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T7 Polymerase Expression of Guide RNAs *in vivo* Allows Exportable CRISPR-Cas9 Editing in Multiple Yeast Hosts

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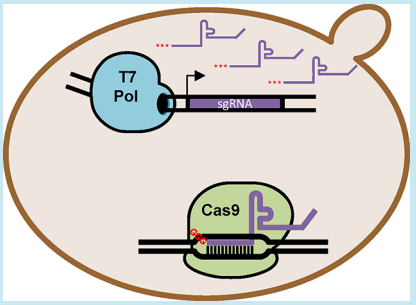
Supporting Information

ABSTRACT: Efficient guide RNA expression often limits CRISPR-Cas9 implementation in new hosts. To address this limitation in fungal systems, we demonstrate the utility of a T7 polymerase system to effectively express sgRNAs. Initially, we developed a methodology in *Saccharomyces cerevisiae* using a modified version of the T7 P266L mutant polymerase with an SV40 nuclear localization signal to allow guide RNA expression immediately downstream of a T7 promoter. To improve targeting efficiency, guide RNA design was found to be tolerant to three mismatches or up to three additional bases appended to the 5′ end. The addition of three guanines to a T7-based guide RNA improved guide RNA expression 80-fold and achieved transcriptional output similar to the strong Pol III *snr52* promoter. Resulting gene editing and dCas9-guided gene regulation with a T7-based guide RNA was on par with the commonly used *snr52* system in *S. cerevisiae*. Finally, 96% and 60% genome editing efficiencies were achieved in *Kluyveromyces lactis* and *Yarrowia lipolytica* respectively with minimal optimization of this system. Thus, T7-based expression of sgRNAs offers an orthogonal method for implementing CRISPR systems in fungal systems.



KEYWORDS: *CRISPR/Cas9, T7 RNA polymerase, yeast*

In recent years, the CRISPR endonuclease system has emerged as a relatively host-agnostic and versatile tool for genome editing and gene regulation.[1,2] However, implementation in new hosts can be labor intensive and challenging due to poor expression of guide RNA.[3−5] Specifically, functional guide RNA in eukaryotes requires high level expression, nuclear localization and proper 5′ and 3′ ends. These traits are often achieved by using strong RNA Pol-III based promoters, such as the U6 promoter in mammalian cells[6] and the *snr52* promoter in *Saccharomyces cerevisiae*.[7] However, many organisms, including certain yeasts, lack strong or well-characterized Pol III promoters. In such cases, Pol II or Pol III systems are often combined with ribozyme cleavage sequences.[4,5,8] Unfortunately, this approach takes significant trial and error and does not bypass the general limitation of the need for an orthogonal, host-agnostic means of expressing guide RNA.

To address these limitations, we demonstrate here that a T7 RNA polymerase system can efficiently express single-guide RNAs (sgRNA) in multiple yeast systems. T7 polymerase is popularly used for *in vitro* transcription sgRNA synthesis;[9−12] however, very few eukaryotic studies use T7 polymerase *in vivo*.[13,14] T7 polymerase can broadly produce well-defined, nuclear RNA transcripts without a 7-methylguanylate cap in eukaryotic organisms when a SV40 nuclear localization sequence is appended.[14−17] Furthermore, it is well-known that the relative strength of these short 17 bp promoter regions can be altered through modifications of the 4−6 bp initially transcribed sequence (ITS) region.[18−20] Additionally, T7 polymerase is an orthogonal system that does not rely on native polymerases, operates as a single subunit, and only needs a T7 promoter for transcription initiation in yeast.[15]

In this work, we develop the T7 polymerase system as an orthogonal and portable system for *in vivo* expression of guide RNAs in yeasts. To do so, we prototype, optimize, and benchmark this system for utility in the model yeast, *S. cerevisiae*, in part since strong sgRNA expression is enabled through the Pol III *snr52* promoter. Next, we demonstrate the portability of this system to two additional and different yeasts of interest in the literature: *Kluyveromyces lactis* and *Yarrowia lipolytica*. *K. lactis* is an industrially relevant organism for which Cas9 editing has been realized through a similar Pol III *snr52* promoter system.[4] *Y. lipolytica* is an oleaginous yeast of industrial interest[21−24] that is much more distantly related to *S. cerevisiae* and has a reported hybrid Pol III promoter for sgRNA expression.[5] For both of these cases, T7 RNA polymerase has not been tested for RNA expression, and the T7 promoter is less bulky than the *Y. lipolytica* (443 bp hybrid promoter) and *K. lactis* (268 bp *snr52* promoter) systems.

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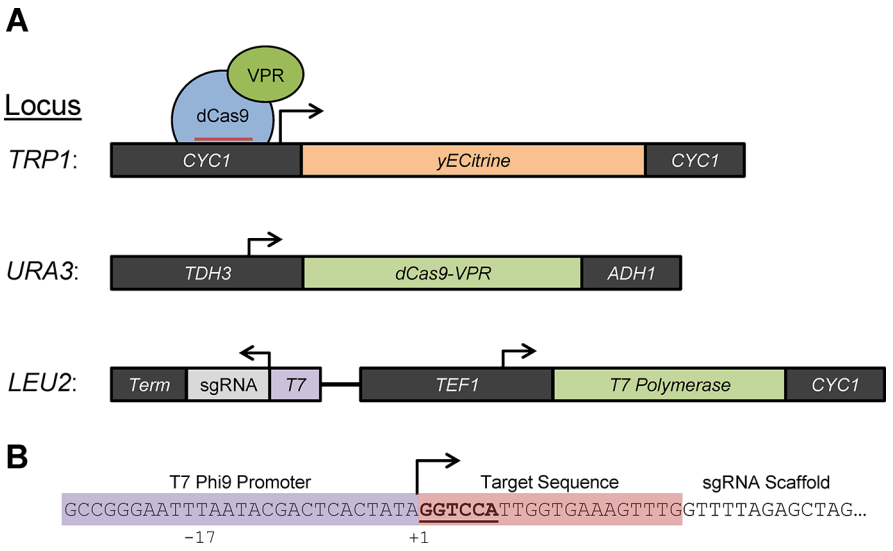

Figure 1. Constructs cloned in this study. (A) All constructs were integrated for testing. The targeting of dCas9-VPR to the promoter region of *yECitrine* activates fluorescence. (B) The T7 promoter (purple) used for this study is, based off the *phi9* promoter in the T7 bacteriophage genome. The −17 bp is annotated to show the typically used T7 promoter. The underlined region is defined as the initially transcribed sequences (ITS) in the +1 to +6 region. The red indicates the 20 bp sequence used by dCas9 for targeting. The remaining sequence contains the structural aspects of the sgRNA scaffold.

