Interaction of Bare dSpCas9, Scaffold gRNA, and Type II Anti-CRISPR Proteins Highly Favors the Control of Gene Expression in the Yeast S. cerevisiae

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ABSTRACT: Type II CRISPR-(d)SpCas9 and anti-CRISPR proteins (AcrIIs) show evidence of coevolution and competition for survival between bacteria and phages. In biotechnology, CRISPR-(d)SpCas9 is utilized for gene editing and transcriptional regulation. Moreover, its activity is controlled by AcrIIs. However, studies of dSpCas9/AcrII-based transcription regulation in Saccharomyces cerevisiae are rare. In this work, we used dSpCas9 as a template to engineer new transcription activators. We found that the most performant activation system requires the use of bare dSpCas9 in conjunction with scaffold gRNA (scRNA). This means that activation domains shall not be fused to dSpCas9 but rather interact with scRNA. We showed that a low amount of sgRNA is not a limiting factor in dSpCas9-driven transcription regulation. Moreover, a high quantity of sgRNA does not improve, generally, activation (and repression) efficiency. Importantly, we analyzed the performance of AcrIIA2, AcrIIA4, and AcrIIA5 in S. cerevisiae in depth. AcrIIA4 is the strongest of the three AcrIIs and also the only one able to induce high inhibition at low concentrations. However, the activation domains fused to dSpCas9 hindered interactions with the AcrIIs as well and limited their control of gene transcription regulation, confirming that bare dSpCas9 is the best solution for building synthetic genetic networks in yeast.

KEYWORDS: synthetic biology, CRISPR-dCas9, type II anti-CRISPR, S. cerevisiae, scaffold gRNA

INTRODUCTION

The clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) protein system is an adaptive and inheritable immune response that exists in prokaryotes and protects them from invading DNA (e.g., phage DNA). Among the six types of CRISPR-Cas systems, type II CRISPR-SpCas9 (from Streptococcus pyogenes) is the most widely used in gene editing because it only requires a single CRISPR-associated protein. Upon binding a single-guide RNA (sgRNA), Cas9 targets double-stranded DNA after recognizing the protospacer-adjacent motif (PAM) NGG or NAG. The complex Cas9:sgRNA works as a ribonuclease protein (Cas9 RNP) that contains two nuclease domains, HNH and RuvC, and induces a double-strand break in the DNA. CRISPR-Cas9 has been broadly utilized as a biotechnological tool in both prokaryotes and eukaryotes for gene editing and, upon deactivation of its nuclease function, transcriptional regulation. Novel transcription factors that can bind almost every DNA sequence are built on dSpCas9, i.e., the nuclease-deficient version of SpCas9 that is realized via two mutations, D10A and H840A, in the HNH and RuvC domains. By fusing dSpCas9 with either activation domains (ADs) or repression domains (RDs), up- or downregulation is achieved if dSpCas9 targets an appropriate site along a promoter sequence. Type II anti-CRISPR proteins (AcrIIs) can be

CRISPR-Cas biotechnological tool and optimize its performance.

Over the past decade, synthetic biology has shown rapid development in various fields, such as metabolic engineering, medicine, agriculture, environmental engineering, and biofuel production. To obtain different functionalities, cells are modified with DNA circuits that are often gene transcriptional networks. Novel transcription factors that can bind almost every DNA sequence are built on dSpCas9, i.e., the nuclease-deficient version of SpCas9 that is realized via two mutations, D10A and H840A, in the HNH and RuvC domains. By fusing dSpCas9 with either activation domains (ADs) or repression domains (RDs), up- or downregulation is achieved if dSpCas9:sgRNA targets an appropriate site along a promoter sequence. Type II anti-CRISPR proteins (AcrIIs) can be

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Figure 1. Transcriptional activation based on dSpCas9-AD:sgRNA. (a) Scheme of our initial transcription activation circuit. A target site, lexOpR, for the dSpCas9-AD:sgRNA activator is placed upstream of the synthetic promoter trnuc_pCYC1core, where two TATA boxes are present upstream of the transcription start site (TSS). Gray arrows represent the promoters in each transcription unit. In the sgRNA structure, the blue line stands for the spacer and the black curve corresponds to the direct repeat. Pol II is an abbreviation for RNA polymerase II. (b) Fluorescence level and activation rate reached by dSpCas9-VP64:sgRNA and dSpCas9-VPR:sgRNA. sgRNA −” and “+” indicate the absence and the presence, respectively, of sgRNA able to bind lexOpR (on the antisense strand). When sgRNA complementary to lexOpR is not expressed, it is replaced by a scrambled sgRNA that does not match any sequence in the S. cerevisiae genome. The activation efficiency was calculated as the ratio between the fluorescence intensity corresponding to the (+) and (−) bars. The “*” symbol indicates a statistically significant difference between the test strain and the negative control. **, p-value < 0.01 and two-tailed Welch’s t-test. Each fluorescence level represents the mean value from at least three independent experiments, i.e., carried out on different days. Error bars are the standard deviation of the mean.

utilized to control and restrain CRISPR-dSpCas9 from binding the DNA, in this way modulating the gene transcriptional regulation.20,21 To date, 21 AcrIIs have been discovered.16 AcrIIA2 and AcrIIA4, found in Listeria monocytogenes prophages,13 have been verified to have the ability to abrogate the capability of (d)SpCas9 to bind the target DNA. Furthermore, AcrIIA2 and AcrIIA4 share an identical inhibitory mechanism: they act as DNA (PAM sequence) mimicry and bind to sgRNA-loaded Cas9 (Cas9:sgRNA) in competition with and more specifically than target DNA, but they cannot dislodge the target DNA itself once the ternary complex Cas9:sgRNA−DNA is formed.22–25 AcrIIA2 and AcrIIA4 have the drawback that both are narrow-spectrum inhibitors that can only function on subtype II-A and inhibit a small number of Cas9 homologs. By contrast, AcrIIA5, discovered in the virulent phages of Streptococcus thermophilus,14 is a broad-spectrum inhibitor for type II CRISPR-Cas systems. AcrIIA5 can suppress gene editing caused by many Cas9 homologs that belong to type II-A, type II-B, and type II-C.26–28 In addition, it is capable of abolishing the function of CRISPR-dSpCas9-mediated gene transcriptional regulation in Saccharomyces cerevisiae.20,21 However, the exact mechanism by which AcrIIA5 functions is still unknown.

