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Technical Note

A Tunable and Expandable Transactivation System in Probiotic Yeast *Saccharomyces boulardii*

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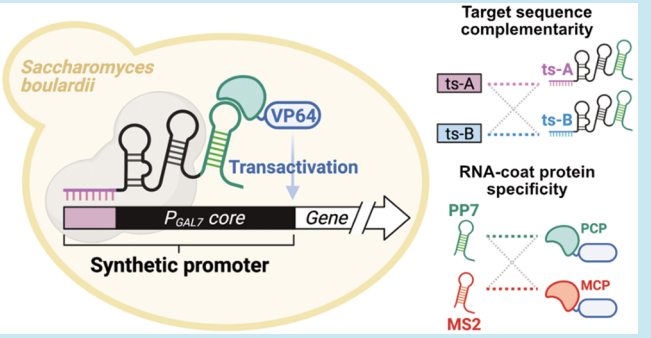
ABSTRACT: Precise transcriptional modulation is a key requirement for developing synthetic probiotics with predictably tunable functionalities. In this study, an expandable and tunable transactivation system was constructed and validated in probiotic yeast *Saccharomyces boulardii*. The use of nuclease-null Cas9 and scaffold RNA (scRNA) directed regulation enabled transactivation under the control of a synthetic promoter in *S. boulardii*. A synthetic promoter consisting of the scRNA target sequence and the core *GAL7* promoter region restricted interference from the native galactose regulon. The system was readily expanded by introducing new target sequences to the promoter and scRNA. Complementarity between the promoter and scRNA, and binding specificity between scRNA and transcriptional activator, served as two layers of orthogonality of the transactivation. In addition, activator expression under the control of an inducible promoter enabled control of the transactivation via chemical inducer. The described system has the potential to enable engineering of probiotic yeast to more precisely perform therapeutic functions.



KEYWORDS: *Saccharomyces boulardii, transactivation, Cas9, scaffold RNA*

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Saccharomyces boulardii (*Sb*) is a probiotic yeast that has shown benefit toward preventing and treating various gastrointestinal disorders.[1,2] Despite the significant genetic similarity between *Sb* and baker's yeast *S. cerevisiae*, *Sb* has shown phenotypic advantages over *S. cerevisiae* as a probiotic, such as better tolerance to gastric environmental conditions.[3,4] As a synthetic probiotic chassis, *Sb* is amenable to genetic engineering and has unique advantages over bacterial probiotics; *Sb* is tolerant of bacteriophages and more compatible than bacteria for the biosynthesis of heterologous proteins requiring post-translational modifications to be biologically active.[5,6] Despite the therapeutic potential of *Sb*, in comparison to putative probiotic bacteria, limited development and applications of genetic tools for *Sb* engineering have been reported to date.[1,5,7]

Transcriptional control engineering is a prerequisite to enable probiotics to predictably perform novel functions or finely modulate existing benefits.[5,8] Nuclease-null CRISPR-associated protein 9 (dCas9) fused with a transcription effector module is a powerful toolkit for controlling transcription in target loci via complementarity with a corresponding guide RNA (gRNA).[9] However, the direct dCas9-effector fusion confines the regulation to only one direction per dCas9. To enhance the intensity of gene activation further as well as enable multidirectional regulation per dCas9, Zalatan et al. recruited transcription effectors to target loci via an RNA-binding protein (RBP)-effector fusion protein and a gRNA extended with modular RNA domains recruiting designated RBPs, named scaffold RNA (scRNA).[10] The dCas9 and scRNA-directed transcriptional regulation system was demonstrated to successfully control target transcriptions in *S. cerevisiae*,[10,11] a brewer's yeast closely related to *Sb*, but has not yet been validated in the probiotic yeast *Sb*. In the current study, we constructed a synthetic transactivation system based on the dCas9 and scRNA-directed regulation system, along with a set of synthetic promoters, and validated it in *Sb* (ATCC MYA-797). Furthermore, we demonstrated its expandability and tunability, namely the ease of increasing and controlling independent synthetic transactivations, which are critical prerequisites to execute complex designed expression programs in synthetic probiotics.[5,8]