Here, we demonstrate that the T7 RNA polymerase system can enable CRISPR-based genome editing and gene expression control at levels commensurate with high-expression Pol III systems and exhibits an easy portability into yeast systems.

RESULTS

T7 Polymerase Can Transcribe Functional sgRNA in *S. cerevisiae*. Initially, we sought to demonstrate that a T7 polymerase system can express a functional sgRNA in *S. cerevisiae*. Specifically, this test employed the nuclease-null dCas9 fused to the VPR activation domain to activate a gene of interest when targeted to a promoter region.[25−27] Here, we studied sgRNA expression by adapting our previous system to activate expression of the fluorescent protein yECitrine driven by a weak *CYC1* promoter integrated into the genome.[26] To do so, we first removed all errant T7 promoter regions, used in the yeast vectors as convenient sequencing primer locations, to build the expression system outlined in Figure 1A. The yECitrine cassette and the dCas9-VPR cassette were integrated into the genome along with a blank *TDH3* promoter and *ADH1* terminator cassette as a control for dCas9-VPR. A sgRNA cassette was created in which both the guide RNA and T7 polymerase gene were expressed in divergent directions. For this experiment, a previously reported T7 polymerase was used containing a SV40 nuclear localization signal placed between amino acids 10 and 11.[15,17] Next, the sgRNA was chosen such that it had two adjacent guanines at the start of the transcript, as necessary for strong expression[19,20] (Figure 1B), and targeted a region −222 base pairs upstream of the proximal TATA-box for*CYC1* promoter based on our prior results.[26] Finally, the cassette was flanked with the T7 promoter from upstream the *phi9* gene found in the T7 bacteriophage genome (GenBank V01146.1) (GCCGGGAATTTAATACGACTCACTATA), including the underlined 17 bp consensus promoter, and a single T7 *phi9* consensus terminator sequence, which was previously used to localize transcript to the yeast nucleus.[17] These constructs were all stably integrated into the genome.

With the first system test in place, we compared activated yECitrine fluorescence levels in a factorial design with cells containing and not containing dCas9-VPR and T7 polymerase along with a scrambled sgRNA (CTACCGCGGTCGA-CCATACC) not predicted to target in the promoter region (Figure 2). Among all the combinations, the only statistically

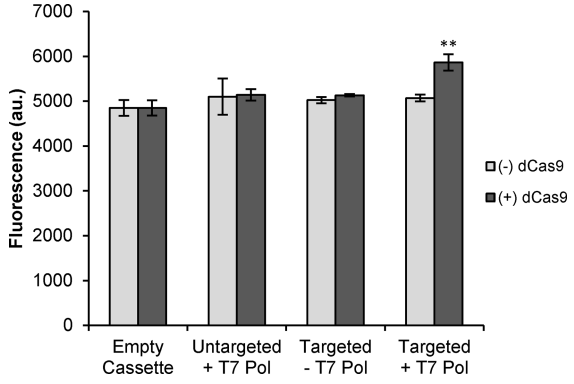

Figure 2. T7 polymerase makes targeting guide RNA. Cells were grown with and without the dCas9-VPR ORF and T7 polymerase ORF. An untargeted scrambled sgRNA was then used as a control. Only when a targeting guide RNA, dCas9-VPR, and T7 polymerase were all expressed is a significant level of activation observed. Error bars represent standard deviation between biological triplicates. One-factor ANOVA with the Tukey's *posthoc* test was used to determine statistical significance. The ** signifies $p < 0.01$ *versus* all other possible pairs.

significant increase in fluorescence was observed when the dCas9-VPR, T7-polymerase, and a targeting sgRNA were present. This resulted in a roughly 1.14-fold increase over the original strain. Therefore, this initial test showed the potential of T7 polymerase to express a functional sgRNA that could correctly target dCas9-VPR to the promoter, albeit at a low efficiency.

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Mutants of T7 Polymerase Enhance sgRNA Expression and Improve dCas9 Targeting. While we were encouraged by the potential for T7 polymerase to drive sgRNA expression, the 1.14-fold activation achieved was far below the 10-fold activation observed from sgRNA using the Pol III *snr52* promoter system.[26] We initially hypothesized that this reduction was due to poor expression of sgRNA. In particular, T7 polymerase produces a high quantity of abortive transcripts when presented with poorly consensus transcription start sites.[28,29] Specifically, mutations within the initially transcribed bases (ITS) (+1 to +6) of the Transcription Start Site (TSS) of TATAGGGAGA can decrease transcriptional output.[19,20] Since the sgRNAs are functional RNAs based on DNA target sequence, they will rarely contain the consensus GGGAGA ITS sequence and thus we sought to decrease the dependence of T7 polymerase on this consensus sequence through evaluating mutant T7 enzymes.

To evaluate mutant T7 promoters on sgRNA expression, we selected three previously identified variants. First, we tested the previously identified P266L variant of a T7 polymerase, analogously P278L in T7 polymerase containing the NLS, shown to decrease abortive transcription.[28] This residue resides in the C-linker region connecting the promoter binding N-terminal domain and the catalytic C-terminal domain and mediates conformational change during the elongation process.[29,30] Previous work shows that a wild-type T7 polymerase performs better with consensus promoter regions than the P266L mutant variant,[28,31] but its performance with the nonideal sgRNA ITS is unclear. Second, we tested a group of mutations previously tested for improved *in vitro* transcription.[31] The "M5" mutations (analogously S442P, N445T, S645P, F861I, and F892Y with the NLS) were identified as thermostable mutations enabling function at elevated (50 °C) temperatures.[31,32] Lastly, the analogous Y651F mutation was identified for its ability to polymerize 2′ modified RNA[33−35] and helped recover some of the processivity loss of the P266L mutant *in vitro*.[31] Using these three sets, we tested their combinatorial effect alongside our NLS-tagged T7 polymerase for increased transcription of sgRNA.

