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Synthetic Biology **Hot Paper**

Controlling Gene Expression in Mammalian Cells Using Multiplexed Conditional Guide RNAs for Cas12a**

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Abstract: *Spatiotemporal control of the activity of CRISPR-associated (Cas) proteins is of considerable interest for basic research and therapeutics. Here, we show that conditional guide RNAs (gRNAs) for Cas12a can be transcribed in mammalian cells by RNA polymerase II, followed by activation via input-dependent processing of the 3′ tail of the gRNA transcript. We demonstrate processing using an RNA strand displacement mechanism, as well as microRNA-dependent processing, and cleavage by a guanine-responsive ribozyme. We further demonstrate that Cas12a along with several independently switchable gRNAs can be compactly integrated on a single transcript using stabilizing RNA triplexes, providing a route towards Cas12a-based gene regulation constructs with multi-input switching capabilities. The principle is shown to work in HEK and mouse fibroblast cells using luminescence, fluorescence, and is also demonstrated for the conditional upregulation of an endogenous gene.*

Introduction

The CRISPR-associated proteins Cas9 and Cas12a are RNA-guided DNA nucleases,[1] while their DNase-dead variants (dCas9 and dCas12a) are programmable DNA binders that can be fused to additional domains for transcriptional activation and repression, base editing, and other functions in mammalian cells.[2] Both for basic research and therapeutic purposes, it is desirable to exert spatiotemporal control on their activity.[3] This can be achieved via inducible or tissue-specific promoters, additional components such as anti-CRISPR proteins, or through switchable CRISPR components whose activity is contingent on control inputs such as light, small molecules, or endogenous RNAs.[3] For switchable control mechanisms, there are three fundamental options: either the Cas protein itself can be made switchable, its associated mRNA, or its guide RNA (gRNA). For interfacing with endogenous RNAs such as miRNAs or other mRNAs, messenger and guide RNAs are natural choices as they can be addressed via predictable Watson–Crick base pairing. Using gRNA as a switchable element has the additional advantage that a combination of multiple different targets and inputs in a single experiment can be achieved comparatively easily. Using Cas protein orthologs, for example, requires delivering a separate protein for each individual target.[4] Delivering multiple gRNAs is much simpler and more compact, i.e., it requires far less total sequence length. Cas12a, specifically, processes its own gRNA arrays, which makes it straightforward to place multiple gRNAs on a single transcript.[5] Using a triplex-forming sequence from the Malat1 noncoding RNA to stabilize the protein coding sequence, Campa et al. recently managed to place Cas12a along with 15 different gRNAs on a single Pol II transcript.[6]

Over the past years, multiple strategies have been developed to engineer switchable gRNAs that respond to a wide range of exogenous and endogenous triggers. Cas9 gRNAs have been switched with small molecules using aptamers embedded in the gRNA structure[7] or using inducible self-cleaving ribozymes at the 5′ end of the gRNA that interact with the spacer sequence.[8] For Cas12a, spacer-interacting riboswitches were implemented.[9] Wang et al. demonstrated microRNA-responsive Cas9 gRNAs by placing microRNA target sites adjacent to the gRNA on a Pol II transcript.[10]

Recently, several papers demonstrated activation of Cas9 or Cas12a gRNAs using toehold-mediated strand displacement.[11] Most of these gRNAs did not have independent input and target sequences, were not implemented in mammalian cells, or required several separate components to be delivered and expressed. Several of these shortcomings have been addressed individually. Our own earlier design of switchable Cas12a gRNAs circumvented sequence constraints using an RNA helper strand that connected an arbitrary input sequence to the switching sequence.[11e] The design developed by Jin et al. implemented switchable Cas9 gRNAs without sequence constraints, but has not been shown to work in either bacteria or mammalian cells.[11c] Recently, two reports have solved two of these problems simultaneously: Collins et al. developed an approach to directly sense natural transcripts using engineered Cas12a gRNAs without the use of helper strands in *E. coli* cells,[11f] while Lin et al. demonstrated activation of Cas9 gRNAs in mammalian cells without formal sequence constraints by using two U6-transcribed helper strands.[11d] So far, however, no design has been shown to address these three issues simultaneously.

Engineering multiple, independently switchable gRNAs for gene expression in mammalian cells is challenging due to

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the large size of the eukaryotic promoter regions, which are much larger than the gRNAs they generate. Production of several gRNAs would require several promoters on a correspondingly large plasmid, or the use of several plasmids in parallel, making true multiplexing difficult.

Herein, we overcome this problem by combining switchable gRNAs with a design strategy that works with a single transcript for the Cas12a protein and its gRNAs. We start out by demonstrating that conditional processing of the 3′ tail of gRNA-containing CAG promoter transcripts is a general method to generate switchable Cas12a gRNAs. In particular, we show activation of gRNA activity using three different mechanisms: first, we use target sites for endogenous microRNAs and externally delivered short hairpin RNAs (shRNAs) to cleave off the 3′ tail, we then use a guanine-inducible self-cleaving ribozyme for this purpose, and finally we engineer a novel pseudoknot-based strand invasion mechanism that switches the conformation of the gRNA handle structure and thus enables cleavage by Cas12a's own RNase activity. Thus separating the sequence region responsible for switching from the active gRNA structure avoids the sequence constraints of our earlier designs.[11e] Furthermore, insulation of the Cas12a coding sequence and multiple switchable gRNA sequences through the repeated use of an RNA triplex structure enables the integration of a complete Cas12a gRNA circuit on a single transcript. The mechanism is shown to work in HEK293 cells and also in NIH/3T3 mouse fibroblasts, suggesting the general utility of our approach.