In this work, we have improved and expanded upon previously reported results12,20 regarding dSpCas9-based transcription factors (mainly activators) in S. cerevisiae. Using an extremely weak synthetic promoter derived from the yeast constitutive CYC1 promoter as a target (whose structure is rather complex since it contains multiple upstream activating sequences, TATA boxes, and transcription start sites29), we found that VP64 led to a higher activation than VPR when it was fused to dSpCas9, although it is a weaker AD than VPR.30 We reckoned that the considerable size of dSpCas9-VPR might prevent, via steric hindrance, proper recruitment of RNA polymerase II to the DNA if the CRISPR-Cas target site is not sufficiently distant from the TATA box. Hence, we showed that bare dSpCas9 together with a scaffold gRNA (scRNA) is by far the best way to engineer strong activators. Moreover, we utilized AcrIIA2, AcrIIA4, and AcrIIA5 to control and inhibit the function of CRISPR-dSpCas9 to modulate or abolish gene transcriptional activation. We deeply analyzed and estimated the potency and efficacy of the three AcrIIs and discovered that each one performs much better on the bare dSpCas9 because any domain fused to dSpCas9, no matter how small, determines an interference in the dSpCas9−AcrII interactions (this is particularly evident with AcrIIA5). This finding might be important for the large-scale utilization of anti-CRISPR proteins inside synthetic gene networks in the future.

RESULTS AND DISCUSSION

CRISPR-dSpCas9-Based Gene Transcriptional Activation in S. cerevisiae. We employed the yeast-enhanced green fluorescent protein (yEGFP)31 as a reporter to assess how different engineering strategies affect the activation efficiency of dSpCas9:sgRNA. First, we designed the transcriptional activation circuit in Figure 1a. yEGFP was produced by a transcriptional activator, yEGFP, which AcrIIA5 functions is still unknown.
24 nt and termed trunc_pCYC1core. We inserted a 27 nt long sequence named lexOpR representing the right part of the complete LexA operator, called lex2Op, preceded by four nucleotides, three of them constituting PAM: 5′-gccaCA-TAACTGTATATACACCCAggg-3′ (the last three guanines had been used here as a PAM too). LexA is a bacterial protein and its operator exists only in prokaryotic genomes.32,33 lexOpR was placed upstream of the TATA boxes of trunc_pCYC1core to provide (because of the presence of the two PAMs) target sites for dSpCas9:sgRNA on both DNA strands. sgRNA we used initially contained a 20 nt long spacer (named sgRNA_lexOpR), whose absence from the yeast S. cerevisiae genome was verified with CRISPRdirect.34 Therefore, under the guidance of sgRNA (in this study, if not otherwise specified, sgRNA is supposed to bind the antisense strand of the target DNA), dSpCas9-AD:sgRNA bound the synthetic promoter and then activated transcription by recruiting RNA polymerase II. Finally, via fluorescence-activated cell sorting (FACS) experiments, we saw an increase in fluorescence. In this initial design, the ADs we used were the strong VP64 and VPR, which were fused to the C-terminus of dSpCas9. The dSpCas9-VP64:sgRNA configuration led to a 2.54-fold activation, whereas dSpCas9-VPR:sgRNA showed a lower activation at 1.54-fold (Figure 1b).

**Engineering Synthetic Promoters for yEGFP Expression.** We modified the synthetic promoter in the initial circuit in Figure 1a in different ways to change the expression of the reporter protein. (1) We inserted more copies (three and six) of lexOpR upstream of the TATA boxes of trunc_pCYC1core (Figure 2a). The distance between two adjacent lexOpRs was always equal to 20 nt. The basal expression of n× lexOpR_trunc_pCYC1core promoters is shown in Figure 2b. Interestingly, an increasing number of lexOpR had the effect of reducing the basal expression, as reported also by Ottoz et al.,35 although they used the full lex2Op. (2) We targeted a second site (t2), which is naturally present (together with its PAM) upstream of lexOpR_trunc_pCYC1core (Figure 2a). sgRNAs complementary to t2 and lexOpR were expressed both separately or simultaneously. (3) We placed a single lexOpR further upstream of the TATA box starting at position −52 with respect to the TSS (we will refer to this TATA box as −52 TATA in the rest of the paper). Precisely, lexOpR was placed 103, 153, and 203 nt upstream of −52 TATA, instead of the initial 40 nt distance (see Figure S1a).

As expected, both dSpCas9-VP64:sgRNA and dSpCas9-VPR:sgRNA produced a higher activation rate with increasing copies of lexOpR (Figures 1b and 2c). In particular, dSpCas9-VP64:sgRNA showed a 2.54-, 2.87-, and 3.36-fold activation for one, three, and six copies of lexOpR, respectively. dSpCas9-
VPR:sgRNA gave a 1.54-, 2.05-, and 2.35-fold activation on the same number of lexOpR. Although the activation efficiency increased linearly in the number of operators, the proportionality constant between the activation rate and the number of operators was extremely small (~0.16, see Figure S2a,b), i.e., the activation rate was enhanced minimally (Figure S2c) by augmenting the number of lexOpR. A better strategy to increase gene expression probably lies in the design/engineering of operators with stronger affinity to sgRNA.

Our results showed that dSpCas9-VP64:sgRNA manifested higher activation than dSpCas9-VP-R:sgRNA on the three synthetic promoters, although VP64 is a weaker AD than VPR. \(^{30}\) It should be noted that VP64 is a small domain composed of only 50 amino acids (5.51 kDa) and VPR is about 10 times larger because it is made of 523 AAs (55.72 kDa). By hypothesizing that the size of VPR may influence, via some steric hindrance, the dSpCas9-VP-R:sgRNA complex assembly, DNA binding, and/or preinitiation complex (PIC) formation at the TATA box, we considered the target site t2 at a distance of 84 nt from −52 TATA (Figure 2a) on the lexOpR_trunc_pCYC1-core. Interestingly, dSpCas9-VP-R:sgRNA produced a 1.97-fold activation by targeting t2, which was stronger than that obtained on lexOpR. A slightly higher value was detected by the simultaneous targeting of the two sequences (2.06-fold), although there was no significant statistical difference compared to the targeting of t2 alone (Figure 2d). dSpCas9-VP64:sgRNA showed a much lower activation (only 1.46-fold) when targeting t2, and no synergistic effects were present when both sgRNA_t2 and sgRNA_lexOpR were expressed because the overall activation was lower than any registered on one or more lexOpR (Figures 1b and 2c,d). Moreover, we modified lexOpR_trunc_pCYC1-core by moving lexOpR further away from −52 TATA (Figure S1a). Our results showed that the distance between the target site and the TATA box influences the function of the dSpCas9-based activator since fluorescence increased by moving lexOpR from 40 to 103 nt upstream of −52 TATA. However, a drop in the activation was already detected by placing lexOpR just 153 nt from −52 TATA. Interestingly, both VP64 and VPR exhibited the same trend with VP64 showing, again, higher performance than VPR (Figure S1b).