Using the same sgRNA design described above, we found that the P278L mutation in T7 (the analogous residue to P266L for T7 RNAP with an NLS) enabled the dCas9-activation system to increase fluorescence 2.44-fold over background compared to the wild-type T7 polymerase (Figure 3A). The M5 mutations only enabled a 1.34-fold activation of yECitrine, but this value increased to 3.42-fold when combined with the P278L mutation. These two sets of mutations largely improved performance over wild-type, though only to 30% of the level of activation enabled with the *snr52* promoter system in *S. cerevisiae*, suggestive of additional optimization required. In nearly each of these experiments, addition of the Y651F mutation decreased sgRNA-mediated activation.

Prior to final optimization, we sought to confirm that the performance improvements seen here were indeed caused by sgRNA expression level. As such, RT-PCR was conducted and compared with corresponding fluorescence from expression activation (Figure 3B). Doing so, we found that the resulting activation of yECitrine corresponded directly to the sgRNA expression level, thus demonstrating the mode-of-action of these mutant T7 polymerases. Interestingly, the correspondence between expression level and activation was nonlinear, agreeing well with previous computational predictions for sgRNA expression on Cas9 efficiency[36] and previous studies in mammalian cells.[37] Therefore, sgRNA still appears limiting despite improvements obtained with these mutant T7 polymerases.

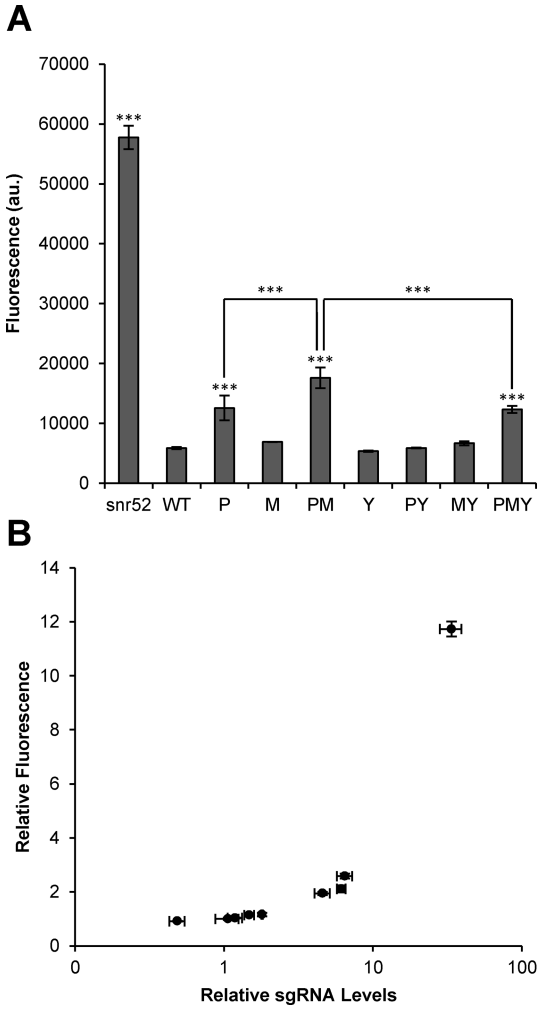

Figure 3. T7 mutants increase fluorescence activation and guide RNA expression. (A) Fluorescence was measured for sgRNA activation using seven different T7 polymerase mutants and compared against the *snr52* promoter system as a positive control. Mutant names were abbreviated for P278L (P), M5 (M), and Y651F (Y), where additional letters indicates a combination of labeled mutations. The P278L/M5 mutant was identified as the strongest. (B) To show these mutants were working to create more sgRNA, qPCR was run on the sgRNA and plotted against fluorescence to show a positive trend between them. For all experiments, error bars represent standard deviation in biological triplicate. ANOVA with the Tukey's *posthoc* test were used to determine statistical significance. The *** indicates $p < 0.001$ relative to wild-type T7 polymerase, except when another pair is specifically indicated. The *snr52* positive control was statistically significant against all possible pairs.

Guide RNA Expression and Performance Can Be Improved through Guanine Additions to the T7 Promoter Sequence. As a second (and complementary) approach for improving sgRNA expression levels using T7 polymerase in *S. cerevisiae*, we chose to modify the promoter sequence and initially transcribed sequence (ITS) region, a region that impacts full-length transcript levels.[19,20,28,29] In the experiments above, the sgRNA was placed in the +1 transcription start site and thus limited the pool of guide RNAs to those starting with "GG" to better match the

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A

Name	Original
Untargeted	CTACCGCGGTCGACCATACC
Targeted	ACTTTAGTGCTGACACATAC
	Appended
aG	GACTTTAGTGCTGACACATAC
aGG	GGACTTTAGTGCTGACACATAC
aGGG	GGGACTTTAGTGCTGACACATAC
	Mismatched
mG	GCTTTAGTGCTGACACATAC
mGG	GGTTTAGTGCTGACACATAC
mGGG	GGGTTAGTGCTGACACATAC

