Engineering reversal of function in glycolytic yeast promoters

Supporting Information

Arun S. Rajkumar[1#], Emre Özdemir[1], Alicia V. Lis[1], Konstantin Schneider[1], Jiufu Qin[1], Michael K. Jensen[1*], and Jay D. Keasling[1-5]

[1] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kgs. Lyngby, Denmark

[2] Joint BioEnergy Institute, Emeryville, CA, USA

[3] Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

[4] Department of Chemical and Biomolecular Engineering & Department of Bioengineering, University of California, Berkeley, CA, USA

[5] Center for Synthetic Biochemistry, Institute for Synthetic Biology, Shenzhen Institutes of Advanced Technologies, Shenzhen, China

[#] Current address: School of Microbiology, University College Cork, Cork, Ireland.

SUPPORTING TABLES AND FIGURES

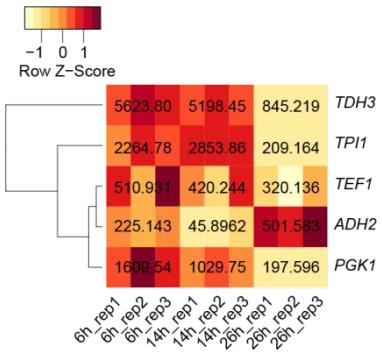

Figure S1. Heatmap of triplicate expression profiles from all 5 native promoters used as benchmark and engineering platforms for main Figures 1D-F. Mean expression values (FPKM) for all genes based on biological triplicates are indicated.

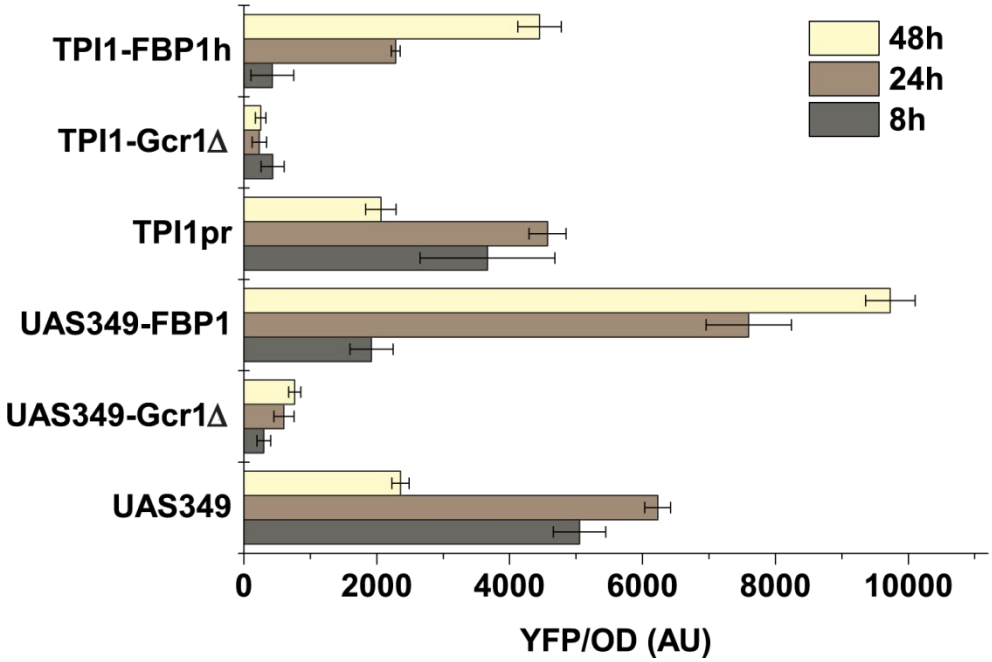

Figure S2. Transcription factor requirements for glycolytic promoter function. Removal of Gcr1 binding sites from promoters *UAS349* or *TPI1pr* (*UAS349-Gcr1D*, *TPI1pr-Gcr1D*) result in a complete loss of promoter activity as measured by YFP fluorescence normalized by OD, either in the presence (8 and 24h measurements) or absence (48h measurement) of glucose in the medium. Data from the engineered promoters *UAS349-FBP1h* and *TPI1-UAS349h* are added for comparison. Data are plotted as mean ± S.D. of 3 biological replicates.

