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Technical Note

Guide RNA Engineering Enables Dual Purpose CRISPR-Cpf1 for Simultaneous Gene Editing and Gene Regulation in *Yarrowia lipolytica*

Adithya Ramesh, Thomas Ong, Jaime A. Garcia, Jessica Adams, and Ian Wheeldon*

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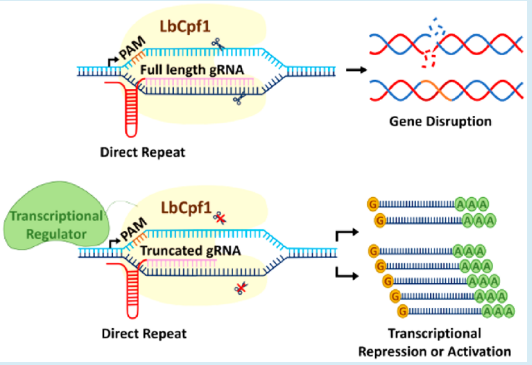
ABSTRACT: *Yarrowia lipolytica* has fast become a biotechnologically significant yeast for its ability to accumulate lipids to high levels. While there exists a suite of synthetic biology tools for genetic engineering in this yeast, there is a need for multipurposed tools for rapid strain generation. Here, we describe a dual purpose CRISPR-Cpf1 system that is capable of simultaneous gene disruption and gene regulation. Truncating guide RNA spacer length to 16 nt inhibited nuclease activity but not binding to the target loci, enabling gene activation and repression with Cpf1-fused transcriptional regulators. Gene repression was demonstrated using a Cpf1-Mxi1 fusion achieving a 7-fold reduction in mRNA, while CRISPR-activation with Cpf1-VPR increased hrGFP expression by 10-fold. High efficiency disruptions were achieved with gRNAs 23−25 bp in length, and efficiency and repression levels were maintained with multiplexed expression of truncated and full-length gRNAs. The developed CRISPR-Cpf1 system should prove useful in metabolic engineering, genome wide screening, and functional genomics studies.



The nonconventional dimorphic yeast *Yarrowia lipolytica* has attracted attention as an industrially relevant host due to its ability to utilize hydrocarbon and other nonsugar feedstocks as carbon sources for the production of high titers of intracellular lipids. Exploiting these phenotypes, metabolic engineers have designed strains that accumulate lipids to over 90% of yeast dry cell weight and titers as high as 85 g/L.[1,2] Modified fatty acid and lipid biosynthesis pathways have also been designed to produce commodity and high value chemicals such as long chain dicarboxylic acids, omega-3 fatty acids, and carotenoids among others.[3−7]

Y. lipolytica's maturation as a host for chemical biosynthesis is in part due to new genetic engineering tools. CRISPR-Cas9 genome editing has played a large part in accelerating metabolic engineering efforts in this and other microbes.[8−12] Targeted genome editing in Yarrowia and other nonconventional yeast is challenging because DNA repair is dominated by nonhomologous end joining, preventing the use of common synthetic biology tools that depend on the high capacity of *Saccharomyces cerevisiae* to perform homologous recombination.[13−16] CRISPR Cas9-based gene regulation and editing have helped mitigate this problem, but multiplexed and multifunctional synthetic biology tools for rapid strain engineering in Yarrowia are still needed. Cpf1, a family of Cas12a bacterial endonucleases, targets to genomic loci in a similar manner to Cas9 but has the advantage of processing its own CRISPR-RNA arrays.[17] The ability to mature its own guide RNAs (gRNAs) from a single transcript can be leveraged for easy multiplexing.[18] Cpf1 also benefits from a T-rich PAM sequence (TTTV) that does not overlap with Cas9 function, and, unlike Cas9, does not require a tracrRNA sequence, which shortens gRNA expression cassettes.[19]

Here, we demonstrate a dual function CRISPR-Cpf1 technology that simultaneously disrupts a gene target and regulates expression at other genomic loci. Length studies of Cpf1 gRNAs show that endonuclease function is lost with spacer sequences of 16 or less nucleotides (nt). We use this effect to control Cpf1 function by expressing guides of different lengths.