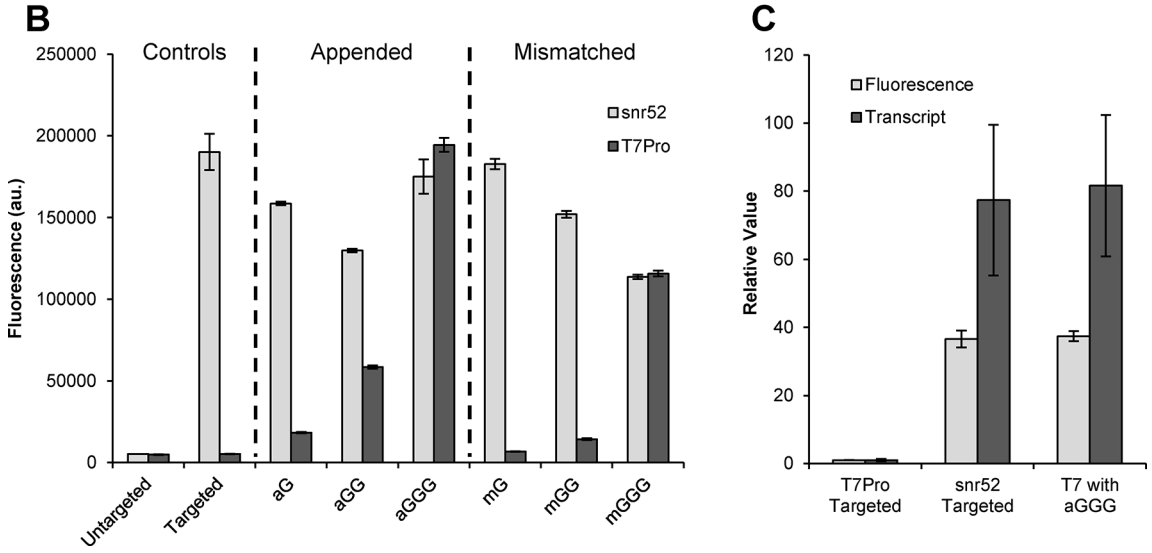

Figure 4. Testing the tolerance of guide RNA for appending bases and mismatches. (A) A list of sgRNA tested with guanine additions to optimize T7 expression. (B) Fluorescence was measured for each of the sgRNA under either the *snr52* promoter or T7 promoter. A scrambled sgRNA sequence was used as negative control. Three guanines were found to be sufficient for T7 expression to equal that of *snr52* expression. (C) qPCR was done on the sgRNA to show that the guanines were indeed increasing sgRNA levels for the T7 system nonlinearly to the fluorescence activation. Error bars represent standard deviation in biological triplicates.

consensus ITS. Here, we sought to test sgRNA tolerance towards mismatches and additional 5′ nucleotides to better generalize sgRNA design considerations for T7 expression. To test this, we chose a sgRNA from our lab (sg1: ACTTTA-GTGCTGACACATAC)[26] that performs well, but lacks the 5′ guanine. We then tested two approaches for altered sgRNA design to better match the consensus ITS of three guanines (GGG).[20] In the first approach, bases were appended to the 5′ end of the target sequence sequentially (Figure 4A), thus creating target sequences that were 20-mer, 21-mer, 22-mer, and 23-mer, respectively. In the second approach, the 20-mer target sequence was mutated such that each target was a 20-mer with 1, 2, or 3 mismatches respectively from the consensus ITS.

When combined with the P278L/M5 mutant for T7 polymerase, both the appended and mismatch sequences enabled an improved sgRNA function, as indicated by an increase in fluorescence (Figure 4B). For the GGG appended to the 5′ end, a 40-fold level of activation was observed, which is on par with the currently used *snr52* promoter system. Similarly, three guanines in the mismatched version also achieved levels commensurate with the *snr52* promoter. Once again, we sought to confirm if these improvements were indeed due to sgRNA expression level and found this to be the case with an over 80-fold increase in sgRNA compared to the original sgRNA and on par with the *snr52* promoter (Figure 4C). On the basis of these results, it is evident that the addition of three guanines to the 5′ end of a targeting sequence appears sufficient to enable high-level sgRNA expression from a mutant T7 promoter in *S. cerevisiae*. Interestingly, the guide RNAs with increased mismatches had a lower efficiency for the *snr52* promoter system. Nevertheless, these results shows that the P278L/M5 mutant variant of T7 polymerase, along with appending guide RNAs with three guanines, can enable efficient T7-based targeting of dCas9 in *S. cerevisiae* on par with the level seen for the traditionally used Pol III *snr52* promoter system.

T7-Expressed sgRNA Can Enable Efficient Cas9-Mediated Genome Editing in *S. cerevisiae*. As a final test of efficacy in *S. cerevisiae*, we tested the ability of T7-expressed guide RNAs to enable genome editing. To do so, we utilized a previously reported guide RNA[7] (named CAN1.Z) to target the *CAN1* gene, thus enabling positive selection on canavanine and quantification of gene editing. Additionally, we designed an additional poly$(T)_6$ containing sgRNA sequence targeting the canavanine gene (named CAN1.T) to test the ability of T7 polymerase to transcribe this sequence that is typically used for Pol III *snr52* termination.[38−41] For this experiment, we integrated the P278L/M5 mutant variant of T7 polymerase and expressed each of these guide RNAs from a plasmid also containing a galactose inducible Cas9 protein. We then tested two different sgRNAs for each target which added up to three guanines for optimized T7 expression: CAN1.ZG (two guanines appended) and CAN1.TG (one mismatch).

Using this assay, we determined that both guide RNAs driven by T7 polymerase were able to effectively edit *CAN1* and yield

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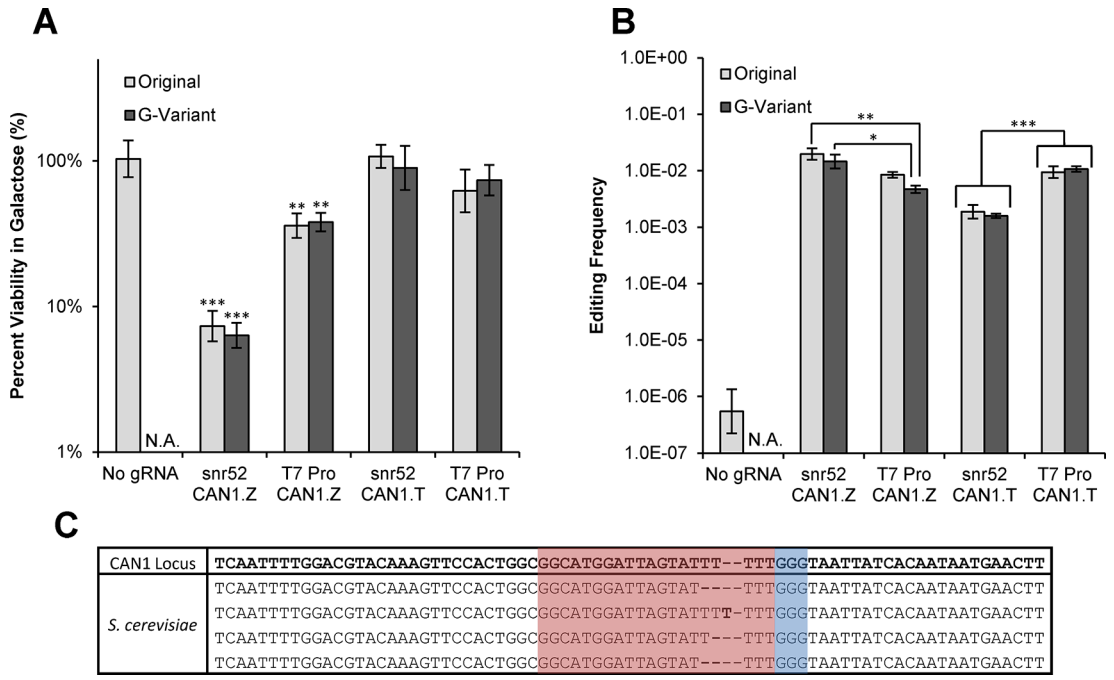