Results and Discussion

MicroRNA- and shRNA-Responsive Cas12a gRNAs

Cas12a gRNAs have previously been placed downstream of protein-coding sequences on mRNAs, where the intrinsic RNase activity of Cas12a excises them from the transcript.[12] To generate an active gRNA molecule, an additional handle sequence needs to be added downstream of the active gRNA sequence to remove the 3′ tail. As a first step towards our implementation of switchable gRNAs, we verified that trimming of a gRNA-containing transcript also works when transcribing Cas12a gRNAs from a CAG promoter without any protein-coding sequence. As a readout, we use a secreted nanoluciferase under the control of a minimal promoter containing seven repeats of a 20 nt long target sequence (t1 or t2) in HEK293 cells, which is in turn activated by an enhanced AsdCas12a-VPR (*Acidaminococcus* DNase-dead Cas12a-VPR) fusion (Figure 1a).[2a] The Cas12a, nanoluciferase, and gRNA are delivered on separate plasmids (Figure S1). As expected, the gRNA is highly active when the 3′ tail is removed by Cas12a's RNase activity, although its "off" activity without removal of the tail is still significantly higher than for a conventional U6-transcribed gRNA with a non-targeting spacer (Figure 1b). We reasoned that processing of the 3′ tail could be engineered to become conditional on external or endogenous molecular inputs, and thus the gRNA would become switchable by that input. As such, any conditional RNA cleavage mechanism would potentially result in switchable gRNAs.

A recent study by Wang et al. has demonstrated that putting two microRNA (miRNA) target sites adjacent to a Cas9 gRNA on a (CAG promoter-transcribed) Pol II transcript results in miRNA-responsive gRNAs,[10] presumably via cleavage of the transcript by the RNA-induced silencing complex (RISC). As our first conditional processing mechanism, we therefore also attempted to control our Cas12a gRNAs by placing microRNA target sites adjacent to the target sequence (Figure 1c). As expected, hsa-miR-20a-5p and hsa-let-7a-5p target sites, whose corresponding microRNAs are expressed in HEK293 cells,[13] lead to a strong activation of gRNA activity. Conversely, a miRNA that is not expected to be present (mmu-miR-294-5p),[10] does not. As in this case, we generally observed a low level of leaky activation for some uncleaved 3′ sequences, while others appeared to be entirely inactive in all experiments. We verified the results using flow cytometry, for which the sequence of a fluorescent protein (mScarlet) was placed downstream of the minimal promoter (Figure S2). Qualitatively, we observe the same behavior as for the nanoluciferase assay, but with an even better on/off ratio and a lower leak for the miR-294 sequence.

Alternatively, we also tested conditional activation using a short hairpin RNA (shRNA) transcribed from a U6 promoter (Figure 1d). The shRNA was designed using software by Gu et al.[14] to target the sequence directly downstream of the spacer sequence of gRNA t1 without a 3′ handle (cf. Figure 1b). At just 1 ng of shRNA plasmid, the gRNA activity already reaches the level achieved with the gRNA construct containing a 3′ handle sequence. We surmise that this high level of activity is likely due to multi-turnover cleavage of gRNA tails by the RISC. We designed two additional shRNAs and placed their targets downstream of two additional CAG-transcribed gRNAs with a t1 spacer sequence (Figure 1e). All gRNAs are activated by their respective shRNA, but not by the other shRNAs. The lower activity of the shRNA 3 construct can likely be explained by a reduced accessibility of the shRNA target sequence on the gRNA transcript (cf. Figure S3a) combined with a slightly negative effect of the shRNA itself on nanoluciferase levels (Figure S3b,c).

The origin of leak activity for different non-processed transcripts is not obvious. There are two major possible causes: First, a non-cleaved gRNA might have some residual activity despite its 3′ tail. Second, a nominally non-processed gRNA could be cleaved erroneously, e.g., by a RISC or another RNA endonuclease. We made two general observations regarding the leak: First, it appears more common for sequences that contain a potential microRNA or shRNA target site, as seen in the difference between gRNAs t1 and t2 (no 3′H structures) in Figure 1b. Second, as can also be seen in Figure 1b, it varies strongly from experiment to experiment. The second observation indicates that a difference in the cell state, e.g., a slightly different growth state during seeding, is relevant to the leak. The first observation could indicate that the comparatively low secondary structure adjacent to the gRNA target site increases the potential leak. Good binding sites for a RISC will generally have such low

