Supporting Information

Synthetic RNA polymerase III promoters facilitate high efficiency CRISPR-Cas9 mediated genome editing in *Yarrowia lipolytica*

Cory M. Schwartz[1], Murtaza Shabbir Hussain[2], Mark Blenner[2], Ian Wheeldon[1,*]

1. Chemical and Environmental Engineering, University of California, Riverside
2. Chemical and Biomolecular Engineering, Clemson University

* Corresponding Author. Email: iwheeldon@engr.ucr.edu

Methods

Strains, media, and culturing

The *Yarrowia lipolytica* strain PO1f (*MatA, leu2-270, ura3-302,xpr2-322, axp-2*)[1] (ATCC no. MYA-2613) was used. Chemically competent DH5α *Escherichia coli* was used for plasmid propagation. *Y. lipolytica* was cultured in either YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or in synthetic complete media (SD) (0.67% Difco yeast nitrogen base without amino acids, 0.079% CSM (Sunrise Science, San Diego, CA), and 2% glucose). Transformants and disruptants of *Y. lipolytica* were selected and screened for on SD-leu, SD-ura, SD-leu-ura, and SD oleic acid (0.67% Difco yeast nitrogen base without amino acids, 0.079% CSM (Sunrise Science, San Diego, CA), 0.3% oleic acid, and 0.2% tween 40) agar plates. *E. coli* was grown in LB medium with 100mg/L ampicillin. *Y. lipolytica* was cultured at 28° C at 225 RPM in 14 mL polypropylene tubes.

Y. lipolytica transformations were performed using the protocol described by Yamane and coworkers[2], with the following modifications: 1) After the final dilution and centrifugation of transformed cells, the cells were resuspended in 200 µl water; and, 2) Half of this volume was plated on appropriate selective media agar plates, and half was used to inoculate a liquid culture of the appropriate selective media for 2 or 4 days. This period of outgrowth was performed by inoculating 2 mL of selective media with 100 mL of transformed cells. After outgrowth, serial dilutions were made and plated on YPD agar plates. A typical transformation was done with 1 µg of plasmid DNA and resulted in approximately $2x10^4$ transformants. Transformation of 1 µg of linear DNA yielded approximately 15 transformants. Growth curves of *Y. lipolytica* were done in SD media by inoculating 25 mL to an initial OD600 of 0.05 in a baffled 250 mL flask and incubating at 28° C at 225 RPM. Samples were removed and diluted (if necessary) and the

OD600 measured at various time points using a Beckman Coulter DU 800 Spectrophotometer (Brea, CA).

Plasmid construction and design

Codon optimized Cas9 was designed based on the sequence of Cas9 found in the p414-TEF1p-Cas9-CYC1t plasmid (Addgene #43802).[3] The amino acid sequence was used as input to Optimizer (http://genomes.urv.es/OPTIMIZER/)[4] with the codon usage table of the CLIB122 strain of *Y. lipolytica*. The resulting sequence was then manually altered, as shown in Figure S1, to allow for synthesis as 3 separate gBlocks by IDT of approximately 1.4kb each.

To generate each CRISPR plasmid, pUC-UAS1B8-TEF(136) (Addgene #44380) from Addgene (Cambridge, MA) was used to recreate pUAS1B8-TEF(136)-hrGFP.[5] This vector was digested with BssHII and NheI, and used with the three fragments of codon optimized Cas9 in a Gibson Assembly reaction.[6] The resulting vector was digested with AatII, and this digestion product was used with the respective sgRNA cassette in a Gibson Assembly reaction to yield the final CRISPR-Cas9 plasmid.

The TEF-HH ribozyme-sgRNA(PEX10)-HDV ribozyme-CYCt fragment was ordered from IDT as a gBlock. For the SNR52-sgRNA cassette, the SNR52 promoter was amplified from PO1f genomic DNA using Cr143 and Cr144 (Table S1). The sgRNA was generated by subjecting the PEX10 TEF gBlock to PCR with Cr141 and Cr142, and these two fragments were assembled into the digested Cas9 backbone via Gibson Assembly. For the $tRNA^{gly}$-sgRNA cassette, the $tRNA^{gly}$ was amplified from PO1f genomic DNA using Cr119 and Cr120. The sgRNA was generated by subjecting the PEX10 TEF gBlock to PCR with Cr129 and Cr130, and these two fragments were assembled into the digested Cas9 backbone via Gibson Assembly. For

the SNR52'-tRNA^{gly}-sgRNA cassette, the SNR52 promoter was amplified from PO1f genomic DNA using Cr143 and Cr146. The sgRNA was generated by subjecting the tRNA^{gly} plasmid to PCR with Cr145 and Cr130, and these two fragments were assembled into the digested Cas9 backbone via Gibson Assembly. For the RPR1'-tRNA^{gly}-sgRNA cassette, the RPR1 promoter was amplified from PO1f genomic DNA using Cr147 and Cr148. The sgRNA was generated by subjecting the tRNA^{gly} plasmid to PCR with Cr145 and Cr130, and these two fragments were assembled into the digested Cas9 backbone via Gibson Assembly. For the SCR1'-tRNA^{gly}-sgRNA cassette, the SCR1 promoter was amplified from PO1f genomic DNA using Cr149 and Cr150. The sgRNA was generated by subjecting the tRNA^{Gly} plasmid to PCR with Cr145 and Cr130, and these two fragments were assembled into the digested Cas9 backbone via Gibson Assembly. The TEF-ribozyme-sgRNA(KU70)-ribozyme-CYCt cassette was ordered from IDT as a gBlock. The SCR1'- tRNA^{gly}-sgRNA(KU70) plasmid was generated from Gibson assembly of a PCR fragment of the SCR1'- tRNA^{gly}-sgRNA(PEX10) plasmid with primers Cr166 and Cr167 and a PCR fragment from amplifying the same template with Cr164 and Cr165 and the digested Cas9 expressing backbone. The SCR1'- tRNA^{gly}-sgRNA(MFE1) was generated in the same way, but with Cr189 replacing Cr167. The linear fragment for *PEX10* disruption was generated by Gibson Assembly of three PCR products; PO1f genomic DNA amplified with Cr111 and Cr112, PO1f genomic DNA amplified with Cr113 and Cr114, and *Y. lipolytica* W29 genomic DNA amplified with Cr115 and Cr116. This fragment was further PCR amplified using Cr111 and Cr114. The pCRISPRyl plasmid was constructed by subjecting the SCR1'- tRNA^{gly}-sgRNA(PEX10) plasmid to PCR by both Cr166 and Cr203 and by Cr164 and Cr165 in separate reactions, and assembling these fragments with the AatII digested Cas9 backbone. The SCR1'-tRNA^{gly}-sgRNA(PEX10 scrambled) plasmid was generated by digesting pCRISPRyl with AvrII,

