins to adjacent CRISPR−Cas complexes, we can use the catalytically inactive *Streptococcus pyogenes* dCas9 together with scaffold RNAs (scRNAs), which are modified sgRNAs with 3′ hairpins that can recruit RNA binding proteins (RBPs).[15] By using orthogonal RNA hairpin−RBP pairs, we can program one target site to recruit the cage and the other target site to recruit the key (Figure 1). Alternatively, we can directly fuse the cage and key to orthogonal CRISPR−Cas proteins.[1]

To test different DNA-dependent Co-LOCKR designs, we constructed a transcriptional reporter system in *Saccharomyces cerevisiae* with multiple distinct CRISPR−Cas target sites upstream of a genomically integrated fluorescent Venus reporter gene. These target sites can be used to colocalize the Co-LOCKR cage and key proteins in close proximity on DNA. Binding of the key to the cage exposes the protein interaction module, a Bim peptide that binds to the Bcl2 protein.[6] We fused Bcl2 to the transcriptional activator VP64 so that cage opening would recruit Bcl2−VP64 to activate Venus reporter expression (Figure 1). Reporter activation thus serves as a proxy for the open state of the Co-LOCKR switch.

**Colocalization on Genomic DNA Can Activate a Co-LOCKR Switch.** We initially prototyped the RNA recruitment strategy using scRNAs with PP7, MS2, and com hairpins together with their cognate RNA binding proteins PCP, MCP, and Com. We used a 2× PP7 scRNA to recruit the key−PCP fusion protein to the upstream target site (J5). PP7 binds PCP as a dimer, so a 2× PP7 scRNA recruits four key−PCP fusion proteins. To recruit the cage to the downstream promoter-proximal target site (J4), we initially tested two strategies: either a 2× MS2 scRNA, which recruits four MCP−cage fusion proteins, or a 1× com scRNA, which recruits one Com−cage fusion protein (Figure 2A).[15] We engineered yeast reporter strains to express all of the protein components of the system: dCas9, Bcl2−VP64, key−PCP, and either MCP−cage or Com−cage. We then delivered a plasmid expressing two scRNAs to each strain: either J5 2× PP7 + J4 2× MS2 to the MCP−cage strain or J5 2× PP7 + J4 1× com to the Com−cage strain. In both cases, we observed Venus fluorescent reporter expression (Figure 2B).

To evaluate the significance of the reporter activation, we need to assess the background signal from colocalization-independent cage opening, which could occur with a recruited cage in the presence of coexpressed key or from a recruited key in the presence of coexpressed cage (Figure 2B). These contributions can be measured by delivering only one guide instead of both guides simultaneously. For the MCP−cage, the background from recruitment of only the key is comparable to the Venus signal obtained when both the cage and key are recruited, suggesting that most if not all of the observed expression arises from colocalization-independent effects where free cage binds the recruited key. In contrast, for the monomeric Com−cage, the background expression levels from recruitment of only the key or only the cage are both significantly smaller than the Venus expression when both the cage and key are recruited.

To confirm that the observed Venus expression with the Com−cage Co-LOCKR is truly colocalization-dependent, we need to consider whether the observed expression is larger than the combined effects of binding of free key to the recruited cage and binding of free cage to the recruited key (Figure S1). In control experiments, we demonstrated that VP64 recruitment to two target sites is additive rather than synergistic (Figure S1), suggesting that the additive sum of individual cage and key recruitment can be used to assess the contribution from colocalization-independent cage opening at both sites. The Com−cage colocalized activity is significantly larger than the sum of the key-only and cage-only activities (Figure 2), suggesting that the Com−cage-mediated Co-LOCKR is a colocalization-dependent switch.