We first screened a series of Cpf1 orthologous from *Acidaminococcus spp.* BV3L6 (AsCpf1), *Lachnospiraceae bacterium* ND2006 (LbCpf1), and *Francisella novicida* U112 (FnCpf1). A single plasmid system containing both the Cpf1 and gRNA expression cassettes was used for gene disruption (Figure 1A). LbCpf1 showed the highest disruption efficiency in the preliminary screen (22 ± 5%; Figure S1) and was used for all subsequent experiments.

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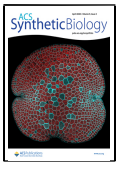

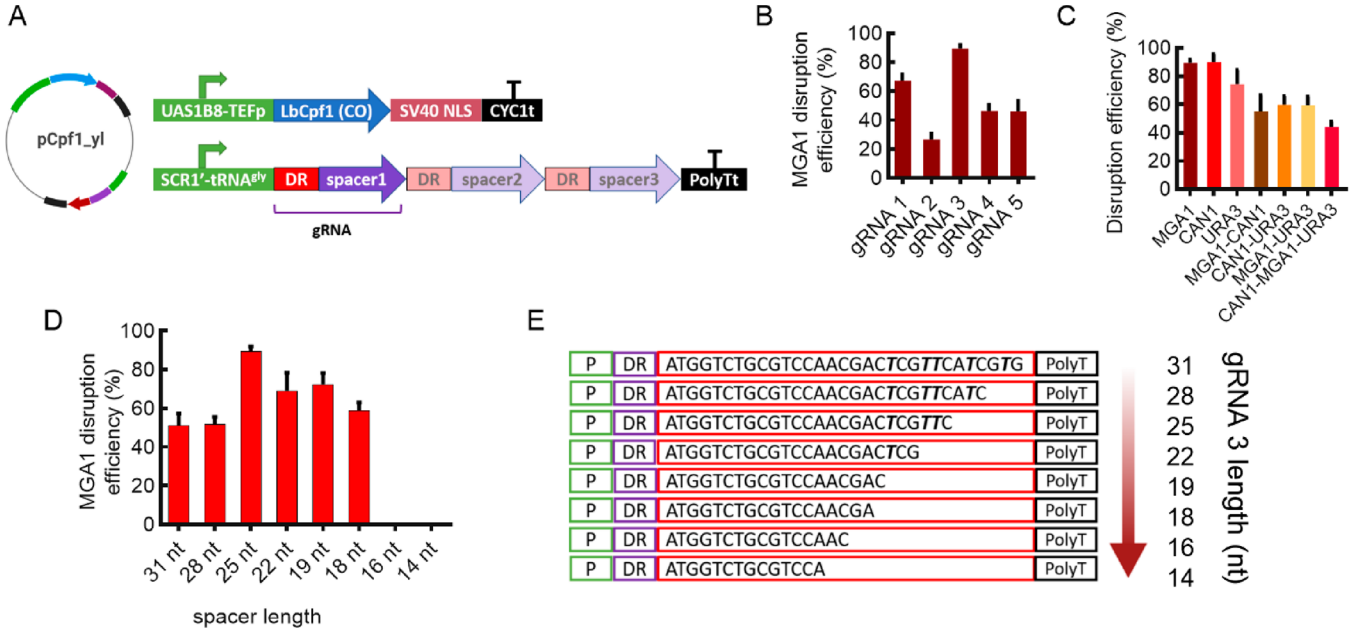

Figure 1. CRISPR-Cpf1 genome editing in *Yarrowia lipolytica*. (A) Schematic of the pCpf1_yl plasmid and expression cassettes for LbCpf1 and gRNA expression. Multiplexed cassettes are made by tiling direct repeat (DR) and spacer sequences. (B) Gene disruption efficiency for five different gRNAs targeting MGA1 in the PO1f strain of *Yarrowia lipolytica*. (C) Efficiency of double and triple disruptions of MGA1, CAN1, and URA3. (D) Effect of gRNA length on gene disruption efficiency, with guide sequences shown in (E). Thymine "T" nucleotides that are bolded and italicized indicate locations within each spacer where truncations were not made due to the presence of the polyT terminator. All *Y. lipolytica* transformants were grown in 2 mL of selective media in culture tubes at 30 °C. Data presented are mean and standard deviation of biological triplicates.

Three genes, MGA1, CAN1, and URA3, whose disruption produces an easily observed phenotype, were used to demonstrate and optimize multiplexed functionality. MGA1 knockout has been implicated in the suppression of pseudohyphal growth in yeast, and null mutants are easily identifiable by a smooth surface colony that is distinct from the wild type rough morphology.[20] CAN1 null mutants are resistant to L-canavanine, which is structurally similar to arginine and toxic to cell growth.[21] Finally, the URA3 gene that is responsible for the *de novo* synthesis of pyrimidines was selected as null mutants are auxotrophic for uracil and resistant to the Ura3 catalyzed product of 5-FOA.[22]

Five gRNAs were designed and tested for each of MGA1, CAN1, and URA3. The best gRNA for each gene achieved disruption efficiencies of 89.5 ± 2.5%, 90.0 ± 5.7%, and 74.4 ± 10% for MGA1, CAN1, and URA3, respectively, after 4 days of outgrowth (Figures 1B and S2). Other gRNAs produced lower disruption efficiencies, but all were successful in creating double stranded breaks in the genome. The observed sequence-dependence of gRNA on endonuclease activity has been demonstrated on a genome-wide scale in *Y. lipolytica* using Cas9.