annealing primers Cr241 and Cr242, and Gibson assembling the resulting fragment into the digested pCRISPRyl.

The homology donor plasmid was achieved by Gibson assembly of 6 fragments. The first two fragments were generated by amplifying pUC-UAS1B8-TEF(136) with Cr224 and Cr231 to generate the backbone, and Cr221 and Cr226 to amplify the Cyc terminator. The third fragment was generated by ordering a gBlock encoding a codon optimized version of a hygromycin phosphotransferase. The remaining 3 fragments were obtained by PCR of PO1f genomic DNA using Cr225 and Cr219 to amplify the TEF promoter, Cr228 and Cr232 for 1kb homology upstream of the *MFE1* CRISPR cleavage site, and Cr229 and Cr230 for 1kb homology downstream of the *MFE1* CRISPR cleavage site. The homology regions were designed in such a way that successful recombination would remove the PAM sequence, and thus avoid further cleavage by Cas9.

Design of 20 nucleotide base pairing sequences was done using several tools. The sequence of the gene to be disrupted was submitted to the sgRNA design tool hosted by the Broad Institute (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design), and the highest scoring sgRNAs were selected for further analysis.[7] Only sgRNAs targeting early in the gene were selected. The final 12 nucleotides of each sgRNA, as well as 3 downstream bases representing the PAM sequence, were then used as an input in a BLAST search on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the whole *Y. lipolytica* genome to ensure no targeting occurred at an undesired location. Each sgRNA was also checked for secondary structure using the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi); those with high levels of secondary structure were discarded.[8] All polymerases, dNTPs, buffers, and

Gibson Assembly Master Mix were ordered from New England Biolabs (Ipswich, MA) and all primers and gBlocks were ordered from Integrated DNA Technologies (Coralville, IA).

Screening for gene disruption and HR

To screen transformants for disruption of the *PEX10* or *MFE1* gene, colonies of interest were randomly selected and streaked onto both YPD and SD oleic media. Growth on YPD but not SD oleic after 2 days at 30°C was taken to indicate gene disruption. For disruption of the *KU70* gene, randomly selected colonies were subjected to colony PCR using primers Cr029 and Cr030, and the product subjected to Sanger sequencing by Genewiz (South Plainfield, NJ). An identified *KU70* disruptant was used as the PO1f *ku70* strain in future experiments. Further confirmation of some of the *MFE1* disruptants was obtained by colony PCR of colonies of interest with Cr084 and Cr085, with subsequent Sanger sequencing of the product. To screen for integration of the HPH cassette into the *MFE1* gene, genomic DNA of selected colonies was isolated using the Yeastar Genomic DNA Kit from Zymo Research (Irvine, CA) after overnight growth in 2ml YPD media. Genomic DNA was subjected to PCR using primers Cr216 and Cr217, and the size of the product was checked on an agarose gel. Each experiment was performed in independent biological triplicates. When agar plates were used for screening, 30 colonies from each replicate were analyzed. When isolation of genomic DNA and subsequent PCR of the gene of interest was used as a screen, between 5 and 8 of each replicate were screened. Error bars were generated from the standard deviation of the three replicates in each case.

Quantitative Reverse Transcription PCR (qRT-PCR)

Y. lipolytica transformants were grown in triplicates in SD liquid media as described above. After 24 hours of growth, cells were re-inoculated into fresh 2 ml SD media at an OD600 of 0.2 and allowed to grow until cultures reached an OD600 of 10, marking early exponential phase. The cells were then harvested and total RNA was extracted using the E.Z.N.A. Yeast RNA kit (Omega Biotek). RNA extracts were placed in aliquots and stored at -80°C until further use. For absolute RT-qPCR, a two-step protocol was employed. 700 ng of total RNA was used for first-strand cDNA synthesis. Oligo(dT) priming was used with the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific). 2uL from the cDNA synthesis mix was subject to qPCR with Maxima SYBR Green/ Fluorescein qPCR master mix (Thermo Scientific). Real time amplification was performed using the CFX Connect Real-Time (Bio-rad). The primers Cr243 and Cr244 were used for amplification. A standard curved was developed using linearized vector containing the gRNA coding sequence to relate C_T values to copy number. The standard curve was used to calculate mRNA copy numbers of qPCR analyzed samples. Actin was used as an internal control gene. Actin amplification was accomplished using the primers Cr245 and Cr246.

Nucleotide sequence of *Y. lipolytica* codon optimized Cas9-SV40

Red underlined bases indicate variation from output of the Optimizer web server[4], to allow synthesis by IDT.