Finally, we assessed whether alternative RNA recruitment strategies could improve switch activation. We tested 1× PP7, 2× PP7, and 1× com scRNAs to recruit key fusion proteins in multiple combinations with 1× MS2, 2× MS2, and 1× com to recruit cage fusion proteins. For all of the MS2-mediated MCP−cage recruitment strategies, background reporter gene activation was relatively high, and there was no significant switch activation above background (Figure S2). The dominant source of background activity is from the "key only" control, which presumably results from recruitment of free, partially open cages by the DNA-tethered key (Figure S1). This background is significantly reduced when the cage is fused to Com, a monomeric RNA binding protein. Using the Com−cage, both 1× PP7 and 2× PP7 produced colocalization-dependent switch activation above background, with more activation from 2× PP7


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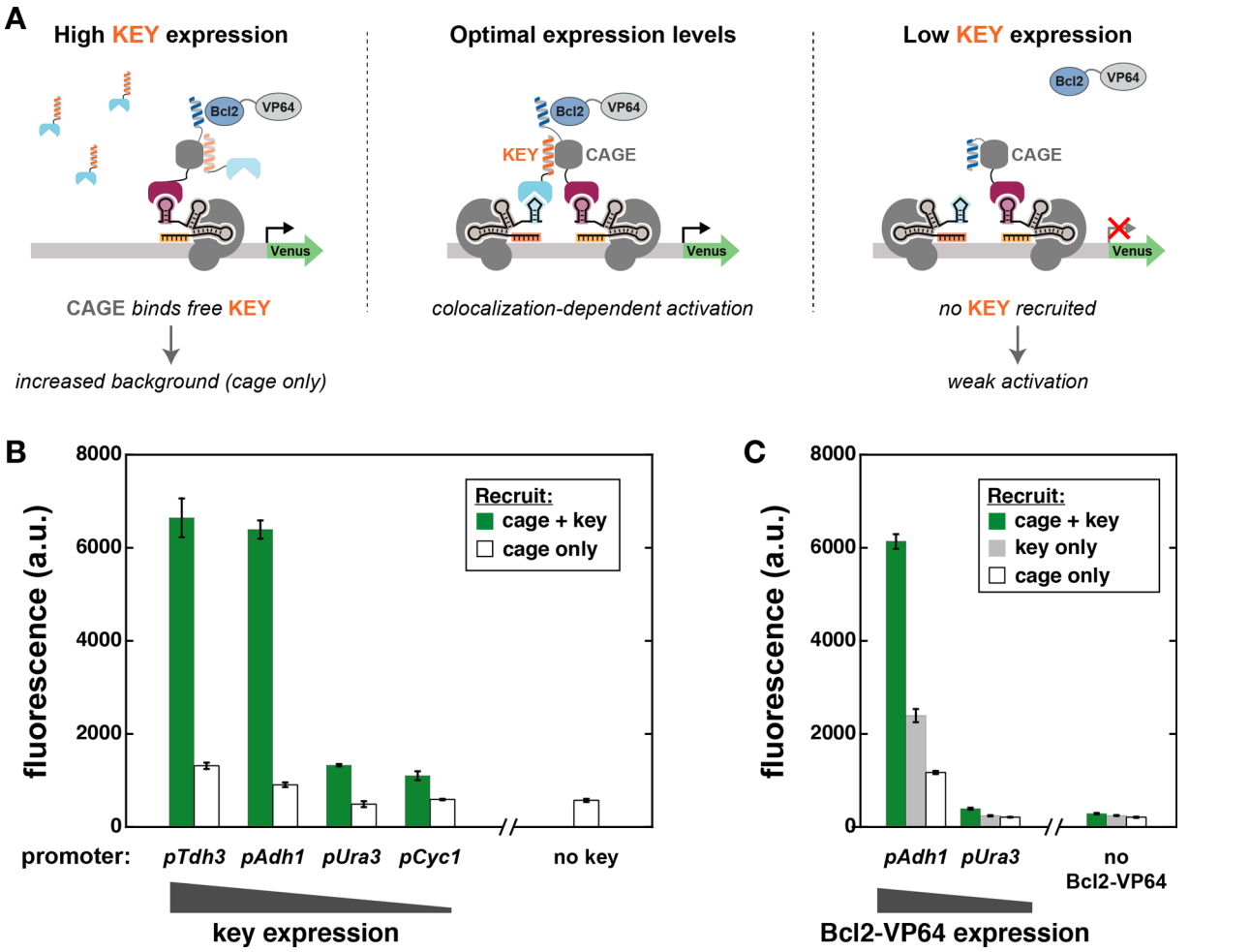