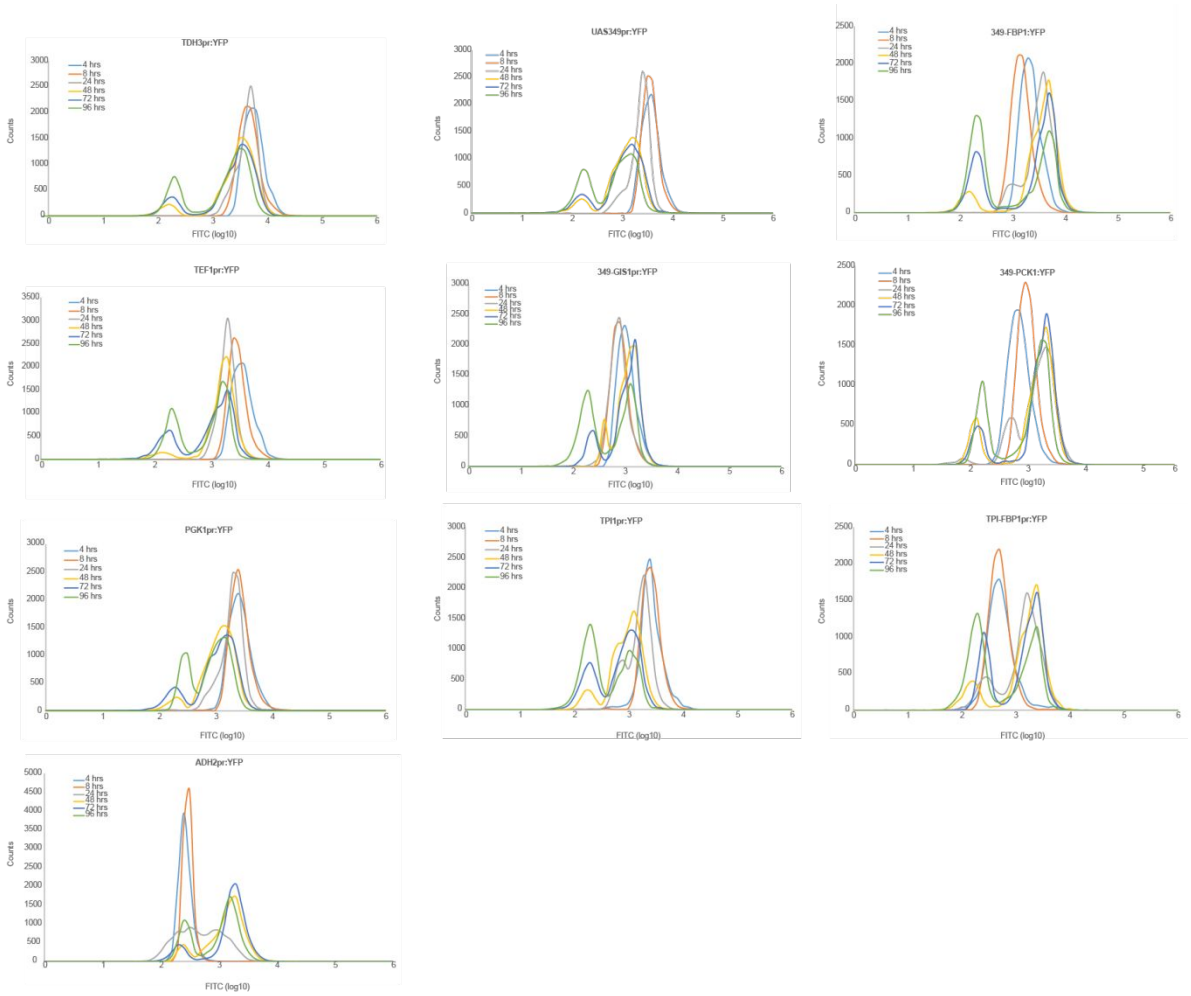

Figure S3. Representative histograms of flow cytometry data for all promoters analyzed in this study. Single cell histograms of flow cytometry data as presented in Figure 1D-F. A total of 10,000 cells from each reporter strain were analyzed. Histograms are plotted with a biexponential scale to render the range of promoter activation. Color code indicate time point of measurement.

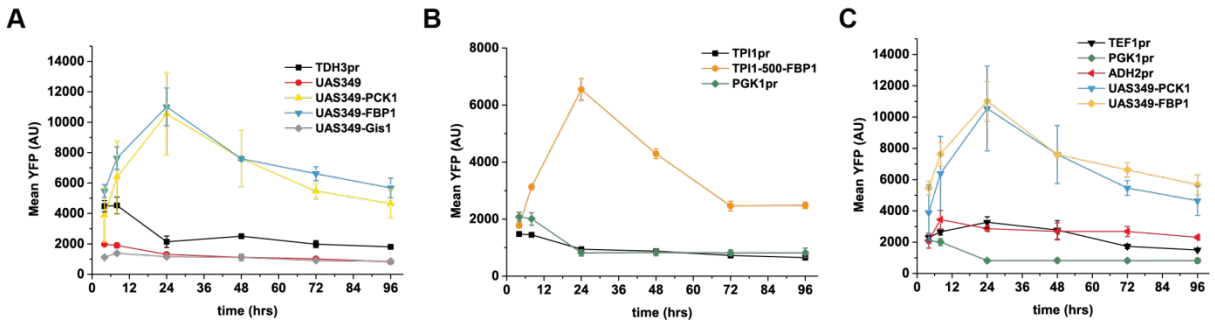

Figure S4. Engineered promoter output with ethanol as a carbon source. (**A**) While *TDH3pr* and *UAS349* have their output significantly reduced compared to when induced in glucose, *UAS349-PCK1h* and *UAS349-FBP1h* have a stronger output than in glucose. They also induce earlier during growth. (**B**) A similar trend is seen for *TPI1-FBP1h* versus *TPI1pr*, the three engineered promoters finally being 2-3 fold stronger than *ADH2pr* (**C**) when it is induced in ethanol. Data are plotted as mean ± S.D. of 3 biological replicates.