[20] The same study also shows that Cas9 activity is influenced by chromatin structure, specifically that the nucleosome occupancy can hinder cutting. We anticipate similar relationships with gRNA sequence and nucleosome occupancy with Cpf1.

The best LbCpf1 gRNAs for each of MGA1, CAN1, and URA3 were used in multiplexed format to generate dual and triple knockouts. ΔMGA1-ΔCAN1 dual knockouts were produced in 55 ± 11% of the observed colonies (30/60, 41/60, 28/60), while the ΔMGA1-ΔURA3 and ΔCAN1-ΔURA3 mutants were generated with 59 ± 6% and 60 ± 6% efficiency (34/60, 33/60, 40/60; 35/60, 40/60, 33/60; Figures 1C and S3). Creating the triple knockout in a single experiment was less efficient with disruption of all genes occurring only 44 ± 4% of the time (40/90, 43/90, 36/90). These results are on par with a recent study of AsCpf1 in Yarrowia.[23]

To characterize the effect of spacer length on LbCpf1 nuclease activity, the best gRNA for MGA1 and CAN1 were picked and the spacer length varied from 31 down to 14 nt. Expression of gRNAs with 23−25 nt spacers in the presence of active LbCpf1 resulted in the highest disruption efficiency for both MGA1 and CAN1. Endonuclease activity decreased in gRNAs longer than 25 and shorter than 23 (Figure 1D,E, S4, and S5). Most notably, cutting function sharply dropped with 14 and 16 nt spacers. None of the 90 screened colonies transformed with CRISPR plasmids expressing truncated spacers showed phenotypic changes associated with MGA1 and CAN1 disruptions, and when 5 colonies were genotyped, none showed the presence of edits. This gRNA length-dependent effect is also seen with Cas9, which requires spacer of at least 16 nt to show detectable levels of gene editing in human cells.[24]

Given the loss of Cpf1 endonuclease function at shorter spacer lengths and evidence that Cas9 binds target DNA but is not catalytically active with spacers 14 nt in length (see ref 24), we explored the possibility of using active LbCpf1 with a fused repressor domain and truncated gRNA as a CRISPR interference (CRISPRi) system.[25,26] If shortened gRNAs can still form a ribonuclear complex with Cpf1 and bind to the genome loci complementary to the spacer sequence, then the system should function as a site-specific gene repressor. Swapping a repressor domain for an activation domain creates a gene activation tool.