ATGGATAAGAAATACTCCATTGGCCTGGACATCGGAACCAACTCCGTGGGTTGGGCCGTGATCA
CCGATGAGTACAAGGTGCCCTCTAAGAAATTCAAGGTCCTGGGCAACACCGACCGACACTCCAT
CAAGAAGAACCTGATCGGCGCTCTGCTCTTCGACTCTGGCGAGACCGCTGAGGCCACCCGACTG
AAGCGAACCGCTCGAAGACGATACACCCGAAGAAAGAACCGAATCTGTTACCTGCAGGAGATCT
TCTCTAACGAGATGGCCAAGGTGGACGACTCTTTCTTCCACCGACTGGAGGAGTCTTTCCTGGT
GGAGGAGGACAAGAAGCACGAGCGACACCCCATCTTCGGCAACATCGTGGACGAGGTGGCCTAC
CACGAGAAGTACCCCACCATCTACCACCTGCGAAAGAAGCTGGTGGACTCTACCGACAAGGCCG
ACCTGCGACTGATCTACCTGGCCCTGGCCCACATGATCAAGTTCCGAGGCCACTTCCTGATCGA
GGGCGACCTGAACCCCGACAACTCTGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTAC
AACCAGCTCTTCGAAGAGAACCCCATTAACGCTTCTGGCGTGGATGCTAAGGCCATCCTGTCTG
CCCGACTGTCTAAGTCTCGACGACTCGAGAACCTGATTGCTCAGCTCCCCGGAGAGAAGAAGAA
CGGTCTGTTCGGAAACCTGATTGCTCTGTCCCTGGGTCTCACCCCTAACTTCAAGTCCAACTTC
GATCTGGCTGAGGACGCTAAGCTGCAGCTGTCTAAGGACACCTACGACGATGACCTGGATAACC
TGCTCGCCCAGATTGGCGACCAGTACGCCGACCTGTTCCTGGCCGCCAAGAACCTGTCTGACGC
CATCCTGCTGTCTGACATCCTGCGAGTGAACACCGAGATCACCAAGGCCCCCCTGTCTGCCTCC
ATGATTAAGCGATACGATGAGCACCACCAGGATCTGACCCTCCTCAAGGCTCTGGTCCGACAGC
AGCTGCCCGAGAAGTACAAGGAGATTTTCTTCGACCAGTCTAAGAACGGCTACGCCGGCTACAT
CGACGGCGGCGCCTCTCAGGAGGAGTTCTACAAGTTCATTAAGCCCATCCTGGAGAAGATGGAC
GGAACCGAGGAACTGCTCGTGAAGCTGAACCGAGAGGACCTCCTGCGAAAGCAGCGAACCTTCG
ACAACGGCTCTATCCCCCACCAGATCCACCTGGGCGAGCTGCACGCCATCCTGCGACGACAGGA
GGACTTCTACCCCTTCCTGAAGGACAACCGAGAGAAGATCGAGAAGATCCTGACCTTCCGAATC
CCCTACTACGTGGGACCCCTGGCCCGAGGAAACTCTCGATTCGCTTGGATGACCCGAAAGTCTG
AGGAGACCATTACCCCCTGGAACTTCGAGGAGGTGGTGGATAAGGGCGCCTCTGCTCAGTCTTT
CATCGAGCGAATGACCAACTTCGACAAGAACCTCCCCAACGAGAAGGTCCTGCCCAAGCACTCT
CTGCTCTACGAGTACTTCACCGTCTACAACGAGCTCACCAAGGTCAAGTACGTGACCGAGGGAA
TGCGAAAGCCCGCTTTCCTGTCTGGAGAGCAGAAGAAGGCTATTGTGGATCTGCTCTTCAAGAC
TAACCGAAAGGTCACCGTCAAGCAGCTGAAGGAGGATTACTTCAAGAAGATTGAGTGTTTCGAT
TCTGTCGAGATCTCCGGCGTCGAGGACCGATTCAACGCCTCTCTGGGTACCTACCACGACCTGC
TGAAGATTATCAAGGACAAGGATTTCCTGGATAACGAGGAGAACGAGGATATTCTCGAGGACAT
TGTCCTGACCCTCACCCTGTTCGAGGATCGAGAGATGATTGAGGAGCGACTCAAGACCTACGCT
CACCTGTTCGACGACAAGGTGATGAAGCAGCTGAAGCGACGACGATACACCGGCTGGGGCCGAC
TGTCTCGAAAGCTGATCAACGGCATCCGAGACAAGCAGTCTGGCAAGACCATCCTGGACTTCCT
GAAGTCTGACGGCTTCGCCAACCGAAACTTCATGCAGCTGATCCACGACGACTCTCTGACCTTC
AAGGAGGACATCCAGAAGGCCCAGGTGTCTGGCCAGGGCGACTCTCTGCACGAGCACATCGCCA
ACCTGGCCGGCTCTCCCGCCATTAAGAAAGGTATCCTGCAGACCGTCAAGGTGGTCGATGAGCT
CGTCAAGGTGATGGGCCGACACAAGCCCGAGAACATTGTCATTGAGATGGCTCGAGAGAACCAG
ACTACTCAGAAGGGTCAGAAAAACTCCCGAGAGCGAATGAAGCGAATTGAGGAAGGTATTAAGG
AGCTGGGATCCCAGATTCTCAAGGAGCATCCCGTGGAGAACACTCAGCTCCAGAACGAGAAGCT
GTACCTGTACTATCTGCAGAACGGTCGAGACATGTACGTCGACCAGGAGCTGGATATCAACCGA
CTCTCCGACTACGATGTGGACCACATTGTGCCCCAGTCCTTCCTGAAGGACGATTCTATCGATA
ACAAGGTGCTGACCCGATCCGACAAGAACCGAGGCAAGTCTGACAACGTGCCCTCCGAGGAGGT