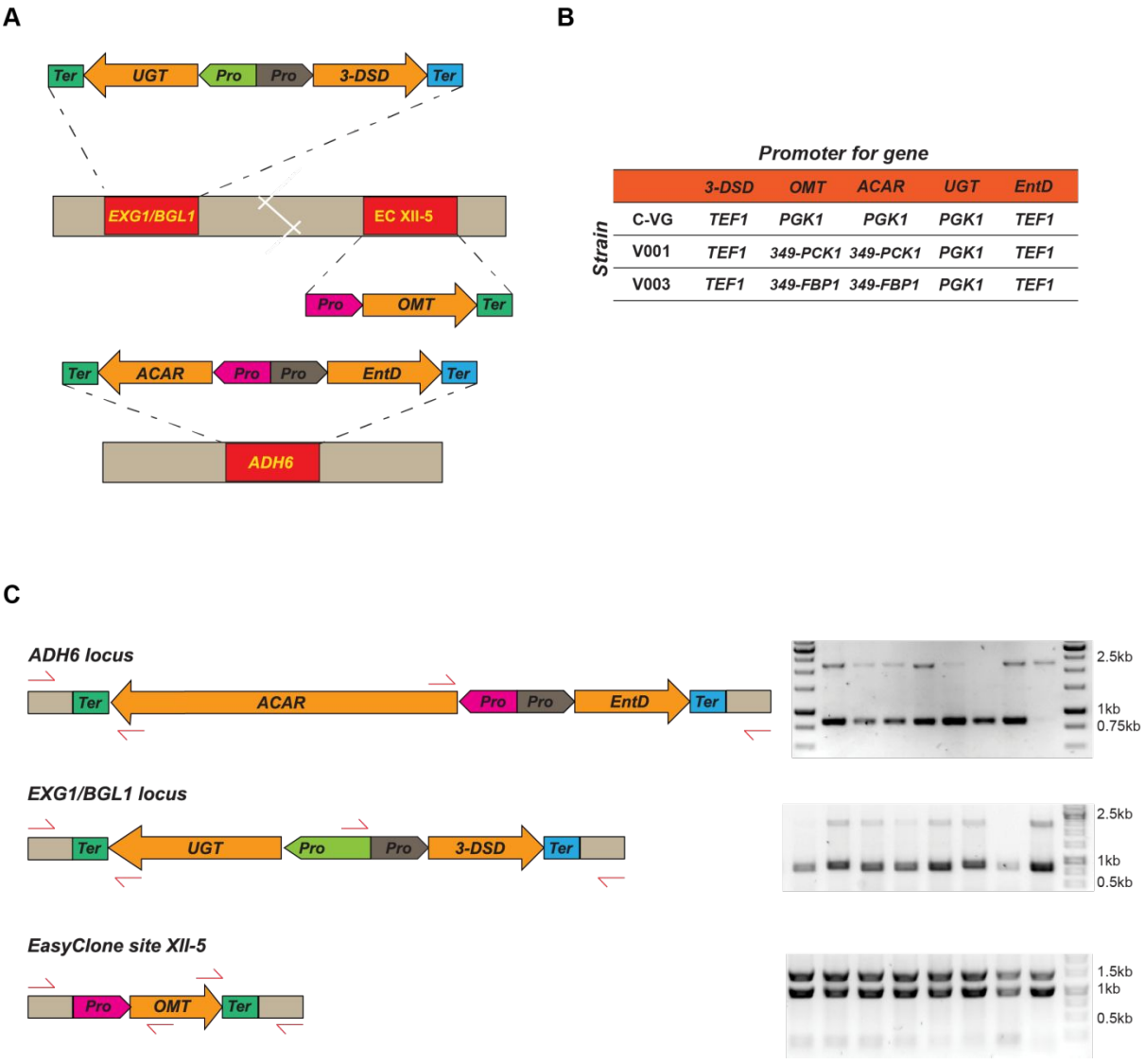

Figure S5. Design and genotyping of vanillin-β-glucoside production strains using synthetic gluconeogenic promoters. (**A**) Schematic outline of the design and assembly strategy for introducing the vanillin-β-glucoside biosynthesis pathway into two native genomic loci in the CEN.PK 113-7D strain background. (**B**) The combinations of genes and promoters used in the three different vanillin-β-glucoside production strains. (**C**) Multiplex genotyping strategy (*left*) for PCR validation of correct *in vivo* assembly and genomic integration of DNA parts as indicated in (A)(*right*).