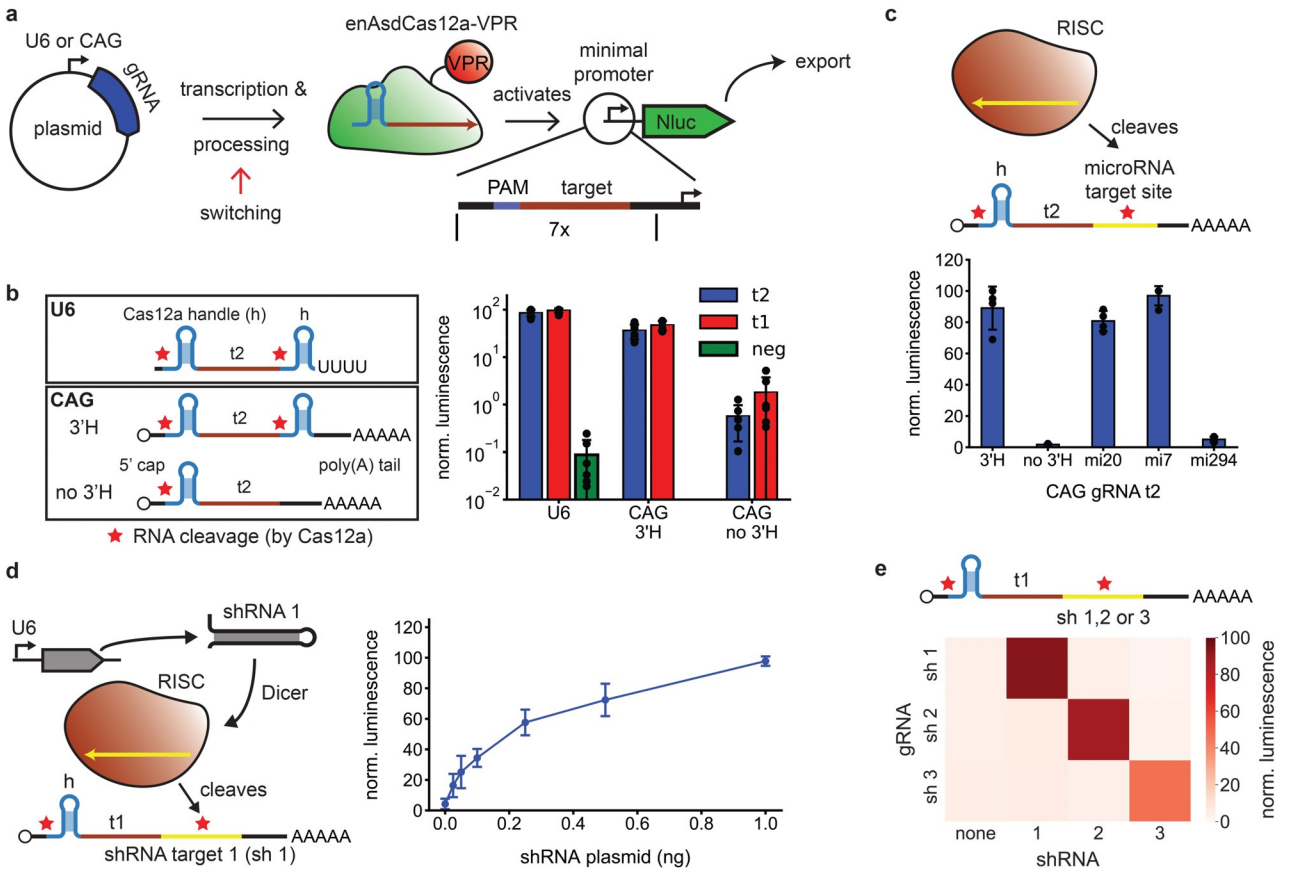

Figure 1. MicroRNA- and shRNA-responsive Cas12a gRNAs. a) Guide RNAs are transcribed using a U6 or CAG promoter. The switching principle used in this study is based on conditional post-transcriptional processing of CAG promoter-transcribed gRNAs. Cas12a gRNA activity is measured via the activation of expression of secreted nanoluciferase by enAsdCas12a-VPR (enhanced *Acidaminococcus* DNase-dead Cas12a-VPR). b) Nanoluciferase assay of the gRNA activity of U6-transcribed gRNAs with three different target sequences, and CAG-transcribed gRNAs with and without a 3′ gRNA handle for two different target sequences. The red star denotes an expected cleavage site, here due to Cas12a's RNA processing activity. t2, t1 = cognate spacer sequences, neg = non-targeting spacer sequence ($N = 6$). c) Nanoluciferase assay of gRNAs in which the 3′ gRNA handle sequence was replaced by a microRNA target sequence ($N = 4$). d) Activation of CAG gRNA t1 by a shRNA transcribed using the U6 promoter normalized to the activity of a gRNA with a 3′ handle sequence ($N = 4$). e) Three gRNAs containing different shRNA targets are activated by three different shRNAs ($N = 5$).

secondary structure, which could make them more vulnerable to unwanted cleavage.[15] However, as we do not observe any sequence dependence for this leak process, erroneous cutting mediated by cellular microRNAs is likely not its cause. Alternatively, the low secondary structure of RISC target sequences could decrease steric hindrances when non-processed gRNAs bind their target site.

Small Molecule-Responsive Cas12a gRNAs Using an Inducible Ribozyme

As a second mechanism, we used transcript processing by ribozymes. Small molecule-inducible ribozymes have been used extensively to make switchable mammalian mRNAs.[16] There, activation of the ribozymes deactivates the mRNA due to degradation in the cytosol after removal of the poly(A) tail. For Pol II-transcribed gRNAs, we expect the opposite effect, namely activation of the gRNA by activation of the ribozyme. In order to test the basic principle, we first inserted a constitutively active hepatitis delta virus (HDV) ribozyme downstream of the gRNA spacer sequence.[17] As for the Cas12a 3′ handle construct (Figure 1b), a HDV ribozyme at the 3′ end of the gRNA indeed leads to activation, while a mutated, inactive ribozyme results in an inactive gRNA (Figure 2a).