Taken together, our results pointed out that cooperativity was basically absent among dSpCas9-AD:sgRNA molecules because no significant increase in fluorescence expression was detected from enhancing the target site number, in agreement with ref 12, where up to 12 copies of the same operator were inserted in S. cerevisiae without any drastic improvement relative to the use of only three or six of them. In contrast, further experiments are necessary to locate the optimal distance between lexOpR and the TATA box (apparently...
not far from 100 nt upstream of −52 TATA) and, presumably, between adjacent target sites to optimize transcription activation.

**Engineering Different Activation Modes.** Beyond fusing either VP64 or VPR to the C-terminus of dSpCas9, we also engineered other dSpCas9-based activation systems in the following ways: (1) attaching VP64 to both N- and C-termini of dSpCas9 and forming, in this way, the VP64-dSpCas9-VP64 chimeric protein (Figure 3a); (2) using scRNA, such that sgRNA is extended with MS2 sequences that recruit the chimeric protein MCP-VP64 (Figures 3b and S3); (3) combining scRNA with a chimeric protein dSpCas9-AD to further increase the number of ADs associated with a single dSpCas9 (Figure 3c); and (4) replacing VP64/VPR with other ADs, e.g., VP16,38 B42,39 and mDR521_80540,41 (Figure S4). The target promoter was lexOpR_trunc_pCYC1core in each case.

We assumed that, by fusing VP64 to both the N- and C-terminus of dSpCas9, a stronger fluorescent signal than that due to dSpCas9-VP64 would be observed due to the possible synergistic effects between the two VP64 copies. Unexpectedly, in the VP64-dSpCas9-VP64 configuration, there was no activation at all when the antisense strand was targeted (Figure 3a). sgRNA complementary to the sense strand provoked a 1.67-fold activation, i.e., it was still much less performant than dSpCas9-VP64 despite the presence of two copies of VP64.
Thus, we can conclude that the fusion of ADs at the two termini of dSpCas9 prevents any synergistic interaction between the ADs, perhaps due to the considerable size of dSpCas9 itself. We cannot exclude that the whole chimeric protein cannot fold properly in this arrangement or that its increased dimension prevents, through steric hindrance, proper PIC formation at the TATA box.

Then, we employed the bare dSpCas9 together with scRNA to recruit VP64 linked to the RNA-binding protein MCP (Figures 3b and S3). In scRNA, we initially used two variants of 1× MS2 that contained a single recruitment site (an RNA hairpin, either the wild-type MS2 hairpin—wt or the f6 MCP aptamer—f6).36 MCP binds to its RNA hairpins as a dimer and is assumed to recruit two VP64 copies. Our results showed that the 1× MS2(wt) scRNA in association with dSpCas9 and MCP-VP64 gave a 5.27-fold activation (Figure S3), slightly more than twice as much (2.07) as dSpCas9-VP64:sgRNA. Afterward, we used the 2× MS2(wt+f6) scRNA that combines two different RNA hairpins: wt and f6.36 The 2× MS2(wt+f6) scRNA construct provides a higher probability of MCP-VP64 independent binding36 and is supposed to recruit four copies of VP64. Zalatan et al. showed that the 2× MS2(wt+f6) construct together with MCP-VP64 produced ∼20-fold higher activation in S. cerevisiae and a double or triple activation in human cells than the dSpCas9-VP64 activator.46 Our results showed that, compared with the dSpCas9-VP64 fusion protein, dSpCas9:scRNA-MCP-VP64 produced a ∼3-fold stronger activation, i.e., 7.54-fold with respect to the negative control, which was the highest activation achieved in S. cerevisiae. It shall be noted, though, that the synthetic promoters used in the two studies are different (e.g., Zalatan et al. made use of the tet operator as a target). Furthermore, in our experiments, no toxicity effects (Figure S5a) nor burdens to cell growth (Figure S5b) were found in the presence of activators. In contrast, previous studies reported slower growth and death of S. cerevisiae cells when chimeric activators were expressed.35,42,43 In these studies, toxicity was ascribed to the action of the foreign AD that was thought to reduce the number of RNA polymerase II molecules necessary for the synthesis of vital genes.44

We sought to improve the performance of the two initial circuits in Figure 1a by expressing 2× MS2(wt+f6) scRNA instead of a basic single-guide RNA. As shown in Figure 3c, this modification induced a greater than 2-fold improvement in both devices: dSpCas9-VP64:scRNA-MCP-VP64 exhibited a 5.37-fold activation, and dSpCas9:VPR:scRNA-MCP-VP64 showed a 3.89-fold activation. Although both had up to five ADs, none were more performant (in terms of the ON/OFF ratio) than the circuit based on the bare dSpCas9 (i.e., dSpCas9:scRNA-MCP-VP64 in Figure 3b), which is evidence in favor of our hypothesis that the direct fusion of ADs to dSpCas9 increases steric hindrance and/or prevents proper protein folding.

The actual activity of VPR in association with dSpCas9 in S. cerevisiae is, nevertheless, controversial. Chavez et al.,30 for instance, showed that the RNA expression of the endogenous S. cerevisiae genes GAL7 and HED1 was enhanced by dSpCas9-VPR up to 78- and 38-fold, respectively, whereas dSpCas9-VP64 managed to produce only a 14- and 9-fold activation of the same genes. As described above, in our study, dSpCas9-VP64 generally performed better than dSpCas9-VPR that was not able to reach a threefold activation in yEGFP expression. The poor performance of dSpCas9-VPR has been reported in other studies as well. Dong et al.45 recently described a method (CRISPR-ARE) wherein SpCas9-VPR reached only a 3.8-fold activation of the mCherry reporter protein expression. Moreover, Deaneer et al.46 reported only a 2.4-fold fluorescence enhancement under the action of dSpCas9-VPR and showed, in a different work,47 that a target site in the proximity (within 20 nt) of the TATA box led to weak activation by dSpCas9-VPR, presumably due to interference with PIC formation. Further, Ferreira et al.48 demonstrated that dissimilar activation efficiencies due to dSpCas9-VPR were probably caused by either different target sites (sequences and positions) or even distinct basal expression levels of the target promoters. Hence, the activity of (d)SpCas9-VPR appears to be strongly associated with the sequence of both target sites and promoters.

Finally, we tested three more ADs that operate in S. cerevisiae, namely, B42, VP16, and mDRS2/805. All of them were fused with the C-terminus of dSpCas9 and showed modest activity (Figure S4). VP16, the strongest among the three ADs, was only slightly more effective than both B42 and mDRS21_805. The gain in fluorescence that VP16 induced (1.83-fold) was nevertheless ∼1.2 times higher than that corresponding to VP64, which further underlined the poor performance of the dSpCas9-VPR chimeric activator.