First, we built expression cassettes of the two core elements, dCas9 and scRNA, under the control of the constitutive promoters P_{TEF1} and P_{SNR52}, respectively. Similar to a previous study,[10] scRNA A-PP7 was composed of a target sequence, dCas9-binding hairpin, and PP7 hairpin which can recruit a

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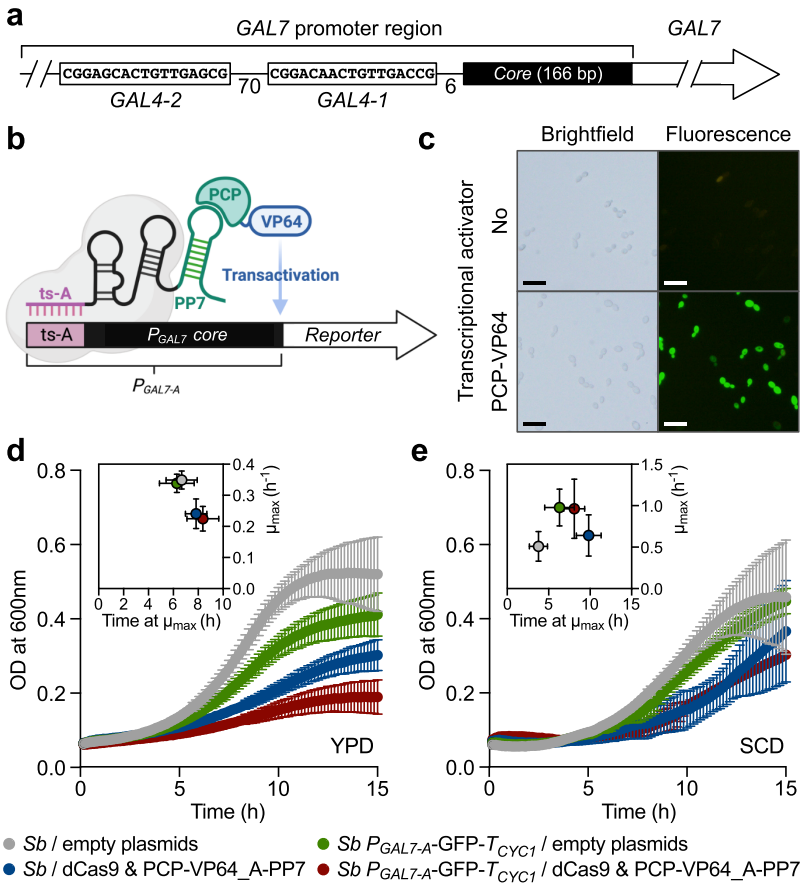

Figure 1. Construction of a synthetic transactivation system in probiotic yeast *S. boulardii*. (a) Structure of the original *GAL7* promoter. *GAL4−1* and *GAL4−2* are two binding regions of the transcription factor Gal4p of the native galactose regulon. (b) Schematic illustration of the transactivation system using nuclease-null Cas9 (dCas9, gray), scRNA (violet, target sequence ts-A; black, dCas9 binding region; green, PP7 sequence recruiting PCP), and transcription activator comprising PCP and VP64. Synthetic promoter $P_{GAL7\text{-}A}$ was constructed by conjugating the ts-A and core *GAL7* promoter. (c) Fluorescence microscopy validation of the transactivation system in *S. boulardii* using yeast enhanced GFP. Scale bars represent 200 nm. (d,e) Expression of components of the transactivation system retarded the growth of engineered strains in the anaerobic condition and both complex (d) and defined (e) media at body temperature.