GGTCAAGAAGATGAAGAACTACTGGCGACAGCTGCTGAACGCCAAGCTGATTACCCAGCGAAAG
TTCGACAACCTGACCAAGGCCGAGCGAGGCGGCCTGTCTGAGCTGGACAAGGCCGGCTTCATCA
AGCGACAGCTGGTGGAGACCCGACAGATCACCAAGCACGTGGCCCAGATCCTGGACTCTCGAAT
GAACACCAAGTACGACGAGAACGACAAGCTGATCCGAGAGGTGAAGGTGATCACCCTGAAGTCT
AAGCTGGTGTCTGACTTCCGAAAGGACTTCCAGTTCTACAAGGTGCGAGAGATTAACAACTACC
ACCACGCCCACGATGCCTACCTGAACGCTGTCGTGGGCACCGCCCTCATCAAGAAGTATCCCAA
GCTGGAGTCCGAGTTCGTCTACGGCGACTACAAGGTCTACGATGTGCGAAAAATGATTGCCAAG
TCCGAGCAGGAGATTGGCAAGGCTACCGCCAAGTACTTCTTCTACTCCAACATTATGAACTTCT
TCAAGACCGAGATTACCCTGGCTAACGGCGAGATTCGAAAGCGACCCCTCATTGAGACCAACGG
AGAGACCGGTGAGATCGTGTGGGACAAGGGACGAGACTTCGCCACCGTGCGAAAGGTGCTGTCT
ATGCCCCAGGTGAACATCGTGAAGAAGACCGAGGTGCAGACCGGAGGTTTCTCTAAGGAGTCCA
TCCTGCCCAAGCGAAACTCTGACAAGCTGATCGCCCGAAAGAAGGACTGGGACCCCAAGAAGTA
CGGAGGTTTCGACTCTCCCACCGTGGCTTACTCTGTGCTGGTGGTGGCCAAGGTGGAGAAGGGC
AAGTCTAAGAAGCTGAAGTCTGTGAAGGAGCTGCTGGGCATTACCATCATGGAGCGATCTTCTT
TCGAGAAGAACCCCATTGACTTCCTGGAGGCCAAGGGATACAAGGAGGTGAAGAAAGATCTGAT
TATCAAGCTCCCCAAGTACTCTCTGTTCGAGCTGGAGAACGGACGAAAGCGAATGCTGGCCTCT
GCCGGCGAGCTGCAGAAGGGAAACGAGCTGGCCCTGCCCTCCAAGTACGTCAACTTCCTGTACC
TCGCCTCCCATTACGAGAAGCTGAAGGGCTCTCCCGAGGATAACGAGCAGAAGCAGCTCTTCGT
GGAGCAGCATAAGCACTACCTGGACGAGATCATCGAGCAGATCTCTGAGTTCTCTAAGCGAGTG
ATCCTGGCTGACGCCAACCTGGATAAGGTGCTGTCTGCTTACAACAAGCACCGAGACAAGCCCA
TTCGAGAGCAGGCTGAGAACATCATTCACCTGTTCACCCTGACCAACCTGGGAGCCCCCGCTGC
CTTCAAGTACTTCGACACCACCATCGACCGAAAGCGATACACCTCTACCAAGGAGGTGCTGGAC
GCCACCCTGATCCACCAGTCTATCACCGGCCTGTACGAGACCCGAATCGACCTGTCTCAGCTGG
GCGGCGACTCTCGAGCCGACCCCAAGAAGAAGCGAAAGGTG