**Figure 4.** High key expression levels increase background activation of the CRISPR Co-LOCKR switch (2× PP7−key and 1× com−cage). (A) If key expression is too high, free key can bind and open the cage, which produces colocalization-independent background when only the cage is recruited. If key expression is too low, there will not be enough key−PCP fusion protein to bind the PP7 RNA hairpin, and there will be little to no colocalization-dependent activation. (B) When the key is expressed from a strong *pTdh3* promoter, we observe an increase in background activation (cage only). When the key is expressed from a weak *pUra3* or *pCyc1* promoter, there is little colocalization-dependent activation (cage + key). (C) Decreasing Bcl2−VP64 expression prevents switch activation. Fluorescence values in (B) and (C) are means ± SD for at least three biological replicates.

(Figure S2). Thus, it appears that providing a high stoichiometry of key to cage can help to promote Co-LOCKR activation, while delivering the cage on a multivalent protein is not effective.

**Direct Protein Fusions to Orthogonal CRISPR−Cas Complexes Can Activate a Co-LOCKR Switch.** We also tested an alternative recruitment strategy with direct fusions to orthogonal CRISPR−Cas proteins. We fused the cage to *S. pyogenes* dCas9 and the key to *Lachnospiraceae* bacterium dCpf1, which has previously been shown to be effective for transcriptional activation in yeast as a dCpf1−VPR fusion.[16] We observed colocalization-dependent activation that was significantly above the background (Figure S3), but the overall activation level was ∼10× smaller than that observed with the RNA recruitment strategy (Figure 2). Unlike mammalian cells, in yeast the direct protein fusions to CRISPR−Cas complexes are often outperformed by RNA recruitment strategies (Figure S3),[15] and the relatively weak activation obtained with direct fusions of the Co-LOCKR switch is consistent with this behavior. We therefore proceeded to focus on the RNA recruitment strategy using the optimal 2× PP7 scRNA to recruit PCP−key and 1× com to recruit Com−cage (Figures 2 and S2).

**Switch Activation Is Sensitive to the Distance between the CRISPR−Cas Complexes.** Using the RNA recruitment strategy, we had initially targeted the cage and key to two relatively close protospacer adjacent motif (PAM) sites positioned 51 bases apart (Figure 2). The 51 base separation between PAM sites includes each 20 base target site, leaving 11 bases between the CRISPR−Cas complexes. To explore the distance dependence of the Co-LOCKR switch, we targeted the key to a range of sites further upstream. Shifting the key five bases upstream to a 56 base separation, which corresponds to a half-turn around the DNA helix, resulted in an almost complete loss in the colocalization-dependent activation, which was recovered with another half-turn shift to a 61 base separation (Figure 3). The same pattern continued with 66 and 71 base separations. Moving the key further upstream resulted in a complete loss of colocalization-dependent activation (Figure 3). A similar periodicity in colocalizing protein effectors to DNA was observed with assembly of the Fok1 dimer with two dCas9−Fok1 complexes.[9]

Colocalization-dependent activation could also be sensitive to the linker length in the CRISPR−Cas complex, as linkers that are too short might not be able to reach their binding partners


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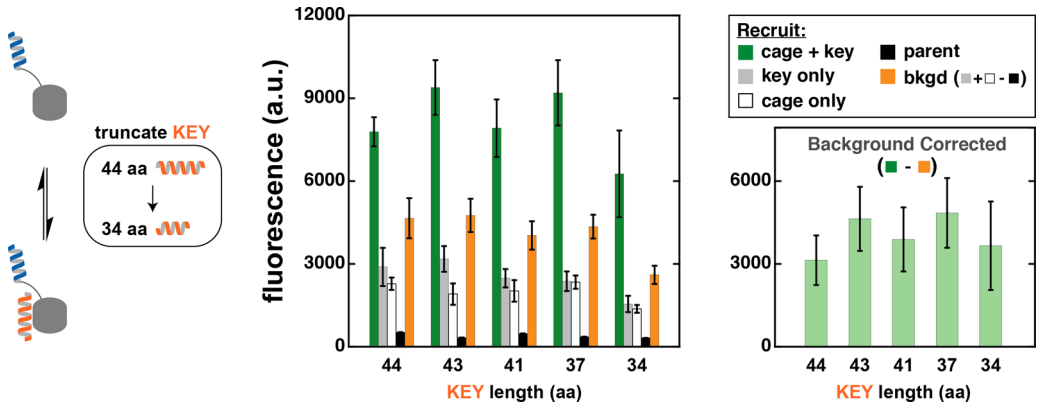