To make the gRNAs respond to small molecules, we next used a guanine-responsive HDV ribozyme originally developed by Nomura et al. for switchable mRNAs (Figure 2a).[17] The nominally best performing ribozyme (GuaM8HDV) initially showed poor performance when inserted directly after the gRNA spacer sequence. While the activated ribozyme (at 100 μM guanine in the medium) has an activity similar to the wild-type ribozyme, there is also strong gRNA activity in the absence of guanine. The wild-type ribozyme shows a slight reduction in activity in the presence of guanine. We suspect that this is due to a mild antiproliferative effect of the added guanine.[18] In order to suppress potential interactions between the gRNA sequence and the ribozyme, we introduced a clamp structure to separate the different

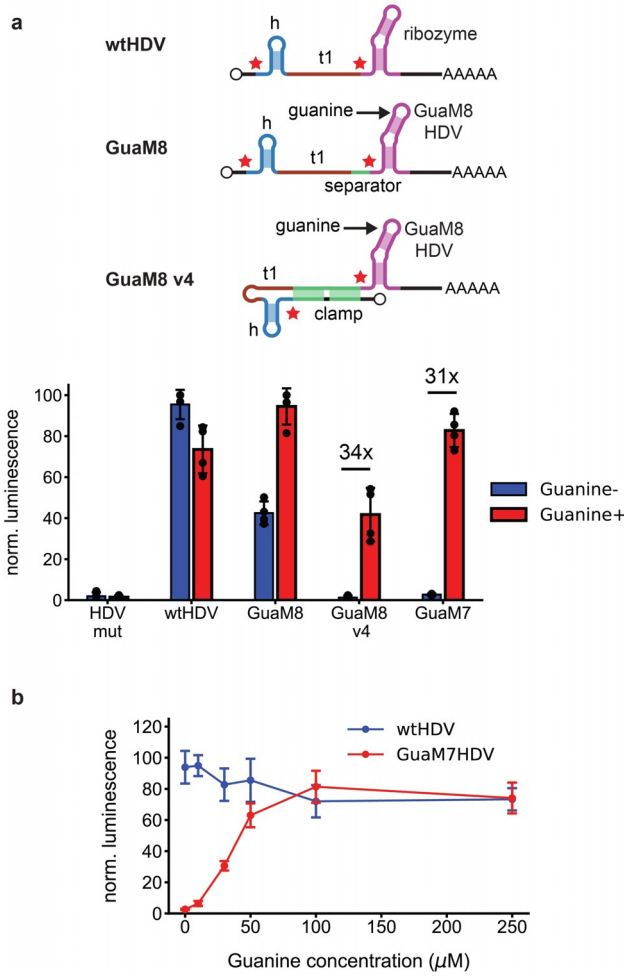

Figure 2. Small molecule-responsive Cas12a gRNAs using an inducible ribozyme. a) Nanoluciferase assay of activity for CAG-transcribed gRNAs containing different wild-type or guanine-responsive ribozymes. The activity was induced with 100 μM guanine. mut = mutated, inactive ribozyme. The red star indicates cleavage of the RNA ($N = 4$). b) Activation curve for gRNAs containing either the wtHDV or the guanine-responsive GuaM7HDV at the 3′ end ($N = 4$).

sequence domains and facilitate correct folding of the ribozyme (Figure S4a,b, GuaM8 v4 in Figure 2a). Due to its relatively short length, we expect the clamp to dissociate on its own after transcript processing by Cas12a. With our best-performing design, an on/off ratio of 34 could be achieved, but the total activity was significantly less than for the wild-type ribozyme.

We also tested an alternative ribozyme (GuaM7HDV), which had a lower activity in terms of mRNA knockdown, but also less leak than GuaM8HDV.[17] Remarkably, in the context of our conditional gRNA GuaM7HDV achieved an on/off ratio of around 31 with full total activity without any clamping or optimization, and with an activity saturating at around 100 μM guanine input (Figure 2b). As discussed in the Supporting Information, the different behaviors of the GuaM8HDV and GuaM7HDV constructs are not obvious from simulations of their secondary structures (Figure S4c), but may be the consequence of different optimization objectives for mRNA inactivation and gRNA activation, respectively (Supplementary Discussion 1).

Activation of gRNA Processing Using Toehold-Mediated Strand Displacement

As shown above (Figure 1), CAG promoter-transcribed gRNAs equipped with a second handle sequence at the 3′ end can be processed into a fully active form via Cas12a cleavage. We had previously demonstrated[11e] that the handle sequence itself can be sequestered into an inactive secondary structure, which can be opened by a trigger RNA via a toehold-mediated strand invasion process. This allows the handle to fold and thus enables Cas12a binding and processing of the gRNA.

We here revisited this concept in the context of the CAG promoter-transcribed gRNAs to facilitate conditional gRNA activation via 3′ handle cleavage. To avoid the sequence constraints of our earlier design, we developed a new switching principle that utilizes the fact that Cas12a recognizes the handle of its gRNA via a pseudoknot structure formed between the 5′ end of the handle and its stem-loop (Figure 3a).[19] Accordingly, we expected disruption of the pseudoknot to be sufficient to prevent Cas12a from binding even when the handle hairpin is otherwise correctly formed. Using the nucleic acid design tool NUPACK, we generated a series of designs for pseudoknot-switchable handle structures (see also Supplementary Discussion 2).[20] We first studied the basic mechanism in in vitro experiments using purified RNA, for which we paired the target sequence of the gRNA with a switch sequence located at its 5′ end to suppress pseudoknot formation (Figure S5a). As expected, the resulting gRNA is inactive and becomes active after addition of a trigger that displaces the switch (Figure S5b,c). As with the other switching mechanisms described above, the separation between the structure of the active gRNA and the sequence responsible for the cleavage mechanism considerably simplifies the design. As the input molecule does not need to interact directly with the sequence of the handle, no sequence constraints on the trigger molecule are incurred. This allows, in principle, to sense any accessible subsequence of a full mRNA transcript (Figure S5b,c).