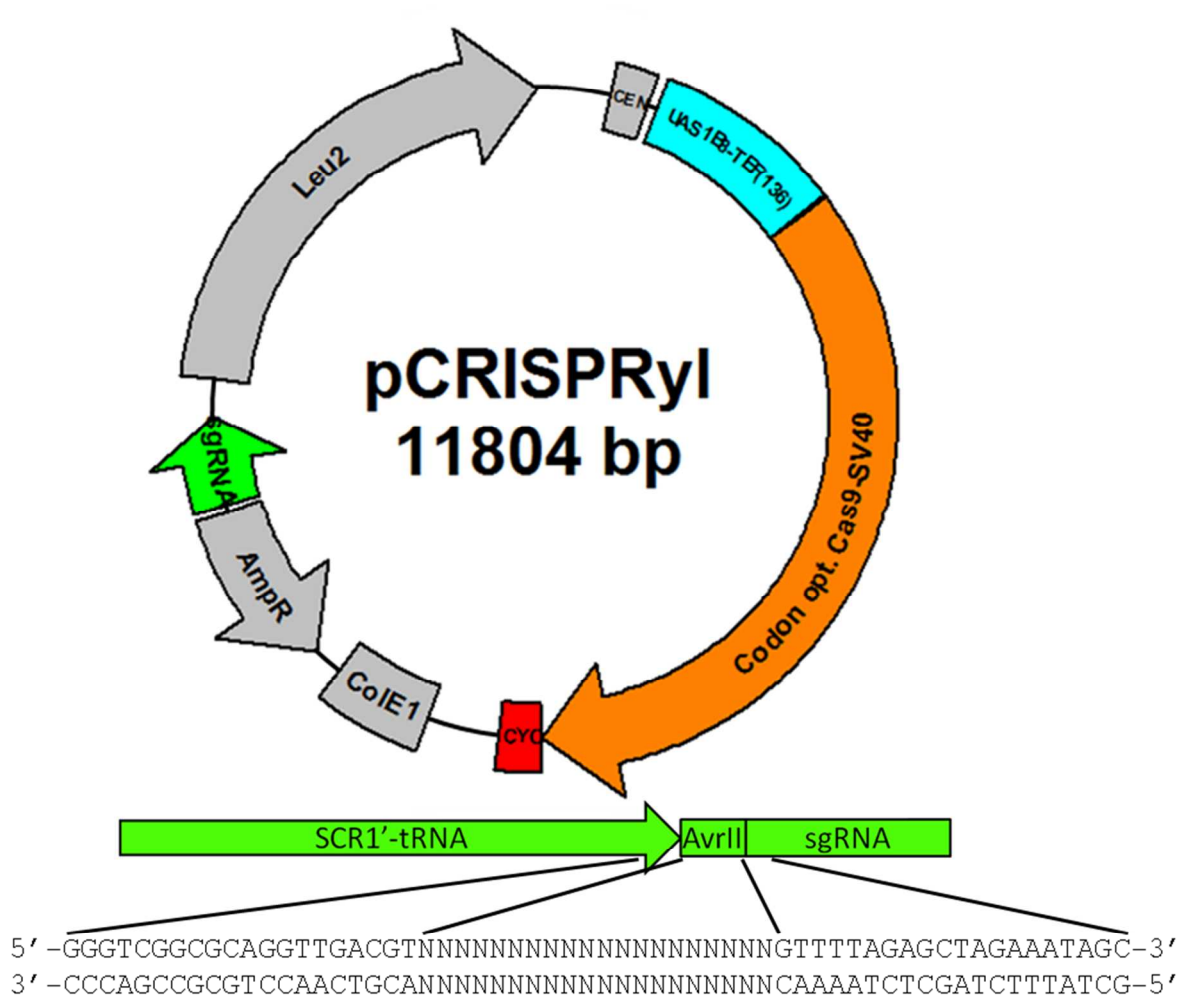


Figure S1. Sequence of *Y. lipolytica* codon optimized Cas9 and plasmid map of pCRISPRyl and cloning site for sgRNA insertion. To insert new sgRNA, digest pCRISPRyl with AvrII and Gibson Assemble 60 bp fragment with desired sgRNA in place of 20 "N".

```
PO1f        GTACAAGGAGGAGCTGGA----------------------GACGGCGTCC
Sample 1    GTACAAGGAGGAGCGATCTCGTATCCAATAGCTCGTTTATAGACACGTCC
Sample 2    GTACAAGGAGGAGCTGG-----------------------GACGGCGTCC
Sample 3    GTACAAGGAGGAGCTGGA----------------------GACGGCGTCC
```

Figure S2. Alignment of sequencing results for screening *PEX10* disruption. Samples 1 and 2 failed to grow on SD oleic media, while sample 3 did grow. Red indicates variation from native PO1f sequence.

TEF sgRNA (PEX10) expression cassette

AGACCGGGTTGGCGGCGCATTTGTGTCCCAAAAAACAGCCCCAATTGCCCCAATTGACCCCAAA
TTGACCCAGTAGCGGGCCCAACCCCGGCGAGAGCCCCCTTCTCCCCACATATCAAACCTCCCCC
GGTTCCCACACTTGCCGTTAAGGGCGTAGGGTACTGCAGTCTGGAATCTACGCTTGTTCAGACT
TTGTACTAGTTTCTTTGTCTGGCCATCCGGGTAACCCATGCCGGACGCAAAATAGACTACTGAA
AATTTTTTTGCTTTGTGGTTGGGACTTTAGCCAAGGGTATAAAAGACCACCGTCCCCGAATTAC
CTTTCCTCTTCTTTTCTCTCTCTCCTTGTCAACTCACACCCGAAATCGTTAAGCATTTCCTTCT
GAGTATAAGAATCATTCAAACCGCCTTGTACCTGATGAGTCCGTGAGGACGAAACGAGTAAGCT
CGTCGTACAAGGAGGAGCTGGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
CGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCATGGTCCCAGCCTCCTC
GCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGAC

SNR52 sgRNA (PEX10) expression cassette

GACCCCGTCTTCAATTACACTTCCCAACTGGGAACACCCCTCTTTATCGACCCATTTTAGGTAA
TTTACCCTAGCCCATTGTCTCCATAAGGAATATTACCCTAACCCACAGTCCAGGGTGCCCAGGT
CCTTCTTTGGCCAAATTTTAACTTCGGTCCTATGGCACAGCGGTAGCGCGTGAGATTGCAAATC
TTAAGGTCCCGAGTTCGAATCTCGGTGGGACCTAGTTATTTTTGATAGATAATTTCGTGATGAT
TAGAAACTTAACGCAAAATAATGACGTGTACAAGGAGGAGCTGGAGAGTTTTAGAGCTAGAAAT
AGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT
TTT

tRNA^{Gly} sgRNA (PEX10) expression cassette

TGAAAAATACCTCTAATGCGCCGATGGTTTAGTGGTAAAATCCATCGTTGCCATCGATGGGCCC
CCGGTTCGATTCCGGGTCGGCGCAGGTTGACGTGTACAAGGAGGAGCTGGAGAGTTTTAGAGCT
AGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
CTTTTTTTT

RPR1'- tRNA^{Gly} sgRNA (PEX10) expression cassette

TTCATTACAGCTATATACCTAGTAAGCCGGGTTATTGGCGTTCAATAAATCATACACTTCTGAA
TCTTTGATTACAGTCATTCCTGACGACTGTCCGATGAAACGGCCTAAAAAAGTTAAAATGTCGG
GAAATTTAGCTCTGGCCTAACGGTCAGACTGCAGTTACCCGGCCTCTCCCTCTGGCCTCGCGTA
CCTGGCTACACAAGACTGCGCTCTGCAGTTCGAATCCAGGTGGGAAATTCGGTG*TGAAAAATAC*
CTCTAATGCGCCGATGGTTTAGTGGTAAAATCCATCGTTGCCATCGATGGGCCCCCGGTTCGAT
***TCCGGGTCGGCGCAGGTT*GACGT**GTACAAGGAGGAGCTGGAGAGTTTTAGAGCTAGAAATAGCA
AGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTT

SCR1'- tRNA^{Gly} sgRNA (PEX10) expression cassette

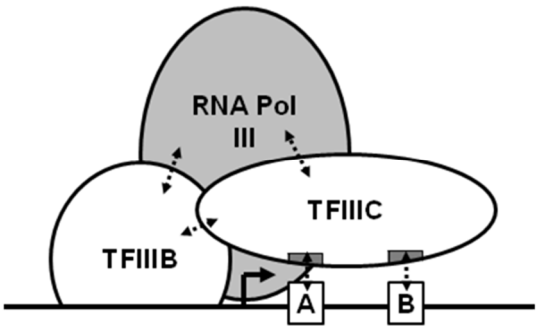
CCCCAGTTGCAAAAGTTGACACAACTCTAGATCTGCTTCCAAATATAGAATCATAACAAGGGTT
AGGGTGTGATTATATAATATTGGTCTTAATTGATGTGCTAGGGCTTTAAAAGTTGGTTAAAATA
ACGCTCTAATGCCTTTTTAATATATTGTCTTTTTCAAAATCTCAAATCGGACACTTCTTCGTGT
ATGAGACTCCATTTTTTGGCTCCGTCACGTGATATGTATTATCAGCTATAGTGGTGTAAACAAA
GTTTTTTACTAGCTGTAATGGCATTTTGTCGGAGTGGTAAATCGCCTTCTTGTTGTGCGTTCGA

GTTCTGGACTCTGCACTGGGCTACTT*TGAAAAATACCTCTAATGCGCCGATGGTTTAGTGGTAA*
***AATCCATCGTTGCCATCGATGGGCCCCCGGTTCGATTCCGGGTCGGCGCAGGTT*GACGT**GTACA
AGGAGGAGCTGGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAA
CTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTT

SNR52'- tRNA^{Gly} sgRNA (PEX10) expression cassette

GACCCCGTCTTCAATTACACTTCCCAACTGGGAACACCCCTCTTTATCGACCCATTTTAGGTAA
TTTACCCTAGCCCATTGTCTCCATAAGGAATATTACCCTAACCCACAGTCCAGGGTGCCCAGGT
CCTTCTTTGGCCAAATTTTAACTTCGGTCCTATGGCACAGCGGTAGCGCGTGAGATTGCAAATC
TTAAGGTCCCGAGTTCGAATCTCGGTGGGACCTAGT*TGAAAAATACCTCTAATGCGCCGATGGT*
TTAGTGGTAAAATCCATCGTTGCCATCGATGGGCCCCCGGTTCGATTCCGGGTCGGCGCAGGTT
GACGTGTACAAGGAGGAGCTGGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGT
CCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTT

Figure S3. Nucleotide sequences sgRNA expression cassettes targeting *PEX10*. For the TEF promoter red indicates the hammerhead ribozyme and blue indicates the HDV ribozyme. For the Pol III promoters, red indicates the A box and blue indicates the B box. The underlined sections indicate the sgRNA, and the bold section is the promoter. Italics indicate the tRNA^{Gly} region in synthetic Pol III promoters.



Promoter	A box	B box	ΔA-B	ΔB-polyT
SNR52	112	70	33	170
tRNA	62	21	32	121
RPR1'-tRNA	192	115	68	215
SNR52'-tRNA	153	112	33	212
SCR1'-tRNA	150	120	21	220

Figure S4. Schematic of Pol III binding and Pol III promoter dimensions. Schematic representation of RNA polymerase III recruitment by TFIIIC transcription factor interacting with with the promoter's A and B boxes. (B) Promoter A and B box characteristics including distance from end of box to beginning of sgRNA, number of bases between different promoter elements,and the distance from the end of the B box to the polyT terminator. Data is shown for the first set of A and B boxes within a synthetic promoter.

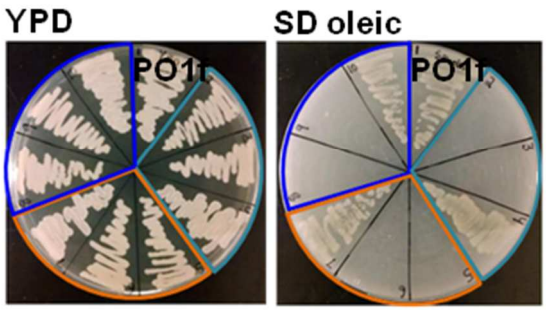


Figure S5. Phenotype of *PEX10* disruptants. Example plates screening of *PEX10* disrupted phenotypes on YPD and SD oleic acid media. PO1f is shown as a control, light blue indicates RPR1'-tRNA^{gly}, orange indicates SNR52'-tRNA^{gly}, and SCR1'-tRNA^{gly} is shown in dark blue.

PEX10 sgRNA	GTACAAGGAGGAGCTGGAGA
Scrambled sgRNA	GTACAAGGAGGAGCGGATAG

Figure S6. Scrambled *PEX10* sgRNA. The active *PEX10* sgRNA is shown aligned with the negative control sgRNA, in which the last 6 nucleotides are rearranged. When the scrambled sgRNA was used in *PEX10* disruption experiments, no disruptions of *PEX10* were identified.