Using Different sgRNA Expression Cassettes. Another variable that considerably affects activation efficiency is the way that sgRNA is expressed and designed. As shown by Farzadpard et al.,12 transcription activation can change when sgRNA targets the antisense rather than the sense strand of the DNA. Therefore, we compared the action of dSpCas9-VP64:sgRNA_bA with that of dSpCas9-VP64:sgRNA_bS on an increasing number of lexAOP upstream of trunc_pCYC1core (Figure 4a) and found that sgRNA_bA always operated more efficiently than sgRNA_bS, no matter how many copies of lexAOP were on the promoter. By contrast, when VPR was fused to dSpCas9, sgRNA_bA performed (slightly) better than sgRNA_bS, but only in the presence of six copies of lexAOP (Figure 5a). The use of different ADs (B42/VP16/mDRS21_805; Figure 5b) and dSpCas9:scRNA-MCP-VP64 (Figure 3b) confirmed that sgRNA_bA assures higher activation than sgRNA_bS. Finally, when targeting the modified lexAOP_trunc_pCYC1core, both dSpCas9-VP64 and dSpCas9-VPR displayed much higher fluorescence by binding the antisense strand (Figure 5c). Taken together, our results indicate that transcriptional activation via the CRISPR-dSpCas9 system is far more efficient by means of sgRNAs complementary to the antisense strand.

We utilized two expression cassettes to synthesize sgRNA (Figure 4b): one employed the RNA polymerase III-dependent promoter, pSNR52,49 and the other hostd pADH1, which is bound by RNA polymerase II, to express the RGR (ribozyme-guide RNA-ribozyme) cassette.50 A comparison of the working efficiency of the two integrative expression systems is displayed in Figure 4c (in the presence of VP64) and Figure 5f (VPR). Out of the six circuits taken into account in our work, pSNR52 guaranteed better performance in three, whereas no significant differences were present in the remaining three gene networks. These results reflect our recent findings on dCas12a,52 where RNA polymerase III-dependent elements produced a slightly better performance inside synthetic circuits. Deaneer et al.50 also showed that sgRNA released from a pTEF1-RGR cassette performed almost identically to that driven by RNA polymerase III-
Figure 5. AcrIIIs inhibiting CRISPR-dSpCas9-based transcription factors. (a) Schematic diagram of AcrIIIs countering transcriptional activation by dSpCas9-AD:sgRNA. (b) Inhibition of the three AcrIIIs on dSpCas9-VP64:sgRNA_bA with an increasing number of lexOpR upstream of trunc_pCYC1core. “−” represents the negative control: no AcrII is expressed and fluorescence is set to 100%. The downward dashed arrows refer to the decrease in fluorescence due to the action of the AcrIIIs. (c) Inhibitory action of the three AcrIIIs on dSpCas9:scRNA-MCP-VP64. The AcrIIIs were expressed by four constitutive promoters of different strength. “gen” refers to genCYC1t-pCYC1noTATA, a synthetic weak promoter. “−” is the negative control, i.e., no AcrII expression (fluorescence = 100%). (d) Comparison of the inhibition of three AcrIIIs on dSpCas9-
VPR:sgRNA_bS. As in (b) and (c), “−” refers to the control circuit without Acr (fluorescence = 100%). (e) Schematic diagram of AcrIIs counteracting the transcriptional repression of dSpCas9-Mxi1:sgRNA. (f) AcrIIs inhibiting dSpCas9-Mxi1:sgRNA that represses the synthetic promoter T5synth8_pCYC1noTATA. The first “−”, without any label inside the bar, is the negative control; AcrIIs are not expressed and the synthesis of yEGFP is repressed by dSpCas9-Mxi1:sgRNA. The last “−”, labeled with “w/o sgRNA” (meaning “without sgRNA”) into the bar, is the positive control (inactive dSpCas9-Mxi1 and fluorescence corresponding to 100%). (g) Cell growth curves (18 h) of the strains containing different AcrIIs. OD600 was measured every 2 h. The “∗∗∗” symbol in black indicates a statistically significant difference between the test strain and the negative control. The “∗∗∗” symbol in gray indicates a statistically significant difference between the two test strains. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001; ****, p-value < 0.0001; and two-tailed Welch’s t-test. The “ns” in gray indicates no significant difference between the corresponding test strains. Each fluorescence level represents the mean value from at least three independent experiments, i.e., carried out on different days. Error bars are the standard deviation of the mean.

Figure 5. continued
AcrIIA4 inhibition overcame 85% with the highest fluorescence reduction (the best one in absolute), reaching 88.63% when pGPD was employed. This value was 1.36-fold higher than the repression achieved on dSpCas9-VP64:sgRNA. A similar predilection for dSpCas9:scRNA-MCP-VP64 was shown by AcrIIA5, whose performance under pTEF2 and pGPD was comparable to that of AcrIIA4 because fluorescence decreased up to 86.56% (*i.e.*, 2.04-fold stronger than on dSpCas9-Vp64:sgRNA). AcrIIA2 showed a less evident improvement when exposed to dSpCas9:scRNA-MCP-VP64, although its highest inhibition (achieved under pGPD) arrived at 78.63%, *i.e.*, 1.46-fold more than on dSpCas9-VP64:sgRNA. On the whole, the three AcrIIIs worked in a dose-dependent manner in *vivo*, with AcrIIA4 showing excellent inhibition of activation at very low concentrations, by binding the bare dSpCas9. Both AcrIIA2 and AcrIIA5, by contrast, were powerful only at a high expression level. AcrIIA4 was always the strongest inhibitor regardless of the promoter that drove the anti-CRISPR expression (Figure S9).

Our previous tests showed a generally high affinity between AcrIIIs and bare dSpCas9 in *S. cerevisiae*. To further confirm the hypothesis that domains fused to dSpCas9 interfere with the action of the AcrIIIs, we harnessed the three AcrIIIs to suppress the chimeric activator dSpCas9-VPR:sgRNA, where the large VPR potentially hindered the interaction of AcrII and dSpCas9. Indeed, the three AcrIIIs reduced the fluorescence only moderately (Figure 5d): 26.79% (AcrIIA2), 22.14% (AcrIIA4), and 28.55% (AcrIIA5).