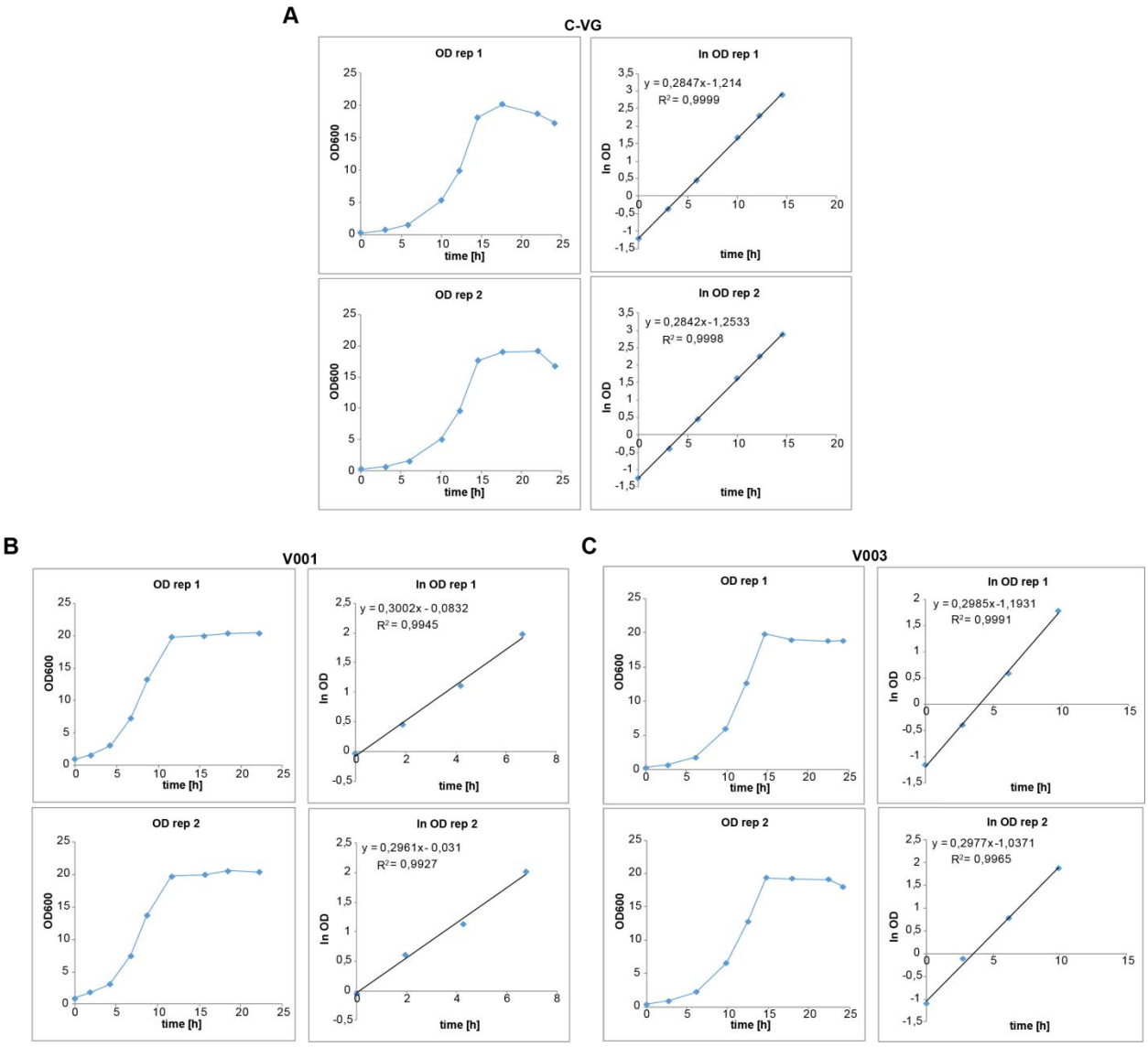

Figure S6. Growth curves and growth rates of vanillin-β-glucoside production strains. **A**) Two biological replicate growth curves (left) of control strain C-VG, and respective growth rates (right) calculated from sampling points falling along the straight line representing the exponential growth phase. **B**) Two biological replicate growth curves (left) of strain V001, and respective growth rates (right) calculated from sampling points falling along the straight line representing the exponential growth phase. **C**) Two biological replicate growth curves (left) of strain V003, and respective growth rates (right) calculated from sampling points falling along the straight line representing the exponential growth phase.

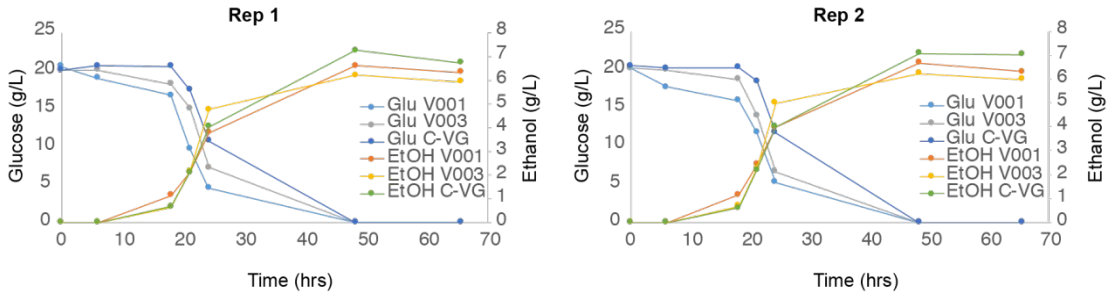

Figure S7. Time course of glucose and ethanol concentrations of vanillin-β-glucoside production strains. Two biological replicates are shown with glucose and ethanol quantifications based on HPLC measured during 65 hrs fermentations (Figure 2B).

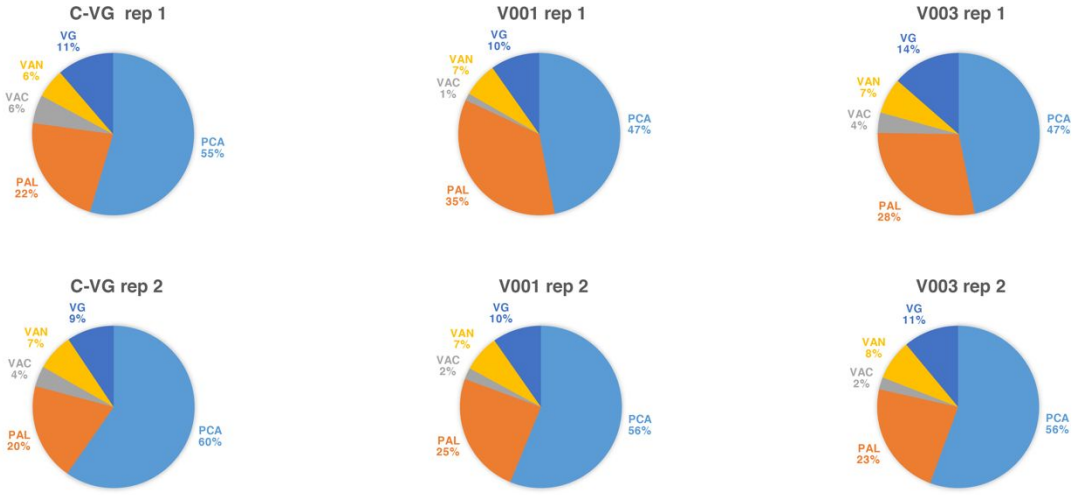

Figure S8. Pie charts illustrating relative distribution of the vanillin-β-glucoside pathway products from two independent replicates for each of the three vanillin-β-glucoside production strains as a percentage of the total products of the vanillin-β-glucoside pathway. The metabolite distributions are sampled at the end of 65 hrs fermentations (Figure 2B).

Table S1. Gene names and significance level as inferred from 3-way comparison with q-value cut-off of <0.05 to identify significantly differentially expressed genes.

Table S2. Transcription factor binding site occurrence in genes associated with carbon metabolism upregulated between the log phase and diauxic shift.

Gene_name	minUAS_f	minUAS_rev_c	ADR1_f	ADR1_r	RDS2_f	RDS2_r	CAT8_f	CAT8_r	SIP4_f	SIP4_r
	CCNNNNNNCCG	**CGGNNNNNNGG**	**YTGGRG**	**CYCCAR**	**YCCNTTNAKCCG**	**CGGMTNAANGGR**	**YCCNYTNRKCCG**	**CGGMYNARNGGR**	**TCCATTSRTCCGR**	**YCGGAYSAATGGA**
PCK1	CCTTTCATCCG	CGGGTGAATGG CGGATAAAGGG	CTGGGG		TCCTTTCATCCG		TCCTTTCATCCG			
FBP1		CGGACGGATGG CGGTCGTGCGG CGGACACCCGG								
MDH2	CCTTTAATCCG CCATTCGGCCG	CGGCATCTCGG CGGCCAAATGG		CCCCAA	CCCTTTAATCCG		CCCTTTAATCCG CCCATTCGGCCG	CGGCCAAATGGA		
PYC1	CCGCTCCACCG									
ICL1	CCATTCATCCG			CCCCAG	TCCATTCATCCG		TCCATTCATCCG		TCCATTCATCCGA	
MLS1	CCATTGGGCCG CCCCTTTCCCG CCGGCGAGCCG	CGGCTCAATG CGGCGAGCCGG CGGCAGTCGGG		CCCCAG		CGGCTCAATGGA	TCCATTGGGCCG	CGGCTCAATGGA		
YIG1	CCGCATTTCCG		TTGGGG							

			TTG GAG							
MAL12	CCTCAA GCCCG									
MAL11		CGGGCT TGAGG								
MAL32			TTG GAG	CTC CAG						

Table S3. Primers used in this study. Overlaps for *in vivo* assembly are in red.