corresponding RBP, PP7 coat protein (PCP). The target sequence at the 5′ end of A-PP7 consists of a random 20 base-pair DNA sequence that is not found in the *Sb* genome (ts-A, Table S2). Synthetic promoter $P_{GAL7\text{-}A}$ was designed by conjugating the ts-A sequence and the core promoter region of *Sb GAL7* promoter. To avoid potential interference by the native galactose regulon,[12] we cloned 166 bp of the *GAL7* upstream region that does not include the two binding regions of the Gal4p transcription factor, namely *GAL4−1* and *GAL4−2* (Figure 1a and 1b).[13] The corresponding transcriptional activator was prepared by fusion of PCP and VP64, a tetrameric repeat of the herpes simplex VP16 activation domain.[14] An expression cassette of PCP-VP64 was constructed with another constitutive promoter, P_{ADH1}.

To validate the functionality of the synthetic transactivation system in *Sb*, a reporter expression cassette of yeast enhanced green fluorescence protein (yeGFP)[15] under the control of the synthetic promoter $P_{GAL7\text{-}A}$ was integrated into an intergenic site of the *Sb* genome (IS1n, Table S2). Expression cassettes of dCas9, A-PP7, and PCP-VP64 were introduced into episomal plasmids with antibiotic selection markers (Table S1). The PCP-VP64 expression cassette was omitted from the negative control strain. Fluorescence microscopy of the experimental and control strains carrying the plasmids showed that the presence of the transcriptional activator PCP-VP64 determines the yeGFP expression under the control of $P_{GAL7\text{-}A}$ in *Sb* (Figure 1c). This demonstrates the functionality of dCas9 and scRNA-directed transactivation in *Sb* and its controllability by the relative activator. Overexpression of the transactivation system components imperceptibly affected the fitness of *Sb* in the routine yeast culture condition (Figure S2) but retarded the growth of *Sb* significantly in simulated intestinal conditions (anaerobic, 37 °C, Figure 1d,e); genomic integration of their cassettes with promoters of moderated and optimized expression levels would be required for future *in vivo* applications to minimize the negative impact of the over-expression.

To demonstrate the expandability of the transactivation system in *Sb*, we prepared another synthetic promoter by exchanging the target sequence of $P_{GAL7\text{-}A}$ and A-PP7 for another random 20-bp sequence (ts-B, Table S2 and Figure 2a). With constitutive expression of PCP-VP64, yeGFP expression under the control of the synthetic promoters was activated when the target sequences of the promoter and scRNA were complementary. Mismatch of the target sequence resulted in significantly constricted fluorescence levels (Figure 2a). The detectable fluorescence levels of wild-type *Sb* suggest that the fluorescence signals from the engineered *Sb* strains carrying mismatched promoter and scRNA were mainly due to autofluorescence in *Saccharomyces* yeasts.[16] We further