As a further test, we checked the action of the three AcrIIIs on a synthetic repressor made of dSpCas9 fused to the small (36 AAs, 4.24 kDa) Mxi1 repression domain. To this aim, we modified a circuit from previous work from our lab° by expressing AcrIIIs under pGPD (Figure 5e). We confirmed the result obtained in ref 20: AcrIIA2 and AcrIIA4 worked identically and provoked an almost full fluorescence recovery (97.79 and 97.35% of the fluorescence expressed in the absence of sgRNA, respectively), whereas AcrIIA5 behaved as a much weaker inhibitor, leading to a fluorescent signal corresponding to only 34.92% of the control (Figure 5f).

To understand whether the expression of AcrIIIs could slow the cell growth of the yeast and consequently perturb their metabolism, we traced 18 h growth curves of several circuits expressing the three AcrIIIs in relation to different promoters (Figure 5g). Compared to the negative control, no obviously slower growth rate was detected on any of the tested strains. Interestingly, in our previous study, we found that AcrIIIs could significantly slow the growth of yeast strains. Moreover, in that context, AcrIIA5 appeared to induce a mild toxicity effect, which was not detected in this study. We explain these apparently contradictory results with reference to the different carbohydrate sources present in the cell solutions in the studies: glucose in this one and galactose (in which *S. cerevisiae* grows more slowly°) in the previous one. Indeed, in ref 20, the anti-CRISPR genes were placed downstream of pGAL1.

Our fluorescence measurement emerged another important feature of the anti-CRISPR proteins as components of synthetic networks, namely their ability to stabilize (*i.e.*, decrease the noise) of a circuit. Upon expression into a highly performant fluorescence activation network (strain bYMM1137), every AcrII was able to reduce the variability in the fluorescence level remarkably, when expressed under pGPD (*i.e.*, in high quantity). Moreover, also the AcrII inhibition strength appeared to play a non-negligible role in decreasing the circuit noise. The strong AcrIIA4, for instance, managed to bring down the noise from 3.02 to 0.62% (almost 5-fold) if expressed by pGPD and to 1.44% (about 2.1-fold) under pACT1 (see Supporting Information Text S1 for an explanation about the noise calculation). From the results presented in Figure S10 and Table S7, we can conclude that an adequate amount, and a reasonable inhibitory strength, of the AcrIIIs can almost completely eliminate fluctuations in the concentration of the dSpCas9-based activator and, as a
consequence, prevent evident fluctuations in the fluorescence level from an activated synthetic promoter.

Taken together, our results seem to suggest that AcrIIA2, AcrIIA4, and AcrIIA5 are negatively influenced by the presence of AD/RD when interacting with (d)SpCas9 in S. cerevisiae. AcrIIA4, however, appeared more tolerant of engineered dSpCas9s than AcrIIA2 and AcrIIA5. AcrIIA5 was the most susceptible to modifications on dSpCas9, even to a small domain fused to dSpCas9. As noted, AcrIIA2 and AcrIIA4 exhibit PAM mimicry to compete with the DNA for binding (d)SpCas9:sgRNA.22–25,35 By contrast, the inhibitory mechanism of AcrIIA5 is still unknown, although some hypotheses have been advanced. For instance, AcrIIA5 can induce sgRNA cleavage,27 inactivate the RuvC domain without preventing DNA binding,28 or associate with the Cas9 surface and disturb the proper positioning of sgRNA within Cas9 without blocking sgRNA loading onto Cas9.29 Our results indicate that AcrIIA5 is a strong inhibitor of the bare dSpCas9 in S. cerevisiae. This appears to contradict the assertion by Song et al.30 according to which AcrIIA5 inhibits only RuvC nuclease activity without impeding DNA binding.

Furthermore, our previous20 and new results show that AcrIIA2 generally works efficiently in S. cerevisiae, with a level of performance not distant from that of AcrIIA4, at a high expression level. Other studies have found that AcrIIA4 is more effective than AcrIIA2 in Escherichia coli and human cells.19 Jiang et al.35 pointed out that SpCas9:sgRNA-AcrIIA2 shows lower thermal stability in vitro (namely, a lower affinity and a higher dissociation rate, $K_D$) than SpCas9:sgRNA-AcrIIA4, which causes a reduced working efficiency for AcrIIA2 for increased environmental temperature. For this reason, we measured the fluorescence of strains expressing AcrIIIs by changing the culture temperature from 29 to 37 °C, with an interval of 2 °C (see Figure S11). Our results indicate that the temperature does not affect AcrII working and cannot be the cause of the discrepancy between the efficiency of AcrIIA2 and AcrIIA4 in vivo.

Theoretical estimation of the ratio between the in vivo $K_D$ of AcrIIA2 and AcrIIA4 with respect to that of AcrIIA4 is given in the Supporting Information—Modeling file. We calculated that $K_D$(AcrIIA2)/$K_D$(AcrIIA4) was equal to 11.75 (i.e., 1.8-fold higher than the value measured in vitro by Jiang et al.35), which could help explain the difference in the inhibition efficiency between AcrIIA2 and AcrIIA4 when they are produced by a weak promoter, though they share the same inhibitory mechanism. $K_D$(AcrIIA5)/$K_D$(AcrIIA4) is lower, reaching only 4.01.

**Synthetic Biosensor Responding to β-Estradiol.** The abovementioned results on dSpCas9-stimulated transcription activation and AcrII-based inhibition circuits led us to construct a β-estradiol-responsive biosensor by coupling AcrII with the HBD(hER), the hormone-binding domain of the human estrogen receptor57 (Figure 6a). Due to its remarkable performance even at low concentrations, the choice of AcrIIIs fell on AcrIIA4, which was expressed via genYC1t-pYCY110-TATA. The activation of yEGFP synthesis was triggered by the potent dSpCas9:scRNA-MCP-VP64 ribonucleoprotein. Upon expression, the AcrIIA4-HBD(hER) chimeric protein binds the heat-shock protein 90 (Hsp90, inherently produced by S. cerevisiae), resulting in a large complex (AcrIIA4-HBD(hER)-Hsp90) that lies in the cytoplasm.31 While AcrIIA4 is kept in the cytoplasm, the dSpCas9-based activator stimulates the fluorescence emission in the nucleus. When β-estradiol is added to the cell culture in nano- to micromolar quantities, the interaction between Hsp90 and the HBD(hER) is disrupted. The new complex AcrIIA4-HBD(hER)-β-estradiol translocates into the nucleus and neutralizes dSpCas9-based activation.