For our implementation in mammalian cells, we moved the switch and toehold domains to the 3′ end of the switched gRNA (Figure 3a), a design which we refer to as a 3′ handle SD gRNA (3′H SD gRNA). Transcription of a trigger from a U6 + 27 promoter[21] alongside the 3′H SD gRNA leads to a 42-fold activation of gene expression via enAsdCas12a-VPR (Figure 3b). Changing even a single nucleotide in the switch domain of the trigger—and thus inhibiting the strand displacement process—reduces this to 15-fold activation, changing two nucleotides reduces activation to 2-fold, while three changed nucleotides reduce it to the background level. A trigger with a non-cognate toehold and a trigger with a completely scrambled sequence fail to activate the 3′H SD gRNA. Activation of the 3′H SD gRNA by the trigger scales approximately linearly with the amount of trigger, but displays considerable variability from experiment to experi-

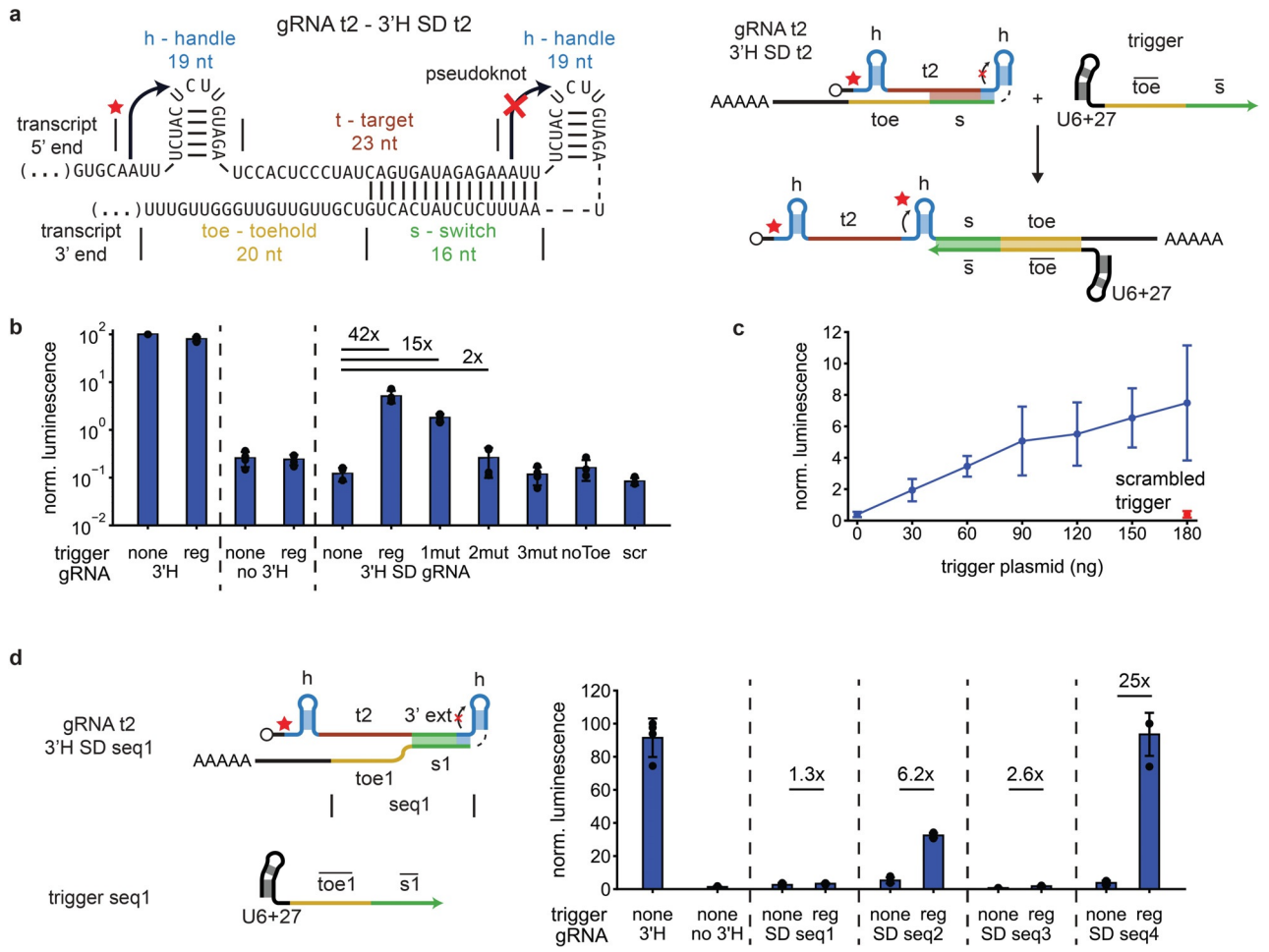

Figure 3. Switching of a 3′ Cas12a handle via strand displacement (3′H SD). a) The switching principle is based on suppression of pseudoknot formation in the 3′ handle. Displacement of the secondary structure by an RNA trigger molecule allows for pseudoknot formation and therefore processing by Cas12a (red star). b) Nanoluciferase assay of the activation of different gRNAs by U6+27-transcribed trigger molecules. (gRNAs:3′H—regular gRNA with 3′ handle, no 3′H—regular gRNA without 3′ handle, 3′H SD gRNA—gRNA with suppressed 3′ handle. triggers: reg (regular)—fully complementary trigger for the 3′H SD design, 1/2/3 mut: trigger with 1/2/3 changed nucleotides in the s domain, noToe—trigger with scrambled toehold, scr—completely scrambled trigger.) ($N=4$). c) Activation of the 3′H SD gRNA shown in (a) with different amounts of trigger ($N=8$). d) Nanoluciferase assay of the activation of 3′H SD gRNAs with different de novo designed trigger sequences (seq 1–4) transcribed by the U6+27 promoter ($N=4$).