CRISPRi studies have shown that transcriptional repression is effective when the endonuclease-repressor fusion is targeted

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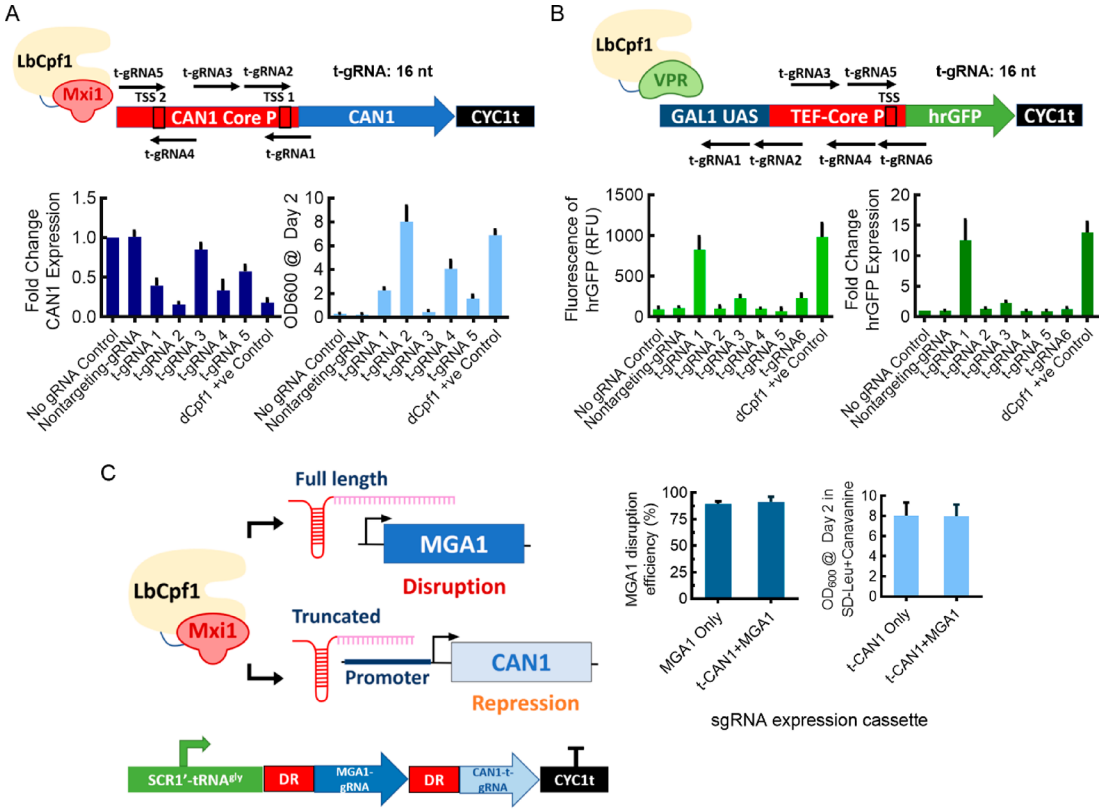