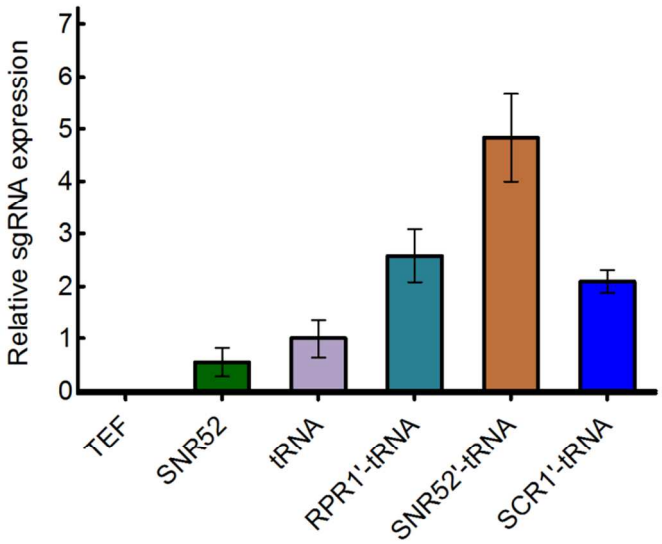


Figure S7. qPCR showing sgRNA levels from each promoter. Total RNA was isolated, converted to cDNA, and sgRNA expression was quantified. Data presented is sgRNA copy number found via qPCR normalized to the amount of sgRNA generated by the $tRNA^{Gly}$ promoter, and represents the results of three biological replicates harvested at an OD600 of 10 (mean ± standard deviation; n = 3). Actin was used as internal control.

sgRNA promoter	*ku70* disruption efficiency
SCR1`-tRNA	100% (14/14)

Figure S8. Disruption of *KU70*. Disruption of *KU70* was screened for by amplifying a section of the gene via PCR and Sanger sequencing the resulting fragment. Representative sequences are shown aligned, with red indicating variation from the sequence of naïve PO1f.

Strain	HR % (Oleic$^-$/Ura$^+$)
PO1f	16% (7/45)
PO1f *ku70*	86% (30/35)

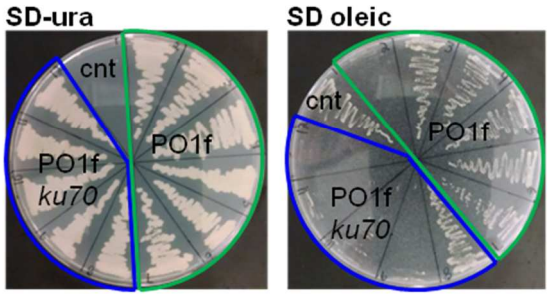


Figure S9. Confirmation of *KU70* disruption phenotype. Fraction of total screened transformants that integrated the linear URA3 cassette with flanking homology via HR, resulting in an Oleic$^-$/Ura$^+$ phenotype. Plates showing streaking on both SD-ura and SD oleic media are shown. "cnt" is the naïve PO1f strain, "PO1f" is PO1f transformed with the linear donor after selection on SD-ura media, and "PO1f *ku70*" is PO1f with *KU70* disrupted transformed with the linear donor. Plates show typical results of screening.

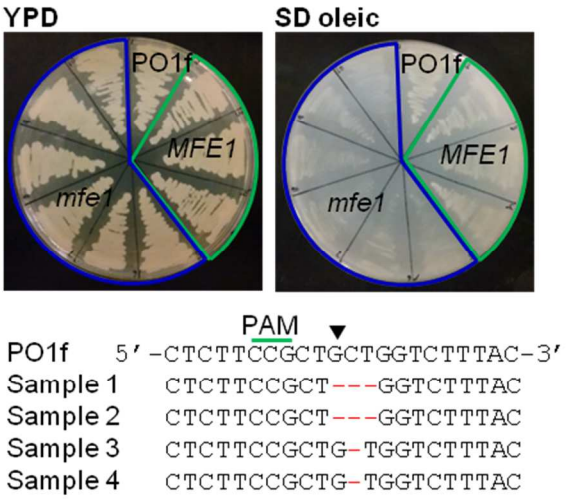


Figure S10. Disruption of *MFE1*. Suspected disruptants of *MFE1* were streaked onto both YPD and SD oleic acid. Those in green have functional *MFE1*, while those in blue have an indel in the *MFE1* gene rendering it inactive. The PO1f strain is streaked as a control. Representative sequencing results of *MFE1* disruption are shown as well, with red indicating variation from the naïve PO1f strain.

Table S1. Growth rates of strains used. The growth rate, calculated during exponential growth phase, of the 4 strains used in this study. Each growth curve was done in triplicate, and the growth rates were averaged and are presented below. The growth rate of each disruption strain was compared to the naïve PO1f strain using a T-test, and none were found to have a statistically significant difference.

Strain	Growth rate (h^{-1})
PO1f	0.421 ± 0.015
PO1f *ku70*	0.361 ± 0.059
PO1f *mfe1*	0.356 ± 0.071
PO1f *pex10*	0.375 ± 0.093