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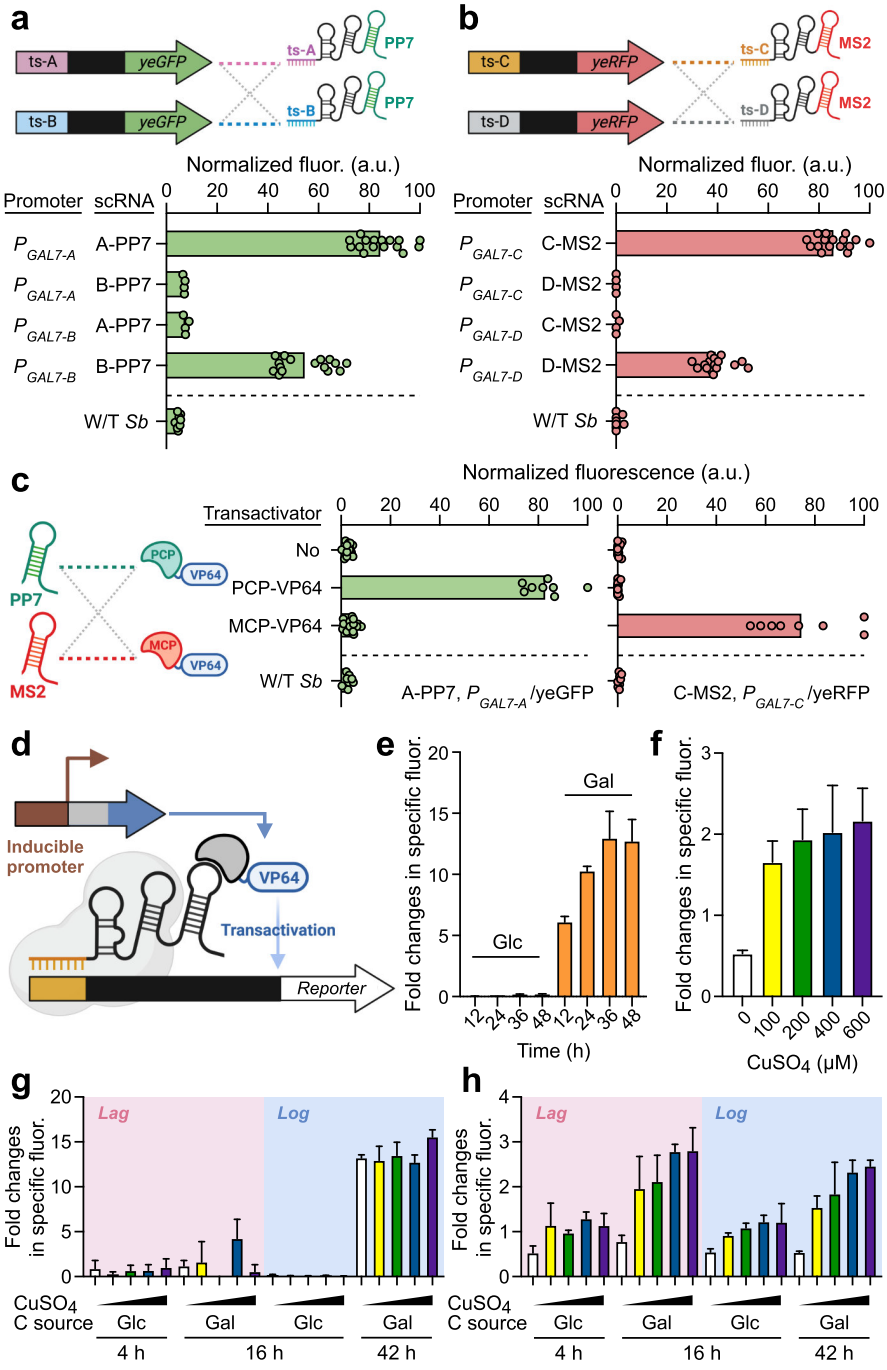

Figure 2. Induction of the synthetic transactivation. (a,b) Expansion of the transactivation system. Yeast enhanced GFP (yeGFP, a) and RFP (yeRFP, b) expression under the control of synthetic promoters demonstrated that the transactivation system operates relying on the target sequence complementarity so that each scRNA activates only the transcription from its designated synthetic promoter. (c) The transactivation specificity depending on the selective interaction between the RNA-binding protein domain of transcriptional activators and the RBP-recruiting hairpin of scRNAs. (d) Schematic of the inducible transactivation mechanism. (e,f) Fold changes in the specific fluorescence signal from the engineered *Sb* strain carrying inducible transactivation systems of P_{GAL1} (yeRFP, e) or P_{CUP1} (yeGFP, f) under varied culture conditions. (g,h) Test of the dual-inducible *Sb* strain expressing yeRFP (g) and yeGFP (h) under the control of P_{GAL1} and P_{CUP1}, respectively. Fluorescence levels were measured at both lag phase (pink) and the middle of log phase (blue) of each culture condition (Figure S7). Glc, glucose; Gal, galactose. $CuSO_4$ concentrations; White, 0 μM; yellow, 100 μM; green, 200 μM; blue, 400 μM; violet, 600 μM. Fold changes in the specific fluorescence level (normalized by $OD_{600\ nm}$) was calculated by comparing with the level of corresponding strains without induction. Error bars represent standard deviations of biological triplicates.