Figure 5. *CAN1* editing in *S. cerevisiae*. (A) Cells were grown concurrently in glucose and galactose, and their relative viable cell counts used to determine the toxicity of galactose induction of Cas9 on the cells (N.A. = Not Applicable). The CAN1.Z guide RNA showed a high level of toxicity, particularly when driven by *snr52*, indicative of a high level of double-stranded breaks. (B) Editing frequency of each of the sgRNA as a percent of canavanine resistant colonies to viable cells. T7 polymerase shows a similar level of editing to *snr52* for the CAN1.Z sgRNA, but a higher level of editing for the CAN1.T sgRNA harnessing a poly(T)$_6$ sequence believed to terminate Pol III expression. (C) A selection of 4 colonies from the T7 Pro CAN1.T G-variant plate, showing that editing is indeed occurring near the guide RNA PAM location. Error bars represent standard deviation between biological (A) triplicates and (B) quadruplicates. ANOVA with the Tukey's *posthoc* test were used to determine statistical significance on log transformed colony counts. The * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ relative to the No sgRNA control unless another pair is specified.

canavanine resistant (CAN^R) colonies (Figure 5). For the CAN1.Z target, the T7 system and the *snr52* system both produced a number of CAN^R colonies with editing frequencies around 0.5% and 2.0% respectively. These values are comparable to previously reported efficiencies in *S. cerevisiae* in the absence of donor/repair DNA[7] and reflect *S. cerevisiae*'s poor nonhomologous end joining efficiency. For the CAN1.T target, the editing frequencies were around 1.1% and 0.2% respectively (Figure 5B). Specifically, we find that the poly(T)$_6$ sequence served as a weak terminator and decreased *snr52* system slightly, without any such detriment to the T7 system. Interestingly, the viability of each strain was different among different sgRNA (Figure 5A), indicative of double-strand breaks becoming detrimental to cell survival. Nevertheless, the T7-expressed sgRNAs enabled a proper genome editing of *CAN1* as determined by insertion and deletions found in the target sequence after sequencing (Figure 5C). Thus, these collective results in *S. cerevisiae* demonstrate a feasible path to express functional sgRNAs at levels on par with that of the most commonly used Pol III system.

T7-Based sgRNA Expression Enables Genome Editing in *Kluyveromyces lactis* and *Yarrowia lipolytica*. As discussed in the introduction, one of the challenges of the CRISPR system is the host-specific elements required to express guide RNAs. To address this challenge, we tested the exportability and generalizability of guide RNA expression *via* T7 polymerase using two industrially interesting fungal organisms: *K. lactis*[4] and *Y. lipolytica*.[21,23] In both of these organisms, we utilized the P278L/M5 mutant T7 polymerase to drive expression of sgRNA containing leading guanines alongside Cas9.

For *K. lactis*, we first tested the T7-transcribed sgRNAs by generating indels in the *NDT80* locus, which was initially tested *via* sequencing to determine successful mutagenesis upstream the PAM site. Subsequently, to quantify editing efficiency, direct homologous recombination to the *NDT80* locus was performed using a system shown previously.[4] Genome editing efficiency was measured as the number of correctly targeted homologous recombination insertions events *versus* random integration events as determined by colony PCR in the targeted *NDT80* locus. Overall, we observed an efficiency of 96% for T7-expressed guide RNAs *versus* 99% for the *snr52* system—a difference that is statistically indistinguishable (Figure 6A).

For *Y. lipolytica*, we employed the T7-transcribed sgRNAs to direct gene knockouts with nonhomologous end joining, which we quantified by targeting the *CAN1* gene. Specifically, cells were cotransformed with a Cas9-sgRNA plasmid and a linear piece of DNA containing the T7 P278L/M5 gene. After this one-step transformation, genome editing efficiency was calculated as the number of canavanine resistant colonies *versus* the total number of colonies picked. Overall, we observed an efficiency of about 60% for knocking out the *CAN1* gene, relative to about 0.3% for the no guide RNA control (Figure 6B). To confirm that these effects were due to sgRNA expressed by T7 polymerase, we sequenced colonies and found an expected indel between the 3−4th base pair away from the PAM sequence, confirming this to be due to the sgRNA expressed from T7. Collectively, these results demonstrate the

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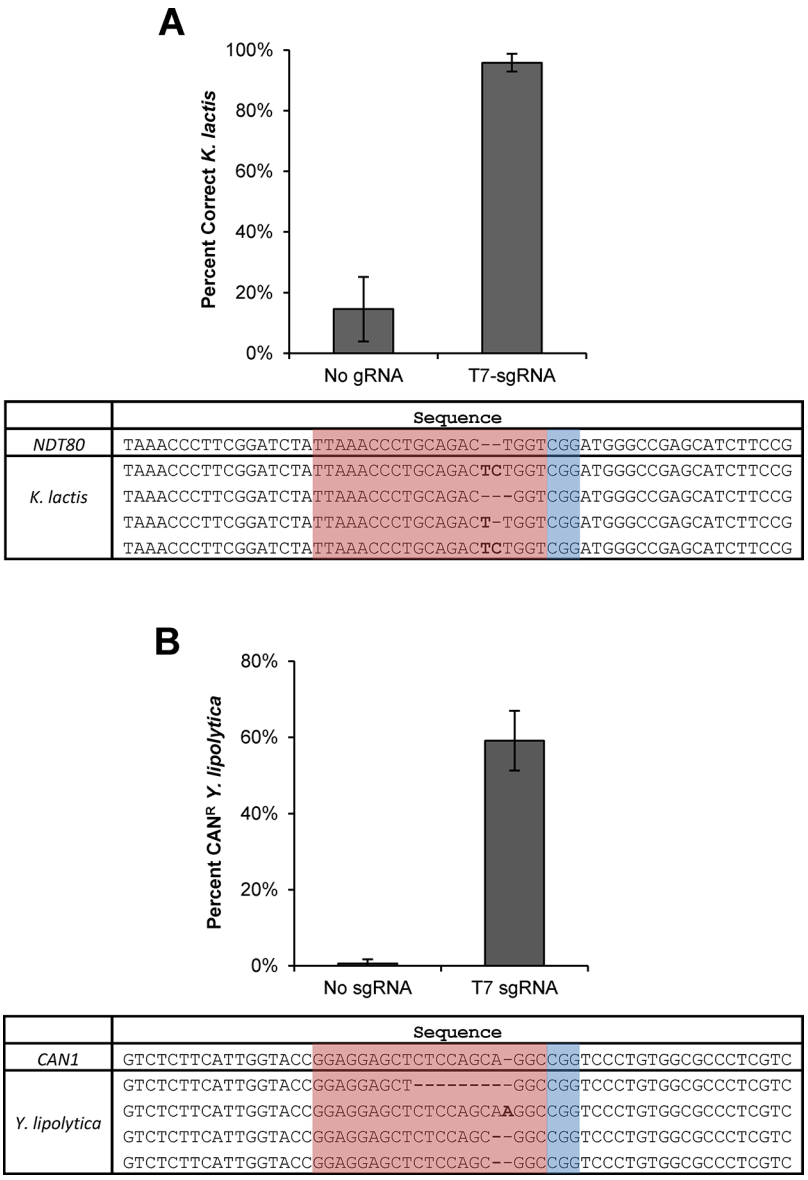