ment (Figure 3c). Although the on/off ratios achieved with activation by strand displacement are comparable to those obtained by the other approaches shown above, with 6–10% the maximum activation level is significantly less than that obtained with a regular control gRNA.

Another drawback of the design described in Figure 3a are its strong sequence constraints, as 12 nt of the 5′ gRNA spacer and the first four nucleotides of the 3′ handle (AAUU) are used as the switch sequence. The sequence constraints can be alleviated by removing the four handle-complementary nucleotides from the trigger (reducing activation by a factor of slightly less than two (Figure S6a)), and by inserting an additional sequence domain between 5′ gRNA target and 3′ handle domain (cf. Figure 3d). The resulting design retains no formal sequence constraints and therefore allows, in principle, sensing of arbitrary RNA inputs without the need for additional adaptor strands. Surprisingly, several iterations of the unconstrained design failed or did not result in appreciable on/off ratios (Supplementary Discussion 3, Figures S7–S9), which we attributed to potential target–toehold interactions.

We therefore designed four 3′H SD gRNAs with different switch and toehold sequences, for which we explicitly excluded undesired interactions of the toehold and the full expected gRNA transcript (Figure S10). Interestingly, the four sequences had a widely varying performance (Figure 3d), ranging from non-functional (seq 1) to an excellent 25-fold activation (seq 4) by the trigger, and with a maximum activity comparable to the level of the positive control.

While the good performance of seq 4 demonstrates that it is possible to utilize our design for toehold-mediated strand displacement for CRISPR-based gene activation in mammalian cells, we were not yet able to find a clear design–performance relationship for the switches. In particular, the low performing sequences 1 and 2 show the most stable folding, while sequence 4 has the worst predicted folding

behavior (Figure S10). At this point, our design approach does not consider the influence of the cellular environment on the folding of the gRNA switches, and we also did not account for interactions with RNA-binding proteins and with the transcriptome. It seems likely that a better understanding of these factors will be required to enable a more reliable design of mammalian 3′H SD gRNAs for arbitrary inputs and targets.

Encoding Cas12a and Multiple Switchable gRNAs on a Single Transcript

Compared to other approaches towards multiplexed gene regulation, in which different target sites are addressed with different stimuli, switchable gRNAs have the major advantage that they do not necessarily require the delivery of multiple large gene constructs. In the examples described so far, however, only a single conditional gRNA was delivered on a separate plasmid than the dCas12a gene. We therefore sought to develop a strategy that allows the integration of the dCas12a coding sequence together with multiple switchable gRNAs on a single transcript.

While it is possible to simply place several regular (non-switchable) Cas12a gRNAs next to each other,[6,22] this straightforward approach fails for our switchable gRNAs (Figure 4a). The gRNA located at the 3′ end of the transcript works as desired, retaining a good on/off ratio and total activity. However, the gRNA at the 5′ end has a drastically