expanded the number of synthetic promoters by exchanging the target sequence of the two synthetic promoters with additional random 20 bp sequences ts-C and ts-D (Table S2), resulting in $P_{GAL7\text{-}C}$ and $P_{GAL7\text{-}D}$, respectively. Corresponding scRNAs were constructed by combining the new target sequences with a dCas9-binding hairpin and MS2 hairpin recruiting another RBP, MS2 coat protein (MCP).[10] Yeast enhanced red fluorescence protein (yeRFP)[15] was placed downstream of the new promoters instead of yeGFP to avoid the confusion between autofluorescence and yeGFP signals.

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The new promoters and scRNAs also showed selective transactivation depending on the target sequence complementary (Figure 2b), indicating that the transactivation system is scalable with its dependency on the target sequence complementarity.

In the identical genetic circumstance, paired dual transactivation with target sequence variants led to a significant difference in the fluorescence level (Figure 2a, 2b, and S2). We hypothesized that the target sequence is a potential modulator for the expression level of the transactivation system by differentiating specificity and efficiency of dCas9 and scRNA-directed regulation, similar to the target sequence effect on the performance of Cas9-directed genome editing.[17] To test this hypothesis, we evaluated the off-target (specificity) and on-target (efficiency) scores of the target sequences in the context of the reporter cassette using scoring models.[17−19] The observed scores did not correlate well with the resulting fluorescence signal patterns (Figure S3), suggesting the presence of additional factors affecting the transactivation, but uncontrollable in the current design, such as copy number instability of the plasmids with the 2 μ origin of replication.[20] Further studies with alternative avenues for the introduction of dCas9 and scRNA with stable copy number and more direct measurement of the target transcription may uncover the potential role of the target sequence as a factor finely tuning the transactivation.

The specific interaction between scRNA and transcriptional activator can serve as another layer of orthogonality in the synthetic transactivation system.[10] We built additional engineered *Sb* strains expressing mismatched scRNA and transcriptional activator pairs (Table S2) and compared their fluorescence levels with those of positive controls. Indeed, the dCas9 and A-PP7 could not activate the yeGFP expression under the control of $P_{GAL7\text{-}A}$ with MCP-VP64, while they expressed the yeGFP with the correct transcriptional activator, PCP-VP64 (Figure 2c). Similarly, dCas9 and C-MS2 activated the expression of yeRFP under the control of $P_{GAL7\text{-}C}$ with MCP-VP64 only (Figure 2c). The highly selective transactivation demonstrates that the designed specificity between the RBP-recruiting hairpin in scRNA and the RBP domain of the transcriptional activator allowed multiple synthetic transactivations to be orthogonal to each other in *Sb*.

We hypothesized that synthetic transactivation in *Sb* could be tuned by manipulating the transcriptional activator expression via an inducible promoter (Figure 2d). To test this hypothesis, inducible variants of the positive *Sb* strains (Figure 2c) were constructed by replacing P_{ADH1} of the MCP-VP64 and PVP-VP64 cassettes with chemical-inducible promoters, P_{GAL1} and P_{CUP1}, respectively (Table S1).[21] As the *Sb* strain of the current study exhibited a detectable galactose-utilizing capacity (Figure S4), we tested the P_{GAL1}-based inducible system as a binary switch, using galactose as a carbon source and an inducer simultaneously. Induction of the transcriptional activator under the control of P_{GAL1} successfully turned on the fluorescent (yeRFP) signal selectively on galactose (Figure 2e and S5). Although the green fluorescence levels of the P_{CUP1}-based inducible system and the $CuSO_4$ concentrations were positively correlated, its dynamic range was significantly narrower than direct induction under the control of P_{CUP1}[21] as well as the P_{GAL1}-based inducible systems (Figure 2f). Considering the noticeably stronger transcription from P_{GAL1} than P_{CUP1},[21] the weak activator expression under the control of P_{CUP1} might more easily lead to the extinguishment of free activators by excessive dCas9 and scRNA expressed under the control of strong constitutive promoters. In addition, the significant overflow metabolism of *Saccharomyces*[22] may have further masked the limited transactivation capacity.