We tested the working of our synthetic biosensor by varying the concentration of β-estradiol from 0 to 2 μM (Figure 6b). Only 1.96 nM β-estradiol caused a 10.08% reduction in fluorescence that was further decreased by increasing the concentration of the hormone up to 125 nM. Here, the titration curve reached a plateau corresponding to a maximal fluorescence inhibition of 56.50%. This result was consistent with the ~2-fold gain in fluorescence expression we obtained from the analogous β-estradiol biosensor in our previous work.20 It should be noted, however, that the circuit in ref 20 presented remarkable differences with respect to that in Figure 6. First, the chosen anti-CRISPR protein was AcrIIA2. Moreover, dSpCas9-Mxi1 was employed as a repressor and targeted the synthetic promoter Tsynth8_pCYC1noTATA, which is about 9-fold stronger than lexOpR_trunc_pCYC1-core. This led to expressing the chimeric protein AcrIIA2-HBD(hER) under the strong TEF2 promoter.

**CONCLUSIONS**

In recent years, the CRISPR-dSpCas9 system has proved to be a useful tool for the de novo construction of transcription factors, which are much easier to handle than zinc finger proteins58 and TAL effectors.59 In this work, we sought to achieve some clarity on the function of CRISPR-dSpCas9 in the yeast S. cerevisiae, particularly as a means of activating gene expression. Moreover, we have carefully analyzed how dSpCas9-based activators and repressors can be controlled by means of three AcrIIs: AcrIIA2, AcrIIA4, and AcrIIA5.

We began with the design and construction of a gene transcriptional activation circuit hosting CRISPR-dSpCas9 with three main basic components. (1) yEGFP,11 whose expression was driven by a synthetic promoter that provided a target site for dSpCas9:sgRNA. yEGFP played the role of a reporter protein to measure the performance of the circuit. (2) A yeast-codon-optimized version of dSpCas9 fused to a nuclear localization sequence (NLS) and an AD.12 (3) sgRNA59 that was the reverse complementary sequence of the target DNA site. We assessed the efficiency and the contribution of each circuit component to the production of the output signal via different engineering operations and modifications.

We harnessed two strong ADs: VP64 and VPR (the limited number of tests we performed on VP16, B42, and mDR521-805 did not return significant results) to assess the performance of chimeric activators such as dSpCas9-AD:sgRNA. Although VPR is stronger than VP64,30 we obtained the highest activation when VP64 was fused to dSpCas9. Targeting the single operator dSpCas9-VP64:sgRNA gave a 2.54-fold activation. By increasing the number of lexOpR (up to 6), fluorescence expression increased as well but not so dramatically (a 3.36-fold increase with VP64 but only a 2.35-fold increase with VPR). Apart from varying the operator number, we also increased the distance between a single lexOpR and the TATA box, and we expressed two sgRNAs binding different DNA sequences at the same time. All of these configurations guaranteed activations between ~1.5- and ~3.4-fold. We suppose that the considerable size of dSpCas9-AD (particularly dSpCas9-VPR) can be a problem for recruiting RNA polymerase II and forming the PIC at the TATA box,
which could explain why VP64 was always more efficient than VPR in this study. Hence, depending on the choice of the AD, the location, the number, and the sequence of the target sites should be carefully chosen and properly engineered, which is not an easy operation.

Thus, we tried different engineering solutions for dSpCas9 to achieve higher activation. We fused VP64 simultaneously at the two termini of dSpCas9 without, however, observing any synergistic effects. Interestingly, when we used bare dSpCas9 and MCP-VP64 together with 1× MS2 (wt) scRNA, which is assumed to be able to recruit two VP64 copies, a 5.27-fold activation was obtained. Then, we found that the best solution was to employ 2× MS2 (wt+fl) scRNA to further enhance the possibility of MCP-VP64 independent binding, which provided the highest activation in this study, 7.54-fold activation. We also made the chimeric dSpCas9-AD interact with scRNA that recruited MCP-VP64. However, this design did not lead to an improvement in the ON/OFF ratio.

Separately, we analyzed the effects of different sgRNA expression systems and the role of the amount of sgRNA on the circuit performance. Initially, we made a comparison between sgRNA_ba and sgRNA_bS. The results clearly indicated that sgRNA allows better performance when binding the antisense DNA strand. Then, we used diverse types of promoters to drive the synthesis of sgRNA, i.e., the RNA polymerase II-dependent promoter pADH1 and the RNA polymerase III-dependent promoter pSNR52. The latter promoter has been described as being much weaker than the former. However, RT-qPCR experiments showed that the amount of sgRNA synthesized by pSNR52 was 3.74-fold higher than that by pADH1. Moreover, in previous work performed in our lab, we have never observed that pADH1-RGR guaranteed higher quantities of the single-guide RNA. In general, we found that pSNR52-sgRNA performed either identically to or better than pADH1-RGR in the presence of both dSpCas9 (this study and ref 20) and dCas12a.

To understand the importance of the amount of sgRNA in dSpCas9-based activation, we placed sgRNA expression systems on episomal plasmids, which assured either a 7.66-fold (pSNR52) or a 14.01-fold (pADH1) higher quantity of sgRNA relative to that due to integrating plasmids. However, a much higher expression level of sgRNA did not lead to any activation enhancement, which is consistent with our previous studies.

Therefore, a higher expression level of sgRNA does not seem to be the key factor to improve and optimize the performance of dSpCas9-based gene regulation in S. cerevisiae. In future studies, it will be worth finding out the optimal amount of both sgRNA and dSpCas9 to enhance gene regulation and diminish the burden to the host cells.

To modulate the action of dSpCas9-based transcription factors in S. cerevisiae, we characterized the activity of three AcrIIs, AcrIIA2, AcrIIA4, and AcrIIA5, in much more depth than in previous works. In E. coli and human cells, AcrIIA4 is a very strong inhibitor, whereas AcrIIA2 looks weaker. Moreover, AcrIIA5 is a broad-spectrum inhibitor that can hinder gene editing caused by many Cas9 homologs. In a previous study, we reported a fairly identical performance of AcrIIA2 and AcrIIA4 in S. cerevisiae, whereas AcrIIA5 appeared to be a moderate inhibitor that caused mild toxicity when expressed by pGAL1 in a galactose-containing medium. In this work, we made a further, more detailed comparison between these three proteins. AcrIIA4 acted more strongly than AcrIIA2 and AcrIIA5 on the activation systems that we constructed. Interestingly, modifications to dSpCas9, such as the fusion of the VPR, appeared to diminish the inhibitory function of the three AcrIIs to some extent. This effect was particularly evident for AcrIIA5, whereas AcrIIA4 appeared to be the most tolerant of changes to CRISPR-associated protein. Furthermore, we demonstrated that only AcrIIA4 is an effective inhibitor of dSpCas9-based transcriptional activation at low concentrations, whereas higher concentrations of AcrIIA2 and AcrIIA5 are required to achieve comparable inhibition. Indeed, when produced by the weak synthetic promoter genCYC1t-pCYC110TATA, AcrIIA4 reduced fluorescence due to dSpCas9:scRNA-MCP-VP64 by 79.38%, whereas AcrIIA2 and AcrIIA5 only cut fluorescence by 43.67 and 64.46%, respectively. By contrast, when expressed under a strong GPD promoter, the three AcrIIs decreased fluorescence to a considerable extent: 78.63% (AcrIIA2), 88.63% (AcrIIA4), and 86.56% (AcrIIA5), and appeared to be able to reduce the circuit noise even dramatically (down to 0.62% in the case of AcrIIA4) compared to the network with the sole activation.