Figure 6. Cas9 Editing in *Yarrowia lipolytica* and *Kluyveromyces lactis*. To test Cas9 editing, the P278L/M5 mutant of T7 polymerase was integrated to each of the strains. (A) A Cas9-sgRNA plasmid was cotransformed with a KanMX cassette containing homology to the *NDT80* locus of *Kluyveromyces lactis*. Values represent the percent of correctly targeted integrations of this cassette as determined by colony PCR of the *NDT80* locus. To test whether indels could also be created, Cas9-sgRNA plasmids were transformed without homology donor and the locus sequenced, where the 20 bp target sequence (red) and PAM sequence (blue) are shown. (B) A Cas9-sgRNA plasmid was transformed into *Yarrowia lipolytica* targeting the *CAN1* locus. The efficiency was determined as the percent of colonies which were canavanine resistant upon replica plating. Error represents standard deviation between three repeats of the experiment. Select *Y. lipolytica* colonies were PCRed and sequenced to observe indels in the locus.

capacity to rapidly export and generalize the use of T7-expressed guide RNAs for genome editing in fungal hosts.

DISCUSSION

Implementing CRISPR-Cas9 systems into different organisms often requires many iterative approaches before achieving efficient guide RNA expression and desired genome editing efficiencies. This work demonstrates that the T7 RNA polymerase system can streamline this approach and provide a facile manner for expressing sgRNAs for yeasts in a manner that does not rely upon endogenous Pol III parts. Thus, this work provides the means for an orthogonal sgRNA expression system. In doing so, this study also revealed some interesting characteristics of guide RNA and T7 polymerase expression in yeast hosts.

First, the tunable nature of T7 promoters enabled a study directly linking guide RNA expression and mediated dCas9 targeting in a manner that is more difficult with Pol III systems.[39] Prior studies have explored the effects of guide RNA expression on targeting efficiencies by introducing additional copies of the guide RNA.[37] Instead, T7-driven guide RNAs through mutant promoters and polymerases deconvolutes any effects splicing efficiency, 5′ end maturation, or genomic copy number variation has on guide RNA expression. Our work demonstrates that a nonlinear increase in sgRNA was needed to observe an increase in fluorescence activation (Figure 3B) and are among the first to validate previous biophysical models of

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Cas9 systems.[36] These same tunable properties enable a facile manner to modulate downstream activation or repression with CRISPRi simply by changing sgRNA levels. It is possible that this approach can serve as a better way to dynamically control dCas9 regulation in multiple organisms.

Second, our study benchmarks T7 polymerase performance in yeast hosts. Previously, only a few studies had reported *in vivo* function of T7 polymerase in yeasts,[15,17,42] presumably due to the lack of a cap-independent translation system. However, our study offered a functional assay for testing T7 polymerase transcription *in vivo* that was tied to a fluorescent readout. The T7 polymerase mutants tested here (P278L, M5, and Y651F) had never been tested in yeast and thus their characterization will be useful for future applications. In addition, this work presents the first time T7 polymerase has been utilized in *K. lactis* and *Y. lipolytica*. Thus, this work further demonstrates the orthogonal and ubiquitous nature of T7 to function in a variety of fungal hosts.

Third, T7-driven sgRNA recapitulates previous observed phenomena regarding the 5′ sgRNA sequence. For one, the sgRNAs employed here were tolerant to mismatches on the 5′ end distal from the PAM site, in line with prior studies on sgRNA.[36,43−45] In addition, our study shows that addition of a third guanine appended to a sgRNA (GGG-N_{20}-NGG) achieved full activity of the sgRNA (Figure 4, "aGGG"). This observation was not entirely expected. A previous study had found that sgRNAs with two guanine additions (GG-N_{20}-NGG) had median activities lower than that of non-mismatched, 20-mer sgRNAs,[9] an inefficency we also saw for our *snr52* controls in the dCas9-VPR experiment (Figure 4, "aGG" relative to "Targeted" for *snr52*). Therefore, the observation that a third added guanine can recover activity may hint at either an inefficiency in Cas9 binding the 22-mer sgRNA or an improper folding structure of sgRNA which destabilizes upon addition of a third guanine.

Through this work, we establish T7 as a platform for sgRNA expression. However, we recognize that this system is not without limitations. Introducing a second protein in addition to Cas9 can be a challenge in organisms with few selectable markers, particularly if they lack strong promoters, as it is unclear how relative T7 polymerase expression impacts performance. To this end, the promoters used in this study were largely based on the strong *TEF1*, *TDH3*, or similar hybrid promoter variants of the respective yeast hosts. In addition, although the SV40 nuclear localization tag used here has been shown to function in a broad range of hosts, nuclear localization can be a challenge in organisms more generically. These limitations notwithstanding, T7-based expression of guide RNA offers significant advantages over the currently used systems based upon its compact, orthogonal, and exportable nature.