To demonstrate the modularity and orthogonality of multiple synthetic transactivations in a single *Sb* strain, we constructed a dual-inducible *Sb* strain by genomically integrating both $P_{GAL7\text{-}A}$-yeGFP-T_{CYC1} and $P_{GAL7\text{-}C}$-yeRFP-T_{CYC1} cassettes (IS1n and IS2n, Table S1 and S2) and combining cassettes of corresponding transactivator-scRNA sets in a single plasmid (Table S1). In the dual-inducible *Sb* strain, the galactose-inducible transactivation system activated the yeRFP expression on galactose exclusively, and the $CuSO_4$ supplementation did not affect the transactivation by galactose. However, the dual-inducible *Sb* did not exhibit significant changes in the level of reporter signals during lag phase of galactose cultures (Figure 2g and S7), probably due to the diauxic shift from glucose preculture.[23] We further assessed the tunability of the dual-inducible system via P_{CUP1}-based transactivation of yeGFP with varied $CuSO_4$ concentrations on both glucose and galactose. Green fluorescence levels were positively correlated with the supplemented $CuSO_4$, and the patterns were similar on both sugars (Figure 2h and S7). P_{CUP1} limited fluorescence signals (yeGFP) on both glucose and galactose in the absence of inducer $CuSO_4$ (Figure 2f and S6), corroborating that the synthetic promoter design effectively restricted potential interference from the native galactose regulon.[13]

In summary, robust transactivation by the dCas9 and scRNA directed transactivation system was validated in *Sb*. The system was expandable and tunable with orthogonality to the native galactose regulon as well as among the synthetic transactivations of the system in *Sb*. Our described system has great potential to be a vital tool for developing next-generation engineered probiotic yeasts with a wide range of predictable and robust therapeutic applications, such as programmable small molecule production, biosensing, and biocontainment.[10,24]

METHODS

Plasmid Construction. Plasmids and primers used in this study are summarized in Table S1 and S3, respectively. Unless otherwise mentioned, Integrated DNA Technologies synthesized DNA fragments, PCR was performed using Q5 High-Fidelity DNA polymerase (New England Biolabs), and DNA products were purified after PCR and restriction enzyme treatment through QIAquick PCR Purification Kit (QIAGEN). *Escherichia coli* Top10 [*F- mcrA D(mrr-hsdRMS-mcrBC) u80lacZDM15 DlacX74 recA1 araD139 D(araleu)7697 galU galK rpsL (StrR) endA1 nupG*] was used as the host system for the plasmid cloning. The final constructs were confirmed through Sanger sequencing (Genewiz).

To construct the constitutive expression cassette of dCas9, codon-optimized coding sequence of dCas9 was amplified from pAG414GPD-dCas9-VPR[9] using primers SK6410 and SK6411 and introduced into pRS425_P_{TEF1}_T_{CYC1}. The cassette P_{TEF1}_dCas9_T_{CYC1} was amplified using primers SK6321 and SK6322 and moved into pRS42H using *Sac*I and *Kpn*I, resulting in pRS42H_P_{TEF1}_dCas9_T_{CYC1} for the episomal constitutive expression of dCas9 in *Sb*.

Coding sequences of the two synthetic transcriptional activators, namely PCP-VP64 and MCP-VP64, were synthe-

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sized together with the SV40 nuclear localization sequence and a terminator (Figure S1), amplified using primers SK6487 and SK6488/SK6489, and introduced into pRS424_P_{ADH1}_T_{CYC1} between *Spe*I and *Kpn*I sites, resulting in pRS424_P_{ADH1}_PCP-VP64_T_{ADH2} and pRS424_ P_{ADH1}_MCP-VP64_T_{ENO2}. The expression cassettes from the two plasmids were amplified using primers SK6321 and SK6322 and transferred into pRS42K using *Sac*I and *Kpn*I, resulting in pRS42K_ PCP-VP64 and pRS42K_MCP-VP64. Scaffold RNA (scRNA) expression cassettes of A-PP7, B-PP7, C-MS2, and D-MS2 (Figure S1) bracketed by *SNR52* promoter and *SUP4* 3′ flanking sequence were synthesized and amplified using primers SK6499 and SK6509. The scRNA cassettes were introduced into the two activator-expressing plasmids or the original backbone, pRS42K.