Table S2. Sequences of primers used in this study

Primer name	Primer sequence (5' to 3')
Cr029	GGAAAATTCAGATCAGATTTGAGAGCAAAGTCCAAC
Cr030	TCAATTCGAACTCGTGTCTTGTTGATATCGTC
Cr084	CAAGGCTGTTGCTAACTACGACTC
Cr085	CTCCTTGGCGAGAGTCTCAC
Cr111	GCTAAAAAAGGGAATATCGGAAATC
Cr112	GCTCCTCCTTGTACTGGTGG
Cr113	CGTCCAAGTTTGCATATCTCG
Cr114	CTCATATCTCCAATGCTAAAATATATTCATG
Cr115	TACGATTTATCCACCAGTACAAGGAGGAGCGTCGACGAGTATCTGTCTGACTC
Cr116	TACACAAACCGAGATATGCAAACTTGGACGGTCGACAAAGGCCTGTTTCTC
Cr119	CACATTTCCCCGAAAAGTGCCACCTGACGTGAAAAATACCTCTAATGCGCCG
Cr120	AATGTCATGATAATAATGGTTTCTTAGACGTCAACCTGCGCCGACCC
Cr129	GATTCCGGGTCGGCGCAGGTTGACGTGTACAAGGAGGAGCTGGAGAG
Cr130	GTCATGATAATAATGGTTTCTTAGACGTAAAAAAAAGCACCGACTCGGTG
Cr141	GTGATGATTAGAAACTTAACGCAAAATAATGACGTGTACAAGGAGGAGCTG
Cr142	ATGTCATGATAATAATGGTTTCTTAGACGTAAAAAAAAGCACCGACTCGGTG
Cr143	CACATTTCCCCGAAAAGTGCCACCTGACGTGACCCCGTCTTCAATTACACTTC
Cr144	ATTATTTTGCGTTAAGTTTCTAATCATCAC
Cr145	TGAAAAATACCTCTAATGCGCC
Cr146	AAACCATCGGCGCATTAGAGGTATTTTTCAACTAGGTCCCACCGAGATTCG
Cr147	CACATTTCCCCGAAAAGTGCCACCTGACGTTCATTACAGCTATATACCTAGTAAGCCGGG
Cr148	AAACCATCGGCGCATTAGAGGTATTTTTCACACCGAATTTCCCACCTGG
Cr149	CACATTTCCCCGAAAAGTGCCACCTGACGTCCCCAGTTGCAAAAGTTGACAC
Cr150	AAACCATCGGCGCATTAGAGGTATTTTTCAAAGTAGCCCAGTGCAGAGTCC
Cr164	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
Cr165	GGTTAATGTCATGATAATAATGGTTTC
Cr166	CACATTTCCCCGAAAAGTG
Cr167	AACTTGCTATTTCTAGCTCTAAAACCCAAGGCCTTATGAAGAGTTACGTCAACCTGCGCC
Cr189	AACTTGCTATTTCTAGCTCTAAAACCGTTTTCGGTGCCTACAAGGACGTCAACCTGCGCC
Cr203	AACTTGCTATTTCTAGCTCTAAAACCTAGGTCAACCTGCGCCG
Cr216	TCGCAATTGTCATTTCTGTCACTATTACC
Cr217	CTCGGTAAGTGTAAGTGTAGTCGAAAG
Cr219	GGAAGTGGCAGTCAGTTCGGGCTTCTTCATCCGCGGTTTGAATGATTCTTATAC
Cr221	CAAGCACTCGACCTCGAGCTAAGGAATAGTCATGTAATTAGTTATGTCACGCTTACATTC
Cr224	AAGCTTGGCGTAATCATGGTC
Cr225	CCAGACCAACTACTCCGCTGCCAAGCTCGCAGACCGGGTTGGCGG
Cr226	GGCACCCTCCTTGGCGAGAGTCTCACCGAAGCAAATTAAAGCCTTCGAGCG
Cr228	GCGAGCTTGGCAGCG
Cr229	TTCGGTGAGACTCTCGCCAAG
Cr230	AACAGCTATGACCATGATTACGCCAAGCTTTGAAGAGCTCGAGCATCTCC
Cr231	GTACCGCATGCTTCCTTGG
Cr232	GTTCGAAGGTACCAAGGAAGCATGCGGTACTCTACAACTTTGAAATTGATGACATTTTC
Cr241	GGGTCGGCGCAGGTTGACGTGTACAAGGAGGAGCGGATAGGTTTTAGAGCTAGAAATAGC
Cr242	GCTATTTCTAGCTCTAAAACCTATCCGCTCCTCCTTGTACACGTCAACCTGCGCCGACCC
Cr243	AGGAGGAGCTGGAGAGTTTTAGAGC
Cr244	CCGACTCGGTGCCACTTTTTC
Cr245	TCCAGGCCGTCCTCTCCC
Cr246	GGCCAGCCATATCGAGTCGCA

Supporting references

(1) Madzak, C., Tréton, B., and Blanchin-Roland, S. (2000) Strong Hybrid Promoters and Integrative Expression / Secretion Vectors for Quasi-Constitutive Expression of Heterologous Proteins in the Yeast Yarrowia lipolytica. *J. Mol. Microbiol. Biotechnol. 2*, 207–216.

(2) Yamane, T., Sakai, H., Nagahama, K., Ogawa, T., and Matsuoka, M. (2008) Dissection of centromeric DNA from yeast Yarrowia lipolytica and identification of protein-binding site required for plasmid transmission. *J. Biosci. Bioeng. 105*, 571–578.

(3) DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., and Church, G. M. (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. *Nucleic Acids Res. 41*, 4336–43.

(4) Puigbò, P., Guzmán, E., Romeu, A., and Garcia-Vallvé, S. (2007) OPTIMIZER: A web server for optimizing the codon usage of DNA sequences. *Nucleic Acids Res. 35*, 126–131.

(5) Blazeck, J., Liu, L., Redden, H., and Alper, H. (2011) Tuning gene expression in Yarrowia lipolytica by a hybrid promoter approach. *Appl. Environ. Microbiol. 77*, 7905–14.

(6) Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. a, and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods 6*, 343–345.

(7) Doench, J. G., Hartenian, E., Graham, D. B., Tothova, Z., Hegde, M., Smith, I., Sullender, M., Ebert, B. L., Xavier, R. J., and Root, D. E. (2014) Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol. 32*, 1262-1267.

(8) Gruber, A. R., Lorenz, R., Bernhart, S. H., Neuböck, R., and Hofacker, I. L. (2008) The Vienna RNA websuite. *Nucleic Acids Res. 36*, 70–74.