Finally, we implemented a β-estradiol-responsive biosensor that worked in a dose-dependent manner and responded to nano-/micromolar concentrations of the input signal. This biosensor was composed of the dSpCas9:scRNA-MCP-VP64 activator and the AcrIIA4-HBD (hER) sensing device. Without the hormone in the cell solution, yEGFP expression was successfully activated. Upon induction with β-estradiol, AcrIIA4-HBD (hER) translocated into the nucleus and counteracted the function of the activator. A 56.60% decrease in fluorescence was observed when the circuit was exposed to 125 μM β-estradiol. This circuit can be used to tune the synthesis of specific endogenous genes or activating pathways by means of β-estradiol. By contrast, we did not manage to implement working Boolean gates in response to theophylline (as shown in the Supporting Information Text S2 and Figures S12–S15).

To summarize, our results indicated that highly performant synthetic gene circuits demand bare dSpCas9 and scRNA. In S. cerevisiae, domains fused to dSpCas9 appeared to hinder the interactions with any AcrIIs and limited the control of transcriptional activation or repression by this means. scRNA turned out to be the best solution to increase activation (by binding MCP-VP64 molecules) and was capable of leading to a high fluorescence level in the presence of a single target site on the DNA, preventing the cumbersome engineering of multioperator-containing promoters. We studied the behavior of three different AcrIIs (AcrIIA2, AcrIIA4, and AcrIIA5) in depth on different configurations of dSpCas9 (e.g., bare or fused to an activation/repression domain). We highlighted that AcrIIA4 was the strongest among the three AcrIIs tested here and the only one that showed high inhibition even at low concentrations. Moreover, we pointed out that every AcrII expressed in high amount (via the GPD promoter) almost completely eliminated fluctuations in the circuit output (fluorescence), making a synthetic network basically insensitive to noise. AcrIIA4 achieved this result even at lower concentrations, i.e., when produced under pACT1 instead of pGPD. Interestingly, we also showed that AcrIIA5, at a high expression level, had performance comparable to that of AcrIIA4. Due to its capability of interacting with many Cas9 homologs, understanding the inhibitory mechanism of AcrIIA5 and how it performs in different species could be extremely useful for building more performant CRISPR/anti-CRISPR-based synthetic gene networks.
**MATERIALS AND METHODS**

**Plasmid Construction.** All of the plasmids used in this work are shown in Table S3. All integrative plasmids are based on the pRSI40X yeast integrative shuttle-vector collection62 (Addgene-35436: pRSI403/His3 marker; Addgene-35438: pRSI404/TRP1 marker; Addgene-35440: pRSI405/LEU2 marker; Addgene-35442: pRSI406/URA3 marker; a gift from Steven Haase). The episomal plasmids for sgRNA expression are based on pRSI424 yeast episomal shuttle-vector (Addgene-35466, TRP1 marker; a gift from Steven Haase).

We followed two methods to construct the new plasmids, as follows: (1) Digestion and ligation. Both backbones and plasmids containing the target fragments were digested overnight. Then, the purified DNA fragments were ligated with T4 DNA ligase (NEB-M0202S) for 8 h at 16 °C. DNA elution from agarose gel was carried out with the AxyPrep DNA Gel Extraction Kit #AP-GX-250. (2) Isothermal assembly.63 Initially, touchdown PCR via Q5High-Fidelity DNA Gel Extraction Kit (NEB-M0202S) was carried out to extract and amplify the DNA sequences of standard biological parts, such as promoters, coding regions, and terminators. Purified PCR products were mixed in equimolar amounts with a cut-opened backbone. The pRSI4XX was digested with SacI-HF (NEB-R3156S) and Acc65I (NEB-R0599S) at 37 °C for 1 h. Then, the two enzymes were inactivated for 20 min at 65 °C before the Gibson assembly program was run on a PCR machine for 1 h at 50 °C.

The synthetic promoters for yEGFP expression were designed on the CYC1 core promoter with a truncated S′ untranslated region (UTR; 24 nt instead of 71 nt), termed trunc_pCYC1core. We inserted one, three, and six copies of the operator, lexOPR, sequence (plus PAMs) gccaCA-TAACGTGATATAACCCAggg upstream of the TATA box (beginning at position −52 with respect to the TSS), which provided target sites for the CRISPR-dSpCas9 system. The distance between this TATA box and the closest lexOPR was 20 nt.

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The construct XbaI-NLS_dSpCas9-Sull−VP64-XhoI came from Addgene-49013 (a gift of Timothy Lu, pMM127). The VP64 that was fused to the C-terminus of dSpCas9 was replaced with the other ADs (VPR, B42, VP16[66-AA long]), and mD251_805 that we used in this study.

The sgRNA expression cassettes were inserted into two different shuttle vectors: pRSI404 (integrative vector, usually present in a single copy after being integrated into the yeast genome) and pRSI424 (episomal vector, generally from 10 to 40 copies of this kind of plasmid can be taken up by a yeast cell).

Three different spacer sequences were placed inside sgRNA: (1) CATAACTGTATATACACCC, a partial lexOPR sequence that binds the antisense strand of tRNA lexOPR−trunc_pCYC1core; (2) TGAGTTGATATACGGTTATG, the reverse complement of the just described partial lexOPR sequence, which targets the sense strand of tRNA lexOPR−trunc_pCYC1core; and (3) ATAGGGCGAATTTGGTACCC, a sequence located upstream of lexOPR−trunc_pCYC1core that binds the antisense strand of the DNA. The negative control for sgRNA (i.e., the scrambled sgRNA) contained, as a spacer, a random sequence (AAGATCTTGTAGAGAGACAA, 20 nt long) that preceded the correct SpCas9 direct repeat. The scRNA expression cassette was designed based on the structure of 2X MS2(wt +6) scRNA from Zalatan et al.36 and synthesized by GENEWIZ Inc., Suzhou, China.

We made use of the yeast-codon-optimized version of AcrI4A and AcrI4A5. An NLS was attached to each anti-CRISPR protein via a GS linker (GGCTCC) (forming the Acr-GS-NLS configuration). In the logic circuits that contained a β-estradiol-responsive device, HBD(hER) was located downstream of Acr via a GS linker again (Acr-GS-HBD(hER)).