**Figure 5.** Tuning of the key length reduces background activation of the CRISPR Co-LOCKR switch (2× PP7:key and 1× com:cage). The 44 aa key is the original key used in previous experiments. When the key is truncated to 34 aa, background activation decreases while the background-corrected colocalization-dependent activation is not significantly affected. The background colocalization-independent activation is calculated from the sum of the "key only" and "cage only" samples as described in Figure S1. Data for parent strains were obtained with the unmodified parent strains yKL014, yKL029, yKL030, yKL031, and yKL032. Fluorescence values are means ± SD for at least three biological replicates.

while linkers that are too long might be too flexible to be effective. However, we varied the linker between the key and PCP from 3 to 45 amino acids and saw little change in colocalization-dependent activation (Figure S4).

**Optimization of the Com−Cage RNA-Mediated Co-LOCKR Switch.** We explored two additional design parameters with the Com−cage Co-LOCKR switch: protein expression levels and cage−key interaction affinity. LOCKR switch activation is sensitive to protein concentration, with high key concentrations driving the switch toward the open state.[7,8] We therefore varied the expression level of the key−PCP fusion protein using a set of promoters with different expression levels (Figure 4); these promoters vary in strength over a >100-fold range (Table S2).[17] Our initial experiments were performed with the relatively strong *Adh1* promoter, which was effective for previous applications of RBP fusion proteins in yeast.[15] We expected that higher key levels might activate the switch without colocalization, while key levels that are too low would fail to bind the scRNA PP7 hairpin at the CRISPR−Cas complex. Consistent with this expectation, the strongest promoter tested, *pTdh3*, results in the highest level of background activation when only the cage is recruited (Figure 4B). *pAdh1* maintains a similar level of colocalization-dependent switch activation but lower background compared with *pTdh3*. The weak promoters *pUra3* and *pCyc1* significantly reduced the background activation but also dramatically reduced colocalization-dependent activation.

Similarly, high expression levels of the Bcl2−VP64 fusion protein could drive the switch toward an open state. We therefore tested whether reducing the Bcl2−VP64 level could decrease the background activation. When we switched from the strong *Adh1* promoter to the weaker *Ura3* promoter, we observed a significant decrease in background activation but also a nearly complete loss of switch activation (Figure 4C).

To improve colocalization-dependent switch activation, we sought to reduce the background by tuning the strength of the interaction between the cage and key. A key that interacts too strongly with the cage might activate the switch without colocalization, while a key that interacts too weakly might not be able to activate the switch. The Co-LOCKR system has already been tuned to weaken the cage−key interface and minimize colocalization-independent activation.[6] To further weaken the cage−key interface, we truncated the key peptide over a range from 44 to 34 amino acids (Figure 5). Similar key truncations have previously been demonstrated to reduce the cage−key affinity in the Co-LOCKR system.[6,7] While most truncations had no significant effect, using the 34 aa key significantly reduced the background activation while maintaining the level of colocalization-dependent activation after correction for background (Figure 5).

## DISCUSSION

We show here that a colocalization-dependent protein switch, Co-LOCKR, can be adapted to act as a sensor for DNA binding. The switch undergoes a DNA-triggered conformational change when the Co-LOCKR cage and key modules are colocalized to CRISPR−Cas complexes at adjacent target sites in the genome. Multiple layers of modularity in the system provide powerful tools for precise control of biological functions: the Co-LOCKR switch can cage a diverse set of functional peptides,[6−8] and the CRISPR−Cas system enables programmable DNA targeting, which means that this switch can in principle be executed at a broad range of different sites in the genome. Further, the Co-LOCKR system could in principle be coupled with other programmable DNA binding systems, such as synthetic zinc fingers, that enable cooperative assembly of protein switch functions.[18]