To construct the transcriptional activator-scRNA-coexpressing plasmid variants with an inducible promoter for the activator expression, *CUP1* promoter (498 bp *CUP1* upstream region) and *GAL1* promoter (476 bp *GAL1* upstream region) were synthesized with T3 and *Bsr*GI sites at 5′ end and T7 and *Spe*I sites at 3′ end. The promoter fragments were amplified using primers SK6321 and SK6322. The amplicons were digested by *Bsr*GI and *Spe*I together with pRS42K_PCP-VP64_A-PP7 and pRS42K_MCP-VP64_C-MS2, purified, and then ligated to replace the original *ADH1* promoter.

Reporter cassettes were constructed in a plasmid as a template of the donor DNA (dDNA) preparation for the CRIPSR-Cas9 directed genomic integration. The yeast shuttle vector pRS426_P_{CCW12}_T_{CYC1} was used as a backbone for the cloning. Synthesized $P_{GAL7\text{-}A}$ was amplified using SK6484 and SK6486 and substituted for the original *CCW12* promoter using *Sac*I and *Spe*I. The target sequence ts-A of $P_{GAL7\text{-}A}$ was replaced by the new 20-bp sequences (Table S2) through FastCloning[25] using corresponding primer sets: SK6570-SK6571 (ts-B), SK6572-SK6573 (ts-C), and SK6574-SK6575 (ts-D). Yeast enhanced GFP (yeGFP) and RFP (yeRFP) coding sequences were amplified from pGADT7-ADH700-yeCherry-YAP1-yeGFP-DHFR[15] using the SK6452-SK6453 and SK6450-SK6451 primer sets, respectively. The amplicons were digested and introduced into the plasmid with a synthetic promoter using *Spe*I and *Bam*HI.

Media and Culture Conditions. *E. coli* strains were grown in 5 mL of Luria−Bertani medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 5 g L^{-1} NaCl) in 14 mL round-bottom culture tubes, at 37 °C and 250 rpm. 100 μg mL^{-1} of ampicillin was supplemented when necessary for selection.

Yeast peptone medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) containing 20 g L^{-1} of glucose (YPD) was prepared for routine *Sb* culture, genome editing, and plasmid transformation. Nourseothricin (100 μg mL^{-1}), geneticin (300 μg mL^{-1}), and hygromycin B (200 μg mL^{-1}) were added to YPD medium when required for selection. Synthetic complete medium, which contains yeast nitrogen base without ammonium sulfate and complete supplement mixture (MP Biomedicals), was used for the inducible transactivation experiments together with 50 mM pH 5.5 potassium hydrogen phthalate buffer. 20 g L^{-1} of glucose or galactose was added as a carbon source. 200 μM $CuSO_4$ was supplemented when required.

Microbial cell growth was monitored by absorbance at 600 nm (A600) using DiluPhotometer (IMPLEN). Consumption of carbon sources were measured by HP 1050 HPLC system equipped with Agilent 1100 Refractive Index Detector (G1362). 0.005 N H_2SO_4 was eluted through Rezex ROA-Organic Acid H+ (8%) column (Phenomenex) at 0.6 mL min^{-1} and 50 °C.