**Yeast Strain Construction.** *S. cerevisiae* CEN.PK2−1C (MATα; his3D1; leu2-3,112; ura3-52; trp1-289; MAL2-8c; SUC2)—Euroscarf-30000A, Johann Wolfgang Goethe University, Frankfurt, Germany—was the parent strain in this study (called either byYMM34 or byYMM111). Regardless of the plasmid type (integrative or episomal), we followed the PEG/LiAc protocol for yeast transformation.64 Around 5 μg of integrative plasmids was linearized at the corresponding auxotrophic marker with an appropriate restriction enzyme or around 2 μg of episomal plasmids was used. Transformants were grown on proper synthetic selective medium plates (2% glucose, 2% agar) for 2–3 days at 30 °C. Correct transformants were stored as 15% glycerol stocks. All of the yeast strains engineered in this work are listed in Table S5.

**Fluorescence Measurement.** To detect the fluorescence expression from constitutive promoters, yeast cells were cultured in SDC (synthetic complete medium, 2% glucose) for 20 h. To characterize the performance of AcrIIA4-HBD(hER) and the theophylline ribozyme—LYbulgeOFF1, yeast cells were precultured in SDC overnight (more than 14 h) and then they were diluted to OD600 ≈ 0.1 in fresh SDC, supplied with a proper concentration of β-estradiol or theophylline, respectively, and finally cultured for additional 7 h until reaching OD600 ≈ 0.8–1.0. β-Estradiol (Sigma-Aldrich—E8875) was dissolved in ethanol to make a 10 mM stock solution. Theophylline (Ehows—58-55-9) was initially dissolved in SDC directly to make a 40 mM stock solution.

Then, it was diluted in fresh SDC to get the desired concentration. Due to the poor solubility of theophylline in water, we need to warm the SDC-theophylline solution slightly, to 37 °C. Theophylline could be dissolved well in 0.1 M NaOH or 0.1 M NaOH and N,N-dimethylformamide (DMF) to make a 200 mM stock solution. However, yeast cells are not tolerant of NaOH or DMF. When we cultured yeast cells in SDC that are supplied with high concentrations of theophylline dissolved in 0.1 M NaOH or DMF, we observed that the cells grew either too slowly or did not grow at all.

Flow cytometry (BD FACSVerse, blue laser 488 nm, emission filter 527/32 nm) was employed to record fluorescence from yeast strains. The BD FACSVerse setup was reproduced at each experiment by measuring fluorescent beads (BD FACSuite CS&T Research Beads—650621). They give two peaks. We placed the first peak (mean value) at approximately 75 AU and the second peak (mean value) at...
approximately 3300 AU. The fluorescent bead measurement was done at the beginning and the end of each experiment to set, initially, the voltage of the FITC/GFP channel and assure that the condition of the equipment was stable during the whole measurement. The results were considered reliable if the relative difference in the fluorescent beads at the end and the beginning of an experiment was lower than 5%. For each sample measurement, 30,000 events were collected at a low flow rate (the threshold rate was kept lower than 2000 evts/s). To eliminate the influence of possible autofluorescence due to the S. cerevisiae cell solution (i.e., not related to fluorescent protein expression), the fluorescence associated with the chassis strain CEN.PK2–1C was measured, in every independent experiment, and subtracted to that of any engineered strain. The final fluorescence level of each strain was evaluated as the mean value of at least three independent experiments. The original data from BD FACsVerse were analyzed with the flowcore R-Bioconductor package.65 A two-sided Welch’s t-test was performed to assess the statistical difference between the mean values of two nonpaired groups.

**RT-qPCR.** Yeast cells were cultured in SDC or SD-TRP (SD without tryptophan) overnight at 30 °C. The following morning, the cells were diluted with 2 mL of fresh SDC or SD-TRP to OD$_{600}$ $\sim$ 0.1. The diluted cells then were grown up to an OD$_{600}$ of $\sim$ 0.8–1.0 (about 7 h). RNA extraction and purification were carried out with the YeStar RNA kit (Zymo Research-R1002). cDNA synthesis was performed with the HiFiScript cDNA Synthesis Kit (CWBIO-CW2569M). Oligo(dT) and random primers were employed to perform reverse transcription of RNA. SYBR Green I dye (Takara-RR820A) was used for running qPCR. 50 ng of cDNA was mixed with 5 μL of TB Green Premix Ex Taq II (Tli RNaseH Plus) (2x), 0.4 μL of 10 μM forward and reverse primers, and H$_2$O up to 10 μL. The following program was run on a Roche LightCycler96: (1) preincubation stage: 95 °C for 30 s; (2) two-step amplification stage: 95 °C for 5 s followed by 58 °C for 34 s, with 45 cycles being performed at this stage; and (3) melting stage: 95 °C for 1 s followed by 85 °C for 15 s, and then 95 °C for 1 s again. ACT1 was chosen as the reference gene and was amplified with forward primer 5'-CGTCTGGATTGGTGGTTCTATC-3' and reverse primer 5'-GGACCACTTTGCFTAGTATTCTG-3'. The target sgRNA was amplified with forward primer 5'-CGGTCTTGGTTTATTACATAT-3' and reverse primer 5'-GGACCACTTTGCFTAGTATTCTG-3'. sgRNA-HDV was amplified with forward primer 5'-TTGAAAAATGGGCACC-GAGTC-3' and reverse primer 5'-GCCATGCGCGGACCCCC-3' (see Table S6). Each sample was present in three replicates and analyzed with the Pfaffl formula66 to calculate its relative amount (R) with respect to the mRNA of the ACT1 gene. By assuming a maximal amplification efficiency (i.e., equal to 2) for both target and reference, R is calculated after measuring the

$$R = 2^{(C_t(\text{reference}) - C_t(\text{target}))}$$

where $C_t$ is the cycle threshold.

**Cell Growth Curve.** Yeast cells were grown in either 2 mL of SDC or in 2 mL of SD-TRP (both media were supplied with 2% glucose) for 20 h at 30 °C. Then, they were diluted approximately 1:100 in 30 mL of the corresponding medium such that the initial OD$_{600}$ was equal to roughly 0.1 (time = 0). From this point on, yeast cells were cultured at 30 °C at a rotational speed of 240 rpm. Every 2 h, 1 mL of cell culture was removed to measure the absorbance at 600 nm with an Eppendorf BioPhotometer. This procedure went on for 18 h, and each measurement was carried out in three replicas. Finally, after collecting all of the absorbance data, the growth curves were analyzed with nonlinear regression and simulated using a logistic grow equation.

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