Name	Sequence	Function
XII_4_up_F	GTATCCGGCTGTTCCTTCATA	Forward XII-4 upstream homology.
ASR_0344_R	CTCGTGATACGCCTATTTTTATAGTGCCATAGTATGTGTGATGGA	Reverse XII-4 upstream homology, 3' resistance marker overlap.
ASR_0347_F	TATTGACCACACCTCTACC$GGCATGCCAACTTTGTACTATTCCTTCCC	Forward XII-4 downstream homology, 5' reporter construct overlap.
XII_4_down_R	TTTCTGCTGTACCTGGATGG	Reverse XII-4 downstream homology.

ASR_0307_F	AAAACAATGAGTAAAGGAGAAGAACTTTTC	Forward primer for amplification of YFP expression cassette
ASR_0345_R	TTTTTCCATCACACATACTATGGCACTATAAAAATAGGCGTATCACGAG	Reverse primer for YFP expression cassette, 3' XII-4 downstream homology overlap
ASR_0294_F	TAAATCTGCTTTAGGTCTAGAGATCTGTTTAGCTTG	Forward primer for resistance marker
ASR_0346_R	CGGGGAAGGAATAGTACAAAGTTGGCATGCCGGTAGAGGTGTG	Reverse primer for resistance marker, 3' XII-4 downstream homology overlap.
ASR_0318_F	AAACAGATCTCTAGACCTAAAGCAGATTTAATAAAAAACACGCTTTTTCAGTTCG	Forward primer for TDH3pr, 5' overlap with resistance marker
ASR_0310_F	AAACAGATCTCTAGACCTAAAGCAGATTTATTAGTCAAAAAATTAGCCTTTTAATT	Forward primer for UAS349 and derivatives, 5' overlap with resistance marker
ASR_0031_R	GTTCTTCTCCTTTACTCATTGTTTTTTTGTTTGTTTATGTGTGTTTATTCGA	Reverse primer for TDH3pr and UAS3 derivatives, 3' overlap with YFP

ASR_0342_F	AAACAGATCTCTAGACCTAAAGCAGATTTAATTTAAACTGTGAGGACCTTAATACATTC	Forward primer for TPI1pr and derivatives, 5'overlap with resistance marker
ASR_0343_R	GTTCTTCTCCTTTACTCATTGTTTTTTTTAGTTTATGTATGTGTTTTTTGTAGT	Reverse primer for TPI1pr and derivatives, 3' overlap with YFP
ASR_0273_F	AAACAGATCTCTAGACCTAAAGCAGATTTAGCACACACCATAGCTTCAAA	Forward primer for TEF1pr, 5'overlap with resistance marker
ASR_0274_R	GTTCTTCTCCTTTACTCATTGTTTTTTGTAATTAAAACTTAGATTAGATTGCTATG	Reverse primer for TEF1pr, 3' overlap with YFP
ASR_0335_F	AAACAGATCTCTAGACCTAAAGCAGATTTAGGAAGTACCTTCAAAGAATGG	Forward primer for PGK1pr, 5'overlap with resistance marker
ASR_0336_R	GTTCTTCTCCTTTACTCATTGTTTTTGTTTTATATTTGTTGTAAAAAGTAGATAA	Reverse primer for PGK1pr, 3' overlap with YFP
ASR_0448_F	AAACAGATCTCTAGACCTAAAGCAGATTTATCTCTCCGGTTACAGCCTG	Forward primer for ADH2pr, 5'overlap with resistance marker
ASR_0449_R	GTTCTTCTCCTTTACTCATTGTTTTTGTGTATTACGATATAGTTAATAGTTGATAGTT	Reverse primer for ADH2pr, 3' overlap with YFP

ASR_0020_F	ACTGACGTCGAAGGCTCTTT	Forward colony PCR primer to verify insertion at XII-4, 5' end. Primes 104bp upstream of site XII-4, and is used with ASR_0307_F.
XII-4-up-out-sq ID897	CGTGAAATCTCTTTGCGGTAG	Forward colony PCR primer to verify insertion at XII-4, 3' end. Primes 100bp downstream of XII-4, and is used with ASR_0110_F.
ASR_0110_F	GCCATCTAAGTTGGGACACAGATAAGCGAATTTCTTATGATTTATGATTTTTATT	Reverse colony PCR primer to verify insertion at XII-4, 3'end.
ASR_0288_R	CGCTTGACATCTACTATATGTAAG	Forward colony PCR primer to verify assembly of promoter-reporter cassette. Also used for sequencing.
ASR_0314_R	GGGATGTATGGGCTAAATGT	Reverse colony PCR primer to verify assembly of promoter-reporter cassette. Also used for sequencing.