Construction of Engineered Yeast Strains. *S. boulardii* ATCC MYA-797 was the host yeast strain in this study. Plasmids and dDNAs were introduced to *Sb* by lithium acetate/single strand carrier DNA/polyethylene glycol method[26] with an elongated heat-shock time of 1 h. Genomic integration of the reporter cassettes was performed via CRISPR-Cas9 genome editing.[27] First, *Sb* was transformed with the Cas9-NAT plasmid (Table S1). Reporter cassettes were amplified from corresponding plasmid templates (Table S1) using primers SK6558 and SK6559, which generated dDNAs with 50-bp overhang regions homologous to the upstream and downstream of the intergenic site IS1n (Table S2), using Phusion HF DNA polymerase (New England Biolabs). A dDNA and the guide RNA expressing plasmid pRS42K-IS1n (Table S1) were simultaneously transformed into the *Sb* carrying Cas9-NAT. For the second integration into another intergenic site IS2n, SK7197-SK7198 primer set was used to generate dDNAs, and pRS42H-IS2n was introduced to supply corresponding gRNA. *Sb* transformants were spread on selective YPD medium plate with nourseothricin and geneticin or hygromycin B for screening. Correct transformants were selected via colony PCR and replica plate.

Fluorescence Signal Measurement. All *Sb* strains were recovered from −80 °C glycerol stocks on YPD agar plate and then precultured in 5 mL YPD with geneticin and hygromycin B, if required. To validate the transactivation system, cells of wild-type and engineered *Sb* strains were harvested from the precultures at 4000 rpm for 5 min and washed with sterilized PBS. The main cultures were performed in YPD with geneticin and hygromycin B, if required, with initial A600 of 0.1. Cells were harvested in the middle of the exponential phase, washed with PBS, and diluted to adjust the A600 to 0.1. 200 μL of the diluted samples were distributed to a black-walled, clear-bottom 96-well plate, and the green fluorescence (Ex, 488; Em, 527) and red fluorescence (Ex, 587; Em, 615) levels were determined using Synergy H1 microplate reader (BioTek). Fluorescence levels were normalized based on the signal from negative wells (200 μL of PBS) and the highest fluorescence level. The main cultures for the inducible transactivation experiments were performed in 5 mL of SCD (glucose) or SCG (galactose) media with a $CuSO_4$ supplement, if required. Fluorescence levels were measured in the middle of the log phase (on glucose, 24 h; on galactose, 70 h). Specific fluorescence levels were calculated by adjusting the normalized fluorescence levels based on A600.

Calculation of Efficiency and Specificity Scores of Target Sequences. Efficiency and specificity scores of the 4 target sequences were calculated in the context of corresponding reporter cassette and the intergenic site IS1n (Figure S2) as well as *Streptococcus pyogenes* Cas9, the origin of the dCas9 in this study. The off-target (specificity) and on-target (efficiency) scores were calculated using the models from Hsu et al.[19] and Doench et al.[17] in Benchling (https://benchling.com). CHOPCHOP (http://chopchop.cbu.uib.no), a target sequence selecting tool based on another model,[18] generated identical efficiency scores.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00384.

List of plasmids used in this study; information on target sequences; list of primers used in this study; design of scRNAs and transcriptional activators; impact of the synthetic transactivation system components on the Sb fitness; efficiency and specificity scores of target sequences; galactose utilization by wild-type *Sb* strains; culture tests of the engineered *Sb* strain expressing yeRFP under the control of the galactose-inducible transactivation system; culture tests of the engineered *Sb* strain expressing yeGFP under the control of the copper-inducible transactivation system; Culture tests of the dual-inducible *Sb* strain (PDF)

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Author Contributions

S.K., B.M., and G.D. designed the experiments. S.K. and B.M. performed the experiments and interpreted the results. S.K., B.M., and G.D. prepared the manuscript. All authors have given approval to the final version of the manuscript.

Author Contributions

⊥S.K. and B.M. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Sb, *Saccharomyces boulardii*; dCas9, nuclease-null CRISPR-associated protein 9; gRNA, guide RNA; scRNA, scaffold RNA; RBP, RNA-binding protein; yeGFP, yeast enhanced green fluorescence protein; yeRFP, yeast enhanced red fluorescence protein; PCP, PP7 coat protein; MCP, MS2 coat protein.

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