METHODS

Media and Strains Construction. *Saccharomyces cerevisiae* strain BY4741 (*Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) (Euroscarf) was routinely cultivated in yeast extract peptone dextrose (YPD) media at 30 °C or yeast synthetic complete (YSC) medium. YPD is composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. YSC dropout medium is composed of 6.7 g/L yeast nitrogen base, 20 g/L glucose or galactose, and CSM dropout supplement (MP Biomedicals, Solon, OH). *Escherichia coli* strain NEB10*β* was used for all cloning and plasmid propagation. NEB10*β* was cultivated at 37 °C in lysogeny broth (LB) supplemented with 100 μg/mL of ampicillin. *Escherichia coli* and *Saccharomyces cerevisiae* were cultivated with 225 rpm orbital shaking and stored at −80 °C in 15% glycerol. Plasmids were transformed to *E. coli* by electroporation in 2 mm cuvettes at 2.5 kV and 25 μF using a Gene Pulser Xcell (Bio-Rad). Plasmids were isolated from *E. coli* using the GeneJET Plasmid Miniprep kit (Thermo). Yeasts were transformed using the EZ Yeast Transformation II Kit (Zymo) according to manufacturer's instructions.

Yarrowia lipolytica PO1f (ATCC MYA-2613) was modified to produce a Δ*TRP1* variant. *TRP1* (YALI0B07667g) was first replaced with a *loxP*-flanked dominant hygromycin marker cassette.[46] This marker was subsequently excised through Cre recombinase expression from a replicative plasmid,[47] followed by plasmid loss in nonselective YPD media to generate PO1f Δ*TRP1*. *Kluyveromyces lactis* strain NRRL Y-1140 (ATCC 8585) was modified to generate a Δ*URA3* variant by integrating *Cas9* driven by the *K. lactis TEF* promoter into the *URA3* locus *via* homologous recombination. The P278L/M5 polymerase was then integrated into the *LEU2* locus *via* homologous recombination for testing.

Plasmid Construction. The sequences of the *Saccharomyces cerevisiae* plasmids used in this study have been included in the Supporting Information. PCR was carried out using Q5 Hot Start (New England Biolabs) according to the manufacturer's instructions. PCR purifications were done with the Qiagen PCR Purification Kit. Gel extractions were done using the Thermo Gel Extraction kit. Plasmids were constructed *via* Gibson assembly. *Saccharomyces cerevisiae* plasmids were based on the yeast shuttle vectors by Mumberg *et al.*,[48] and *dCas9-VPR* was amplified from Addgene plasmid # 46920.[49] The *GAL1* promoter and Cas9 plasmid sequence is included in the Supporting Information. T7 mutants were made by PCR amplification and Gibson assembly. The T7 promoter was from 27 bp upstream the *phi9* gene found in the T7 bacteriophage genome (GenBank V01146.1), and the T7 terminator sequence from a previous study.[17] Both were cloned by annealing DNA oligos (Integrated DNA Technologies) and Gibson assembling them into the backbone. The initial sgRNA was amplified from a previous study[26] and cloned *via* Gibson assembly. Subsequent guide RNAs were cloned *via* Gibson assembly of single-stranded oligos. A more in-depth cloning strategy has been included in the Supporting Information. Correct assemblies were verified using Sanger Sequencing.

For the *Yarrowia lipolytica* experiments, the pUCS3-8dTEF base vector was constructed by replacing the *URA3* auxotrophic marker cassette in pUCS1-8dTEF[47,50] with a *TRP1* marker cassette. The T7 P278/M5 protein sequence was codon optimized for *Yarrowia lipolytica* expression (Blue Heron Biotechnology), synthesized as a gBlock (Integrated DNA Technologies), and then cloned into AscI/PacI digested pUCS3 to create *TRP1*-marked pUCS3-8dTEF-T7PM. pCRISPRyl[5] was a gift from Cory Schwartz and Ian Wheeldon (Addgene plasmid # 70007). To modify pCRISPRyl for T7-dependent guide RNA expression, the region between the *bla* (ampicillin resistance) marker and the *LEU2* auxotrophic marker was replaced by a synthetic sequence containing a T7 promoter, gRNA scaffold, and phi 10 terminator. Two variants of this plasmid were constructed: untargeted negative control pCRISPRyl-T7pro-SpeI containing only a unique SpeI restriction site between the T7 promoter and gRNA scaffold, and targeted pCRISPRyl-T7pro-CAN1 containing a 21 nucleotide *CAN1* (YALI0B19338g) specific sequence

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(gGGAGGAGCTCTCCAGCAGGC) between the T7 promoter and gRNA scaffold. Full sequences for T7 P278/M5 and gRNA cassettes are provided in the Supporting Information.

For *Kluyveromyces lactis*, a 2 μ plasmid for sgRNA expression was made by Gibson assembling a *K. lactis URA3* auxotrophic marker cassette and a stabilizing pKD1 element[51] into the *S. cerevisiae* sgRNA plasmid. The target sequence *NDT80* (gGTTAAACCCTGCAGACTGGT) was then cloned *via* Gibson assembly downstream either the T7 promoter or *snr52* promoter. For the no sgRNA control, the plasmid was linearized *via* PCR excluding the target sequence and ligated together with T4 DNA Ligase (New England Biolabs). Sequences are available in the Supporting Information.

Flow Cytometry for dCas9-VPR Activation. The strains used to test fluorescence activation had the weak *CYC1* promoter driving the fluorescent protein yECitrine in the *TRP1* locus, the strong *TDH3* promoter driving either an empty vector or the dCas9-VPR protein in the *URA3* locus, and the cassette containing T7 polymerase and the guide RNA in the *LEU2* locus. The plasmids used to integrate the dCas9-VPR and T7 cassettes, as well as the protocol for integration, are contained in the Supporting Information.

To run the experiment, yeast strains were picked from glycerol stocks stored at −80 °C and grown in YSC-His media at 30 °C to saturation. From this, cultures were seeded into YSC-His media at OD600 = 0.005 and grown 14 h to early exponential phase to about OD600 = 0.2−0.5. Fluorescence from strains expressing yECitrine was measured with a FACS Accuri (BD Biosciences) using a YFP fluorochrome and a sampling time of 3 s, which corresponded to about 20 000 to 50 000 events per sample. Day to day variability was mitigated by running all comparable strains on the same day. FlowJo (Tree Star Inc.) was used to gate on forward and side scatter for the population and compute geometric mean fluorescence values for each population of cells. Reported values are the average of geometric mean fluorescence of independent biological triplicates. Statistics were done *via* one-way ANOVA in R followed by the Tukey's *posthoc* test. Statistical significance is indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Quantitative PCR. A full protocol has been included in the supplemental. Briefly, yeast strains were grown in biological triplicate to exponential phase before being analyzed for fluorescence and RNA extracted. Gene specific primers for the sgRNA and the housekeeping gene *ALG9* were used for reverse transcription, with qPCR run using SYBR Green. Averages of fluorescence and qPCR data was then compared among biological replicates.