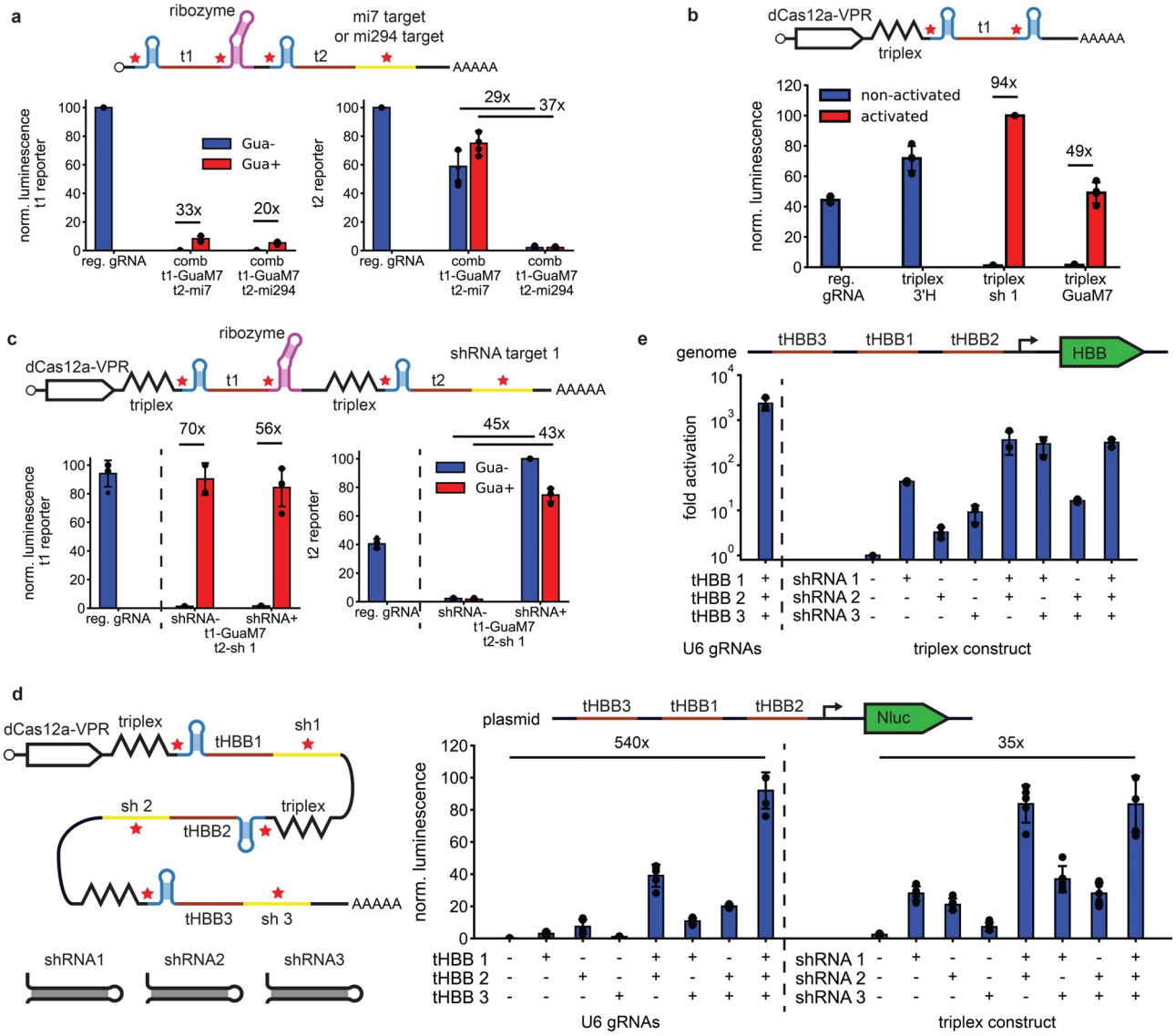

Figure 4. Placing multiple switchable gRNAs on a single transcript. a) Nanoluciferase assay for two switchable gRNAs placed next to each other on a single CAG promoter transcript ($N=4$). b) Nanoluciferase assay for Cas12a and switchable gRNAs placed on the same transcript and separated using a Malat1 triplex. activated = with shRNA 1 for sh 1 construct, with 100 μM guanine for GuaM7 construct ($N=4$). c) Nanoluciferase assay for Cas12a and two switchable gRNAs placed on the same transcript, all of them separated by a Malat1 triplex ($N=4$). d) Nanoluciferase assay for Cas12a and three switchable gRNAs placed on the same transcript, all of them separated by a Malat1 triplex. All gRNAs target the same minimal promoter ($N=5$). e) RT-qPCR measurement of the HBB mRNA level for three switchable gRNAs placed on the same transcript. The fold activation is measured relative to the triplex construct without shRNAs ($N=3$).

reduced activity in the on state. We surmise that the removal of the poly(A) tail during transcript processing leads to an enhanced degradation of the RNA, which affects the function of the 5′ gRNA domain more than that of the 3′ gRNA. This interpretation is in agreement with a set of experiments in which we systematically varied the 3′ sequence context (Figure S11 and 12, Supplementary Discussion 4).

A similar problem occurs when trying to integrate the Cas12a coding sequence and its gRNAs on a single transcript: after excision of the gRNAs, the mRNA is degraded due to the lack of a poly(A) tail. Campa et al. recently resolved this issue in the context of conventional gRNAs by inserting a protecting RNA triplex structure from the murine Malat1 noncoding RNA between the Cas12a coding sequence and a gRNA array.[6] We tested whether our switching principle was compatible with this approach by separating conditional Cas12a gRNAs from the enAsdCas12a coding sequence in the same manner (Figure 4b). Exhibiting a 94-fold and 49-fold activation by shRNA/RISC or guanine/GuaM7HDV, respectively, the constructs perform even better than our previous gRNA-only constructs.

As shown above, unlike for regular gRNAs, simply placing several of our conditional gRNAs next to each other is not a viable approach to multiplexing. We reasoned that protection from degradation by insulating RNA triplex structures might also improve the performance of multiple switchable gRNAs arrayed on a single transcript. We therefore placed an additional Malat1 triplex between two switchable gRNAs with a shRNA target site and the GuaM7HDV ribozyme. As desired, the gRNAs on the resulting construct can be activated independently by shRNA and guanine (Figure 4c). A slight reduction in activity for the shRNA-activated gRNA in the presence of guanine is in line with our observations for the wtHDV ribozyme (Figure 2a). The repeated use of the Malat1 triplex therefore appears to be a viable approach to integrate the Cas12a coding sequence along with a switchable gRNA array on a single transcript.

We also tested the constructs used in Figure 4b,c in NIH/3T3 mouse fibroblasts (Figure S13a,b) to assess whether our approach would work in other cells than HEK293. Both the individual gRNAs and the integrated constructs were found to be functional in the 3T3 cells.

We extended this multiplexing approach and placed three conditional gRNAs that respond to the three activation mechanisms on one transcript (Figure S14). All three gRNAs are activated by their corresponding input, but not by the other inputs. The shRNA-activated gRNA is less active when all three gRNAs are activated simultaneously, possibly because trigger transcription, which is also U6-based, interferes with shRNA transcription. The efficiency of the strand displacement-activated conditional gRNA, for which we used sequence 4 of Figure 3d, is much lower than for the original construct. This is not surprising, as this sequence was designed for the sequence context of the original construct. Unlike the shRNA- or ribozyme-based activation, the strand displacement mechanism is highly sensitive to the sequence of the rest of the transcript and therefore is likely to require additional optimization for each new construct.