ASR_0083_SEQ	TCACCATCTAATTCAACAAGAATTG	Sequencing primer for promoter.
ASR_V001	ACGAGCCTGAGACAAGCC	EXG1_US_F
ASR_V002	TATTGACCACACCTCTACCGGCATGTTTAGTTGGTAATTAACTAGAAAAAGAAAG	EXG1_US_R, overlap with ADH1 terminator
ASR_V003	CGTGGACTTGTCACGTGGTGCCTAGGCGAATTTCTTATGATTTATGATTTTTATT	Forward primer for tADH1, overlap with UGT1
ASR_V004	TTTTTCTAGTTAATTACCAACTAAACATGCCGGTAGAGGTGTG	Reverse primer for tADH1, overlap with EXG1_US
ASR_V005	TACTTTTTACAACAAATATAAAAC AATGCATATCACAAAACCACAC	Forward primer for UGT1, overlap with PGK1pr
ASR_V006	AAATCATAAATCATAAGAAATTCGCCTAGGCACCACGTGACAAGT	Reverse prime for UGT1, overlap with tADH1
ASR_V007	CGGCGTGTGGTTTTGTGATATGCATTGTTTTATATTTGTTGTAAAAAGTAGATAA	Reverse primer for PGK1, overlap with UGT1
ASR_V008	AACATTTTGAAGCTATGGTGTGTGCAAGAAGTACCTTCAAAGAATGG	Forward primer for PGK1, overlap with TEF1
ASR_V009	ACCCCATTCTTTGAAGGTACTTCTTGCACACACCATAGCTTCAAA	Forward primer for TEF1,

		overlap with PGK1
ASR_V010	GCTCCTTAGTGTCACCCATTGTTTTTTGTAATTAAAACTTAGATTAGATTGCTATG	Reverse primer for TEF1, overlap with HsOMT
ASR_V011	ATCTAAGTTTTAATTACAAAAAACAATGGGTGACACTAAGGAGC	Forward primer for HsOMT, ovelap with TEF1
ASR_V012	TCCTTCCTTTTCGGTTAGAGCGGATTTATGGACCAGCTTCAGAAC	Reverse primer for HsOMT, overlap with tCYC1
ASR_V013	TCCAGGTTCTGAAGCTGGTCCATAAATCCGCTCTAACCGAAAAGGA	Forward primer for tCYC1, overlap with HsOMT
ASR_V014	CTAAAATGAGCGGACTGAGGGCGACTTCTCAAGCAAGGTTTTCAGTATAATG	Reverse primer for tCYC1, overlap with EXG1_DS
ASR_V015	ATTATACTGAAAACCTTGCTTGAGAAGTCGCCCTCAGTCCGCTC	EXG1_DS_F, overlap with tCYC1
ASR_V016	GTTGTTTAAGTTCTTTTATCCTTCCTTAGATAACA	EXG1_DS_R
ASR_V017	AATACGCACACATACGCGCAT	US CPCR_F, 91bp US of EXG1_US
ASR_V018	CCAAGGAGTGTCAACGGTT	US_CPCR_R,57 bp US of UGT's Stop codon, product 840bp

ASR_V019	GTCATCGACGTTATCTCTACTATAG	DS_CPCR_F, 105bp into PGK1
ASR_V020	ATGTTCTTTCGTTTGGATGAGG	DS_CPCR_R, 84bp DS of EXG1_DS, product 1.95kb for original config,2.4kb with DSD
ASR_V021	TTCCAAGCTCGATCACCGG	EXG1_jn_F, 79 bp DS of UGT's Start codon
ASR_V022	GGAGTCCGAGAAAATCTGGAAGAGTA	EXG1_jn_R,63 1126bp DS into TEF1, product for original config
ASR_V023	TTTTCATTTTCAACTTGGTAATGAC	ADH6_US_F
ASR_V024	TATTGACCACACCTCTACCGGCATGGATTTTGGCTTTTCTTGTTGTTG	ADH6_US_R, overlap with ADH1 terminator
ASR_V025	TTTGGAATTGTTACAATTGTTATAAGCGAATTTCTTATGATTTATGATTTTTATT	Forward primer for tADH1, overlap with ACAR
ASR_V026	CACAACAACAAGAAAAGCCAAAATCCATGCCGGTAGAGGTGTG	Reverse primer for tADH1, overlap with ADH6_US
ASR_V027	TACTTTTTACAACAAATATAAAACAATGGCTGTTGATTCACCAG	Forward primer for ACAR, overlap with PGK1pr

ASR_V028	AAATCATAAATCATAAGAAATTCGCTTATAACAATTGTAACAATTCCAAATC	Reverse prime for ACAR, overlap with tADH1
ASR_V029	TCTCATCTGGTGAATCAACAGCCATTGTTTTATATTTGTTGTAAAAAGTAGATAA	Reverse primer for PGK1, overlap with ACAR
ASR_V030	GTAGTTTTCATATCGACCATTGTTTTTTGTAATTAAAACTTAGATTAGATTGCTATG	Reverse primer for TEF1, overlap with EntD
ASR_V031	ATCTAAGTTTTAATTACAAAAAACAATGGTCGATATGAAAACTACGC	Forward primer for EntD, ovelap with TEF1
ASR_V032	TCCTTCCTTTTCGGTTAGAGCGGATTTAATCGTGTTGGCACAGC	Reverse primer for EntD, overlap with tCYC1
ASR_V033	CATAACGCTGTGCCAACACGATTAAATCCGCTCTAACCGAAAAGGA	Forward primer for tCYC1, overlap with HsOMT
ASR_V034	CTACATTTATCAAGAGCTTGACAACTTCTCAAGCAAGGTTTTCAGTATAATG	Reverse primer for tCYC1, overlap with ADH6_DS
ASR_V035	ATTATACTGAAAACCTTGCTTGAGAAGTTGTCAAGCTCTTGATAAATGT	ADH6_DS_F, overlap with tCYC1
ASR_V036	GATAATTGTCTCGAGCCAAA	ADH6_DS_R
ASR_V037	TACCTCACCTGAGTTTTGCTT	US CPCR_F, 91bp US of ADH6_US