***CAN1* Mutagenesis in *Saccharomyces cerevisiae*.** An integrated T7 polymerase P278/M5 strain was grown in YPD and made competent according to the instructions for the EZ Yeast Transformation (Zymo). Then, 25 ng of p415-GAL1-Cas9 containing the guide RNA of interest was transformed according to a 1:4 scaled down version of the manufacturer's instructions. Following incubation, cells were pelleted and the transformation mix removed. Cells were resuspended in YSC-Leu-His media containing 40 g/L of glucose and grown for 48 h to saturation to select for the Cas9 plasmid and repress the *GAL1* promoter. Next, cells were pelleted and the glucose media removed with pipetting. Pellets were then resuspended in YSC-Leu-His media containing 20 g/L of galactose and incubated at 30 °C for an additional 48 h. Following incubation, cells were plated directly onto YSC-Leu-Arg plates containing 20 g/L of glucose and 60 μg/mL of canavanine. To determine the number of viable cells, cells were diluted 1000× and plated onto YSC-Leu-Arg plates containing 20 g/L of glucose without canavanine. Colonies were then counted on the plates with and without canavanine.

To determine the toxicity of galactose induction of Cas9, the strains containing the p415-GAL-Cas9 plasmids were transformed as above and grown 48 h in YSC-Leu-His and 40 g/L of glucose. Cultures were then split equally in half, pelleted, and resuspended in an equal volume of YSC-Leu-His media containing either 20 g/L of glucose or 20 g/L of galactose. Cells were allowed to incubate at 30 °C for an additional 48 h before being diluted 1000× and plated onto YSC-Leu-Arg plates containing 20 g/L of glucose without canavanine. Colony counts were then compared between cells incubated in galactose or glucose.

Prior to statistical analysis, colony counts were $\log_{10}$ transformed in order to make the variances between them similar. Statistics were then done *via* one-way ANOVA in R followed by the Tukey's *posthoc* test. Statistical significance is indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. For the growth experiments, $n = 3$ independent biological replicates were run. For the editing efficiency experiment, $n = 4$ independent biological replicates were used. Significance was measured against the no sgRNA control, unless otherwise specified in the figure.

***CAN1* Editing in *Yarrowia lipolytica*.** *TRP1*-marked 8dTEF-T7PM linear DNA was isolated by NotI digestion of pUCS3-8dTEF-T7PM. The resulting T7 polymerase cassette was then cotransformed into *Y. lipolytica* PO1f Δ*TRP1* with either pCRISPRyl-T7pro-SpeI (untargeted) or pCRISPRyl-T7pro-CAN1 (*CAN1*-targeted) using the Frozen EZ II yeast transformation kit (Zymo Research). Transformations were plated on YSC-Leu-Trp, and plates were incubated for 3 days at 28 °C. All transformants were harvested YSC-Arg-Leu-Trp liquid media in a 14 mL culture tube, and the resulting high-OD cultures were incubated in a shaking incubator for 2 days at 28 °C/225 rpm. Cultures were then diluted and plated on YSC-Arg-Leu-Trp to isolate single colonies. 53 single colonies from these YSC-Arg-Leu-Trp plates and a single colony of PO 1f Δ*TRP1* negative control were then streaked onto YSC-Arg plates with 100 mg/L canavanine and incubated for 4 days at 28 °C. This experiment was completed in biological triplicate, starting from cotransformation. Randomly selected colonies from YSC-Arg plates with 100 mg/L canavanine and a single PO 1f Δ*TRP1* colony (wild-type *CAN1*) from a nonselective YSC-Arg plate were subjected to colony PCR and Sanger sequencing to confirm the induction of CRISPR-mediated indels at the gRNA-targeted sequence within *CAN1*.

***NDT80* Editing in *Kluyveromyces lactis*.** The *K. lactis* strain containing an integrated T7 polymerase P278/M5 mutant and Cas9 in the *LEU2* and *URA3* locus, respectively, was grown in YPD and made competent using a yeast lithium acetate transformation. Next, 1 μg of *KanMX* donor with 1000 bp of upstream and downstream homology to *NDT80* was added along with 250 ng of plasmid containing the sgRNA. After transformation, the cultures were allowed to recover for 3 h in YPD in a shaking incubator at 30 °C. Cells were then spun down and resuspended in 1 mL YSC-Ura media with 200 mg/L G418. 500 μL of this mixture was then plated onto YSC-Ura with 200 mg/L G418 agar plates and allowed to grow for 3−5 days at 30 °C. After this growth period, individual colonies were randomly picked for colony PCR confirmation of the

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integrated donor using one primer specific toward the cargo and one primer flanking the *NDT80* locus, such as to specifically identify homologous recombination events. Colony PCRs were performed by picking the colonies into 10 μL of 20 mM NaOH, 0.1% Triton solution. The colonies were then boiled at 100 °C for 20 min and 1 μL of sample was added to 19 μL of Q5 PCR master mix following manufacturer's instructions (New England Biolabs). This experiment was completed in biological triplicate, starting from cotransformation. For sequence confirmation of Cas9 generated indels in the *NDT80* locus, the same procedure was performed but without the addition of the linear donor.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00461.

Supplied are the oligo sequences used, sgRNA cloning scheme, sgRNA sequences, strain integration methods, qPCR protocol, and plasmid sequences (PDF)

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Author Contributions

N.M. and H.A. designed the study. N.M. designed the experiments, constructed the plasmids, and analyzed the data for the *Saccharomyces cerevisiae* experiments. J.W. designed the experiments, constructed the plasmids, and analyzed the data for the *Yarrowia lipolytica* testing. In addition, J.W. constructed the *Kluyveromyces lactis* strain containing Cas9 and offered valuable advice on working with and the design of experiments for *K. lactis*. K.R. designed, constructed, and tested the plasmids used for Cas9 editing in *K. lactis* and analyzed the data. M.G. ran the RT-qPCR experiment on the sgRNA levels for each of the T7 mutant variants and aided N.M. in the construction of the *Saccharomyces cerevisiae* plasmids. L.L. aided J.W. in the construction and testing of plasmids in the *Yarrowia lipolytica* experiments. N.M. and H.A. wrote the manuscript, and N.M., J.W., and K.R. put together the Supporting Information.

Notes

The authors declare no competing financial interest.

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