The best-performing DNA-triggered Co-LOCKR switch utilized an RNA-recruitment strategy with the Com−cage (Figures 2 and S3). Multiple CRISPR−Cas complex separation distances between 51 and 71 bases are effective, but within this window the relative orientation along the DNA helix appears to affect the activity (Figure 3). While all of our designs produced some colocalization-independent activation, we found that this background could be reduced by weakening the cage−key interaction affinity. The most effective switch used a truncated 34 aa key and reduced the total background signal by 1.7-fold (Figure 5). Future work could potentially minimize this background further by strengthening the cage−latch interface together with more precise adjustments of the expression levels and cage−key affinity.[6,7]

Engineering a DNA-triggered Co-LOCKR switch requires balancing interaction strengths and expression levels so that the switch opens only when the cage and key are colocalized. Alternative approaches to obtain DNA-dependent protein activity face similar challenges in balancing affinities and


https://dx.doi.org/10.1021/acssynbio.0c00012
ACS Synth. Biol. XXXX, XXX, XXX−XXX

concentrations. For example, we could minimize the unbound fraction of a DNA-binding effector protein by expressing it at only a few copies per cell and engineering sufficiently high affinity so that it binds its DNA target even at low concentrations. Practically, however, it is challenging to precisely control expression levels at only a few copies per cell, and at such low concentrations the affinities would need to be quite tight to achieve full occupancy at the DNA target. With the Co-LOCKR system, we can express the components at high enough levels to bind the DNA target, but the switch ensures that the concentration of unbound, functionally active protein is minimized.

There are multiple systems in which minimizing the activity of unbound effector proteins could be advantageous. For example, with epigenetic modifiers, expression of effectors fused to DNA binding domains can lead to nonspecific modifications across the genome.[3] With a DNA-triggered Co-LOCKR, it should be possible to minimize the amount of active enzyme that is not bound to its DNA target. To achieve this goal, future work will need to develop a switch that triggers assembly of an enzyme. LOCKR switches that cage peptide fragments of split proteins have been engineered,[19] suggesting that a DNA-triggered epigenetic modifier should be achievable. A conceptually similar approach using split proteins fused to DNA binding domains has been shown to be effective for regulating the activity of the methyltransferase Dmnt3.[20,21] Because the affinity of a split protein interface may not be readily tunable, the LOCKR system could expand the toolbox of proteins that could be conditionally regulated as split proteins.

Another system in which minimizing active unbound protein would be advantageous is for engineering of long-range loops in DNA to probe the relationship between genome structure and function. It is possible to engineer DNA loops using constitutively active protein interaction domains fused to DNA binding domains,[5,22,23] but free unbound interaction domains can compete with loop formation.[5,24] With a DNA-triggered Co-LOCKR switch, it may be possible to minimize the concentration of free interaction domains, as any unbound complexes should remain caged and inactive. DNA-triggered switch opening would expose the interaction domain, which should promote interactions between DNA-bound complexes.

Finally, there are a broad range of potential applications of CRISPR−Cas guide RNAs as programmable elements in synthetic genetic circuits. CRISPR−Cas systems have been engineered to generate logic gates and transcriptional cascades.[25−30] The Co-LOCKR switch that we have implemented here acts as a simple two-input AND gate, where each input is an scRNA. While NOR gates have been described previously and multiple NOR gates can be linked to construct AND gates,[29] there are practical limitations for delivering large numbers of circuit components to a biological system. A Co-LOCKR-based AND gate provides a potentially simpler alternative to achieve this function.

The DNA-triggered CRISPR Co-LOCKR switch combines DNA-, RNA-, and protein-based logic to achieve sophisticated functional outcomes in a biological setting. The switch effectively functions as an allosteric sensor, with DNA binding triggering a conformational change at a distant site in the complex. These synthetic tools for allosteric control and spatial regulation of biochemical activity should provide new routes toward precise and tunable control of biological systems.