ASR_V038	CTCCATTGATTGATAAGTATGTTTCTGA	US_CPCR_R,53 bp US of ACAR's Stop codon, product 845bp
ASR_V039	AGTATCAACCACTAAAGCGAAAAAC	DS_CPCR_R, 84bp DS of ADH61_DS, use with DS_CPCR_F for EXG1, product 1.92kb for original config
ASR_V040	CTTGCTCATCTTCAGCAAACAATTGAG	ADH6_jn_F, 79 bp DS of ACAR's Start codon, use with EXG1_jn_R for 1.17bp in original config
ASR_V041	AACACACATAAACAAACAAAAAAACAATGCATATCACAAAACCACAC	Forward primer for UGT1, overlap with TDH3 core
ASR_V042	TGTGGTTTTGTGATATGCATTGTTTTTTTGTTTGTTTATGTGTGTTTATTCGA	Reverse primer for TDH3 core, overlap with UGT1
ASR_V043	AACACACATAAACAAACAAAAAAACAATGGCTGTTGATTCACCAG	Forward primer for ACAR, overlap with TDH3 core
ASR_V044	TCTGGTGAATCAACAGCCATTGTTTTTTTGTTTGTTTATGTGTGTTTATTCGA	Reverse primer for TDH3 core, overlap with ACAR

ASR_V045	AACATTTTGAAGCTATGGTGTGTGCTTAGTCAAAAAATTAGCCTTTTAATT	Forward primer for '349', overlap with TEF1
ASR_V046	ATTAAAAGGCTAATTTTTTGACTAAGCACACACCATAGCTTCAAA	Forward primer for TEF1, overlap with '349'
ASR_V047	CAATCTGGCGGCTTGAGT	Forward primer for XII-5-up
ASR_V048	AACATTTTGAAGCTATGGTGTGTGCGCTGATGTGACACTGTGACAAT	Reverse primer for XII-5-up, overlap with TEF1 promoter
ASR_V049	TTTATTGTCACAGTGTCACATCAGCGCACACACCATAGCTTCAAA	Forward primer for TEF1, overlap with XII-5-up
ASR_V050	ATGGCGAGTTTGGAAGGCATTGTTTTTTGTAATTAAAACTTAGATTAGATTGCTATG	Reverse primer for TEF1, overlap with 3-DSD
ASR_V051	ATCTAAGTTTTAATTACAAAAAACAATGCCTTCCAAACTCGCC	Forward primer for 3-DSD, overlap with TEF1
ASR_V052	TCCTTCCTTTTCGGTTAGAGCGGATTTACAAAGCCGCTGACAGC	Reverse primer for 3-DSD, overlap with tCYC1
ASR_V053	GCTGTCGCTGTCAGCGGCTTTGTAAATCCGCTCTAACCGAAAAGGA	Forward primer for tCYC1, overlap with 3-DSD

ASR_V054	GCTTGCTGTCAAACTTCTGAGTTGCTTCTCAAGCAAGGTTTTCAGTATAATG	Reverse primer for tCYC1, overlap with XII-5-down
ASR_V055	TTATACTGAAAACCTTGCTTGAGAAGCAACTCAGAAGTTTGACAGC	XII-5-down_F, overlap with tCYC1
ASR_V056	CTGCGATACCTTTTGTGAT	XII-5-down_R
ASR_V057	CGATCCAGAACTCGAGCT	US_CPCR_R, use with 899(137bp up of up, so 454+137), 337 bp into 3-DSD, 1354bp product, use with EXG1_jn_F for 1.8kb?
ASR_V058	ACACAGCAGCCCATCAGG	DS_CPCR_F, use with 900 (676+97), 45bp upstream of 3-DSD's Stop, 991bp product
ASR_V063	ACCCCATTCTTTGAAGGTACTTCTTGCTGATGTGACACTGTGACAAT	Reverse primer for XII-5-up, overlap with PGK1 promoter
ASR_V064	TTTATTGTCACAGTGTCACATCAGCAAGAAGTACCTTCAAAGAATGG	Forward primer for PGK1, overlap with XII-5-up
ASR_V065	TTCTTTGCTCCTTAGTGTCACCCATTGTTTTATATTTGTTGTAAAAAGTAGATAA	Reverse primer for PGK1, overlap with HsOMT

ASR_V066	TACTTTTTACAACAAATATAAAAACAATGGGTGACACTAAGGAGC	Forward primer for HSOMT, overlap with PGK1
ASR_V067	AAATCATAAATCATAAGAAATTCGCTTATGGACCAGCTTCAGAAC	Reverse primer for HsOMT, overlap with tADH1
ASR_V068	TCCAGGTTCTGAAGCTGGTCCATAAGCGAATTTCTTATGATTTATGATTTTTATT	Forward primer for tADH1, overlap with HsOMT
ASR_V069	GCTTGCTGTCAAACTTCTGAGTTGCCATGCCGGTAGAGGTGTG	Reverse primer for tADH1, overlap with XII-5-down
ASR_V070	TATTGACCACACCTCTACCGGCATGGCAACTCAGAAGTTTGACAGC	Forward primer for XII-5-down, overlap with tADH1
ASR_V073	ATTAAAAGGCTAATTTTTTGACTAAGCTGATGTGACACTGTGACAAT	Reverse primer for XII-5-up, overlap with '349'
ASR_V074	AACACACATAAACAAACAAAAAAACAATGGGTGACACTAAGGAGC	Forward primer for HsOMT, overlap with TDH3 core
ASR_V075	TTTATTGTCACAGTGTCACATCAGCTTAGTCAAAAAATTAGCCTTTTAATT	Forward primer for '349', overlap with XII-5-up

ASR_V076	GAGTAATAGCAGCACAGTCTG	US_CPCR_R, 298 bp ds of OMT's Start codon, use with 899 (so 454+137+ 984+298)-a 1873bp product with PGK1, 1323bp with 349s
ASR_V077	CCAGGTTCTGAAGCTGGTC	DS_CPCR_F, 24 bp us of OMT's Stop codon, yields a 989bp product with primer 900
ASR_V078	GCTCCTTAGTGTCACCCATTGTTTTTTTGTTTGTTTATGTGTGTTTATTCGA	Reverse primer for TDH3 core, overlap with HsOMT
ASR_V076_alt	GTTGTCAGCTAACAAAACAGTAC	US_CPCR_R for 349 cassette, 510bp into OMT's Start codon
ASR_V081	CTAAAGCTATGTTGGGTGTTG	Primer inside ACAR, covers 1.5kb us of its Stop codon, to use to amplify gene for sequencing