Thus far, our processed gRNAs targeted seven repeats of the same sequence in the nanoluciferase promoter on our reporter plasmid. When targeting endogenous genes, each target sequence will typically only occur once, but transcriptional activation via enAsdCas12a-VPR using only a single binding site tends to be weak. We therefore copied a short region that naturally occurs in the HBB (hemoglobin subunit beta) gene in front of our nanoluciferase reporter and picked three Cas12a target sites in this region (Figure 4d).

We then placed the corresponding three gRNAs with three orthogonal shRNA target sites onto a single transcript, representing a type of fuzzy AND gate for the three shRNAs (Figure 4d and Figure 1e). In Figure 4d, we compare the activation achieved by conditional switching with different combinations of the three shRNAs to the activation achieved with conventional U6-transcribed gRNAs. The maximum activation level achieved in the presence of all three gRNAs is the same in both cases, but a larger leak activation results in a lower dynamic range for our conditional gRNA array compared to the U6 gRNAs (35-fold instead of 540-fold). Notably, activation for shRNA1 is much larger than for the corresponding U6 gRNA, and shRNA 1 and 2 activate as strongly as all three shRNAs combined. We also compared activation by shRNAs 1 and 2 as compared to all three shRNAs over a range of gRNA dosages, showing that activation by all three gRNAs is significantly better at least in two out of four cases (Figure S15).

We finally tested the same conditional gRNA construct for activation of the genomic HBB gene itself (Figure 4e), which we read out via RT-qPCR. The activation is similar as for the Nluc construct, although activation by shRNA 3 is much stronger. Also the activation by a combination of shRNA 1 and 2 or 1 and 3 is much stronger than for any individual shRNA (approx. 500 fold as opposed to approx. 50 fold for shRNA 1), indicating that all shRNAs activate their target gRNAs as designed. However, activation by all three shRNAs is not stronger than for either of the two combinations shRNA 1/2 or shRNA 1/3, similar as observed with shRNA 1 and 2 for the Nluc reporter.

Conclusion

We have developed an approach for switchable gene activation via dCas12a-VPR in mammalian cells that is based on the conditional cleavage of Pol II transcripts containing Cas12a gRNAs. Processing of the transcripts removes any 5′ and 3′ sequence context and thus results in fully active gRNAs, which can be utilized by dCas12a-VPR. Conditional cleavage has been achieved by inserting miRNA/RISC target sites, a co-factor-dependent ribozyme, and an auxiliary gRNA handle structure that can be switched with a strand displacement mechanism, resulting in cleavage by Cas12a's intrinsic RNase activity. We surmise that also other cleavage mechanisms would work in this context in a similar way. Notably, our approach can be extended to integrate multiple conditional gRNAs and even the coding sequence of Cas12a on the same transcript, for which the different sequence domains have to be protected from degradation using RNA triplexes.

While RISC and ribozyme-induced cleavage have been previously employed to exert posttranscriptional control of gene expression, toehold-mediated strand displacement of the pseudoknot structure characteristic of Cas12a gRNAs is a novel switching principle that allowed us to utilize arbitrary input trigger RNA sequences without any sequence constraints. At this point, we still found considerable variability in the performance of our mammalian strand-displacement gRNAs, however, and no clear design–function relationship yet. Our use of Pol II transcripts for strand displacement circuits is distinct from other approaches that typically use U6 transcripts. While the additional 5′ and 3′ sequences complicate the design process, we believe that increased stability and colocalization with, for example, mRNAs will ultimately prove to be beneficial for sensing endogenous transcripts. We expect that improved sequence design that takes into account folding of the complete SD gRNA construct and also the boundary conditions set by the cellular environment such as the presence of the transcriptome and RNA-binding proteins will lead to more predictable behaviors.

Compared to earlier designs of switchable guide RNAs that worked in vitro and in bacteria, our approach geared towards the control of eukaryotic gene expression has two significant advantages: First, the sequence domain responsible for activation of the gRNA is spatially separated and independent from the gRNA sequence itself, which enables a modular combination of multiple control domains in one gRNA transcript. The second advantage of the approach is the compactness of the genetic constructs required for implementing conditional gRNA circuits. All components for the evaluation of potentially quite complex logic expressions can be placed on a single transcript, which can be generated from a single promoter and from a single plasmid. This also considerably simplifies the delivery of such circuits to mammalian cells.

The general approach outlined here is likely to work for Cas9 gRNAs in much the same way. While strand displacement-activated gRNAs are specific to Cas12a, since they are based on handle pseudoknot formation and Cas12a's RNase activity, aptazyme-based activation should be straightforward to adapt. MicroRNA-activated Cas9 gRNAs were already implemented in earlier work.[10] As shown in the present work, also in this case placing the coding sequence for Cas9/dCas9 on the same transcript as its microRNA-switchable gRNAs could simplify the delivery of a complete conditional gRNA circuit considerably.

In conclusion, we have demonstrated an approach to design switchable Cas12a gRNAs responding to a wide variety of stimuli via conditional RNA cleavage. The switching principle is compatible with a compact integration of all necessary components on a single transcript, which facilitates the realization of multiplexed gene regulatory circuits for mammalian cells that can be delivered on a single plasmid.

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Conflict of Interest

The authors declare no conflict of interest.

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