## METHODS

**Yeast Strain Construction.** Yeast (*S. cerevisiae*) transformations were performed with the standard lithium acetate method. The parent haploid yeast strain for reporter gene experiments was SO992 (W303; *MAT***a** *ura3 leu2 trp1 his3*). Complete descriptions of all yeast strains generated in this work are provided in Table S1. Reporter genes and protein expression constructs (Table S2) were integrated in single copy into the genome. Guide RNA expression constructs (Table S3) were delivered on CEN/ARS plasmids. Protein sequences and gRNA target site sequences are provided in the Supporting Information.

**Reporter Gene Design.** The pJ1-Venus reporter gene is a modified version of a previously described 1XtetO-Venus reporter gene.[15] The upstream tetO target site was replaced with a short 163 base array of dCas9 target sites, spaced 10 bases apart, from a sequence originally designed for a bacterial reporter system.[31] pR4-Venus and pR5-Venus are derivatives of pJ1-Venus with three or five bases inserted upstream of the dCas9 target site (J4) used for cage targeting. These shifted promoters allowed an additional set of dCas9 target site spacing distances to be tested (Figure 3). pR6-Venus is a modified version of the 1XtetO-Venus reporter gene that retains the tetO target site and inserts a dCpf1 target site 47 bases upstream. pR6 + 5, pR6 + 10, pR6 + 15, and pR6 + 20 are derivatives of pR6 with 5, 10, 15, or 20 bases inserted upstream of the tetO site, which allows a range of dCpf1−dCas9 spacings to be tested (Figure S3). Complete sequences of the reporter genes are provided in the Supporting Information.

**Co-LOCKR Fusion Proteins.** Cage and key protein designs for the Co-LOCKR switch have been described previously.[6] Cage proteins were fused to the C-terminus of RBPs and dCas9; C-terminal fusions of transcriptional regulators to RBPs and dCas9 are effective,[15] and LOCKR cages have been effective as C-terminal fusions *in vivo*.[7,8] Key proteins were fused to the N-terminus of RBPs and dCpf1, as this orientation was effective for key proteins in prior *in vivo* applications.[6−8]

**Flow Cytometry.** After transformation of guide RNA plasmids, yeast strains were grown overnight at 30 °C in minimal media (SD complete, SD − Ura, SD − His, or SD − Ura − His). Overnight cultures were diluted 1:25 and grown for an additional 4−5 h. Fluorescent protein expression levels were measured with an LSRII flow cytometer (BD Biosciences). To select single yeast cells, we applied a gate using the SSC-A versus FSC-A plot. Median fluorescence values were recorded from the gated populations. Values reported in the plots are means ± SD of the median fluorescence values for at least three measurements (biological replicates).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00012.

Calculation of background reporter gene activation; alternative RNA recruitment modules; direct dCas9 fusions; effect of varying key linker length; tables of yeast strains, plasmids, and gRNA target sites; and sequences of reporter genes and proteins (PDF)



https://dx.doi.org/10.1021/acssynbio.0c00012
ACS Synth. Biol. XXXX, XXX, XXX−XXX

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### Notes

The authors declare the following competing financial interest(s): M.J.L., S.E.B., R.A.L., and D.B. are inventors on patents related to this work.

## ACKNOWLEDGMENTS

We thank Dustin Maly, David Shechner, Rhiju Das, Ryan Kibler, and members of the Zalatan and Baker groups for comments and discussion. We thank Jason Fontana for assistance with the design of the J1 reporter gene. R.L.K. was supported by Genome Sciences NIH Training Grant T32 HG00035. R.A.L. was supported by Bruce and Jeannie Nordstrom, thanks to the Patty and Jimmy Barrier Gift for the Institute for Protein Design Directors Fund. M.J.L. was supported by a Washington Research Foundation Innovation Postdoctoral Fellowship and a Cancer Research Institute Irvington Fellowship from the Cancer Research Institute. S.E.B. was supported by a Career Award at the Scientific Interface from the Burroughs Wellcome Fund (1017008). This work was supported by a Career Award at the Scientific Interface from the Burroughs Wellcome Fund (1010814 to J.G.Z.), NIH R35 GM124773 (J.G.Z.), and Open Philanthropy (to D.B.).

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https://dx.doi.org/10.1021/acssynbio.0c00012
*ACS Synth. Biol.* XXXX, XXX, XXX−XXX