Figure 2. Truncated gRNAs enabled CRISPRa/i and dual functioning LbCpf1. (A) CRISPRi repression of CAN1 with truncated gRNAs and LbCpf1-Mxi1. Repression of CAN1 with t-gRNA1, -2, -4, and -5 enables growth in a canavanine challenge assay. qPCR confirms reduced CAN1 mRNA levels correspond with increased growth. (B) CRISPRa activation of hrGFP with truncated gRNAs and LbCpf1-VPR. hrGFP expression, as measured by flow cytometry from a TEF core promoter with GAL1 UAS is low. Activation by CRISPRa with t-gRNA1 increases GFP fluorescence and hrGFP mRNA level. Basal autofluorescence was subtracted from all reported fluorescence values. Results in A and B are compared to negative controls with no gRNA and a nontargeting gRNA, as well as a positive control of CRISPRi/a enabled by deactivated Cpf1 (dCpf1) and full length gRNAs. (C) Simultaneous gene disruption and transcriptional repression using LbCpf1-Mxi1. A dual gRNA expression system producing t-gRNA2 for CAN1 and a gRNA for MGA1 disruption effectively repressed CAN1 while editing MGA1. All *Y. lipolytica* transformants were grown in 2 mL of selective media in culture tubes at 30 °C. Data presented are mean and standard deviation of biological triplicates.

within ∼200 bp upstream of the transcription start site (TSS).[25−27] We identified the putative TSS for CAN1 with the help of the YeasTSS online tool[28] and designed a series of five truncated gRNAs (t-gRNAs) with spacers 16 nt in length that span a short region surrounding the TSS (Figure 2A). In the case of CAN1, two putative TSS's were identified and targeted. A canavanine growth challenge revealed that the coexpression of LbCpf1 and t-gRNA2 enabled cell growth with cultures reaching an OD_{600} of 8.0 ± 1.3 after 48 h, cell density significantly higher than the negative controls, one with no gRNA and a second with a scrambled gRNA that does not match a loci within the genome (OD_{600} = 0.29 ± 0.03 and 0.23 ± 0.04, respectively; comparison $p < 0.0001$, $n = 3$). In total, four out of five t-gRNAs (t-gRNA-1, -2, -4 and -5) showed a significant difference in growth to the negative controls ($p < 0.05$; $n = 3$). qPCR analysis of CAN1 transcript levels confirmed the repression effect, the two cultures that exhibited high resistance to canavanine (t-gRNA2 and -4) also had low levels of CAN1 mRNA with only 15.4 ± 3.1% and 33.5 ± 12.9% expression compared to the negative control. Importantly, sequencing of the region surrounding the targeted PAM sites revealed that endonuclease activity was not the cause of CAN1 downregulation (Figure S6). These results also compare well to a study of a deactivated FnCpf1-based CRISPRi study in *Y. lipolytica*.[29]

Our previous CRISPR activation (CRISPRa) studies with deactivated Cas9 fused to the synthetic transcriptional activator VPR also revealed that function varies with distance from the TSS.[30] Again, we used a series of t-gRNAs that span a region upstream of the gene of interest, in this case an engineered GFP expression cassette integrated at the XPR2 locus of *Y. lipolytica* PO1f (Figure 2B). Six t-gRNAs were designed that span ∼150 bp of the GAL1-TEF_{core} synthetic promoter that drives expression of the integrated cassette. CRISPR plasmids expressing one guide and LbCpf1 were transformed into PO1f and random colonies were selected for flow cytometry and qPCR analysis. A CRISPRa plasmid that expressed no gRNA, as well as one that expressed a nontargeting gRNA, were used as negative controls. One out of six gRNAs (t-gRNA1) showed significant activation at nearly 10-fold above the negative controls. None of the other gRNAs showed any appreciable levels of activation. Sequencing the regions surrounding the targeted PAM site for the best performer, revealed no edits (Figure S6).

For both the CRISPRi and CRISPRa studies, we also performed positive control experiments using deactivated LbCpf1 (Cpf1 D832A; dCpf1). In these experiments, dLbCpf1 was coexpressed with the full-length t-gRNAs that showed the best result for activation and repression. Random colonies were subjected to canavanine toxicity challenge (for CRISPRi), flow

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cytometry (for CRISPRa), and qPCR analysis. These experiments showed that our developed CRISPRi/a system that uses active Cpf1 and truncated gRNAs performs just as well traditional technologies (Figures 2A and 2B).

Together, the length study data and CRISPRi/a demonstrations show that LbCpf1 endonuclease activity can be controlled through gRNA expression. This presents the opportunity to create arrays of guides that target different gene editing functions (disruption, activation, and repression) to sites throughout the genome. To this end, we designed a dual function CRISPR-Cpf1 system by simultaneously expressing a full-length spacer for one gene, MGA1, and a truncated 16 nt spacer for a second, CAN1. The dual expression system was successful. After 2 days of outgrowth in selective media, cultures were subjected to a canavanine toxicity challenge, phenotyped, and genotyped for MGA1 disruption. Dual expression did not affect Cpf1 and CRISPRi function; MGA1 disruption occurred at 92.4 ± 6.1% efficiency and growth in the toxicity challenge was equivalent to the control (Figure 2C). We also note that Mxi1 and VPR fusion to LbCpf1 had no effect on nuclease activity with full length gRNAs (Figure S6).

In studying the effect of Cpf1 gRNA length on endonuclease activity we identified a switch point in function. Spacers 16 nt in length bind to the target site but do not produce double stranded breaks. Spacers greater than 16 nt and up to 31 nt activate LbCpf1 activity. These results are consistent with analyses of Cpf1 crystal structures. Specifically, that the 5′-stem loop of the direct repeat is necessary and sufficient for the formation of a ribonuclear complex, and that the endonuclease domains interact with the genomic target at the 23rd and the 18th positions of the spacer.[31−33] Given this, we speculate that gRNA shorter than 18 nt are unable to activate endonuclease activity but maintain sufficient homology to attach the ribonuclear complex to the locus of interest. Here, we leveraged this effect to express Cpf1 CRISPR-RNA arrays with gRNAs of different lengths, along with LbCpf1 fused to an activator for CRISPRa, or a repressor domain for CRISPRi, to enable multifunctional genome editing. Synthetic biology tools that enable rapid and multiplexed genome modifications are needed to overcome a bottleneck in nonconventional yeast strain engineering. The dual function CRISPR-Cpf1 system shown here adds to the tools needed to address this challenge.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00498.

> Methods; Cpf1 nucleotide sequences; yeasts strains, plasmids, and primers used in this study; initial screening LbCpf1 and FnCpf1 endonuclease activity in *Y. lipolytica*; MGA1, CAN1, and URA3 single and double disruptions and phenotypes; gRNA length study for the disruption of CAN1; sequence alignments of MGA1 and CAN1 targeted by various gRNA lengths; sequence alignments of CAN1 and MGA1 showing indels resulting from Cpf1 endonuclease activity; sequence alignments of CAN1 and hrGFP promoters targeted by truncated gRNAs; gene disruption efficiency effected LbCpf1 fusions with transcriptional regulators; method comparison to other CRISPR-Cpf1 tools in *Y. lipolytica* (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Ian Wheeldon − *Chemical and Environmental Engineering and Center for Industrial Biotechnology, Bourns College of Engineering, University of California Riverside, Riverside, California 92521, United States;* orcid.org/0000-0002-3492-7539; Email: iwheeldon@engr.ucr.edu

Authors

Adithya Ramesh − *Chemical and Environmental Engineering, University of California Riverside, Riverside, California 92521, United States*

Thomas Ong − *Chemical and Environmental Engineering, University of California Riverside, Riverside, California 92521, United States*

Jaime A. Garcia − *Department of Physics and Environmental Science, St. Mary's University, San Antonio, Texas 78228, United States*

Jessica Adams − *Molecular and Cell Biology Department, University of Connecticut, Storrs, Connecticut 06269, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.9b00498

Author Contributions

AR and IW conceived the study, analyzed the data, and wrote the paper. TO, JAG, JA, and AR conducted the experiments. All authors edited the manuscript.

Notes

The authors declare no competing financial interest.

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