ASR_V082	CTCATGTAACAAGTTAGAGAAAGAC	Primer approx 2.1kb ds of ACAR's Start Codon, use to amplify gene for sequencing
ASR_V083	CCACCGAAGTTGATTTGCTT	
ASR_V084	GTGGGAGTAAGGGATCCTGT	

Table S4. Strain list

Strain	**Genotype**	**Reference**
CEN.PK 113-7D	*MATa MAL 2-8^{C} SUC2*	Euroscarf
ScASR.0076i	CEN.PK 113-7D *XII-4(kanMX-TDH3pr-YFP-tVPS13)*	This study
ScASR.0077i	CEN.PK 113-7D *XII-4(kanMX-UAS349pr-YFP-tVPS13)*	This study
ScASR.0232i	CEN.PK 113-7D *XII-4(kanMX-UAS349-FBP1h-YFP-tVPS13)*	This study
ScASR.0233i	CEN.PK 113-7D *XII-4(kanMX-UAS349-PCK1h-YFP-tVPS13)*	This study
ScASR.0241i	CEN.PK 113-7D *XII-4(kanMX-UAS349-Gis1-YFP-tVPS13)*	This study
ScASR.0242i	CEN.PK 113-7D *XII-4(kanMX- UAS349-Gcr1D,-YFP-tVPS13)*	This study
ScASR.0131i	CEN.PK 113-7D *XII-4(kanMX-TPI1pr-YFP-tVPS13)*	This study

ScASR.0236i	CEN.PK 113-7D *XII-4(kanMX-TPI1-FBP1h -YFP-tVPS13)*	This study
ScASR.0245i	CEN.PK 113-7D *XII-4(kanMX- TPI1-Gcr1D-YFP-tVPS13)*	This study
ScASR.0094i	CEN.PK 113-7D *XII-4(kanMX-TEF1pr-YFP-tVPS13)*	This study
ScASR.0125i	CEN.PK 113-7D *XII-4(kanMX-PGK1pr-YFP-tVPS13)*	This study
ScASR.0223i	CEN.PK 113-7D *XII-4(kanMX-ADH2pr-YFP-tVPS13)*	This study
C-VG	*MATa MAL 2-8^{C} SUC2 XII-2(pTEF1-HsOMT,pPGK1-UGT)XII1(pTEF1-PPT,pPGK1-ACAR)XII-5(pTEF1-3DSD) Δbgl1::loxP Δadh6::KanMX*	Strucko et al [1]
ScASR.V001	CEN.PK 113-7D *ADH6(349-PCK1-ACAR-tADH1 TEF1pr-EntD-tCYC1) EXG1(PGK1pr-UGT-tADH1 TEF1pr-3-DSD-tCYC1) XII-5 (349-PCK1-OMT-tADH1)*	This study
ScASR.V003	CEN.PK 113-7D *ADH6(349-FBP1-ACAR-tADH1 TEF1pr-EntD-tCYC1) EXG1(PGK1pr-UGT-tADH1 TEF1pr-3-DSD-tCYC1) XII-5 (349-FBP1-OMT-tADH1)*	This study

Table S5. Plasmid list

Name	**Features**	**Reference**
pCfB2312	*TEF1pr-Cas9-CYC1t-kanMX*, CEN/ARS plasmid	Stovicek et al [2]
pTAJAK-71	· pESC-NatMXsyn-USER	Jessop-Fabre et al. [3]
pASR.G0005	pTAJAK-71 *SNR52pr-gRNA.ADH6-tSUP4* gRNA sequence: GACATTaagatCGAAGCATG	This study

pASR.G0007	pTAJAK-71 *SNR52pr-gRNA.EXG1 XII-5-tSUP4* gRNA sequence: AGATACTacgaTTATGACCA	Strucko et al [1]
pXII1-23	*PGK1pr-ACAR-tADH1 TEF1pr-EntD-tCYC1*	Strucko et al [1]
pXII2-54	*PGK1pr-UGT-tADH1 TEF1pr-OMT-tCYC1*	Strucko et al [1]
pXII5-01	*TEF1pr-3DSD-tCYC1*	Strucko et al [1]

Table S6. Transcription factor binding site motifs used for promoter engineering

Site	**Sequence**	**Reference**
CSRE from PCK1pr	ACGGGTGAATGGAGATCTGG	Soontorngun et al [4]
CSRE from FBP1pr	CCGGACGGATGGAATCGCCG	Soontorngun et al [4]
Gis1 binding site	TAGGGAT	Zhang et al [5]

Table S7. Sequences of all synthetic promoters engineered in this study. Engineered binding sites are marked in boldface.

Promoter	**Sequence**
TDH3pr	GAATAAAAAACACGCTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGAATACGT AAATAATTAATAGTAGTGATTTTCCTAACTTTATTTAGTCAAAAAATTAGCCTTTTAATTCTGCTGT AACCCGTACATGCCCAAAATAGGGGGCGGGTTACACAGAATATATAACATCGTAGGTGTCTGG GTGAACAGTTTATTCCTGGCATCCACTAAATATAATGGAGCCCGCTTTTTAAGCTGGCATCCAGA AAAAAAAAGAATCCCAGCACCAAAATATTGTTTTCTTCACCAACCATCAGTTCATAGGTCCATTC TCTTAGCGCAACTACAGAGAACAGGGGCACAAACAGGCAAAAAACGGGCACAACCTCAATGGA GTGATGCAACCTGCCTGGAGTAAATGATGACACAAGGCAATTGACCCACGCATGTATCTATCTC ATTTTCTTACACCTTCTATTACCTTCTGCTCTCTCTGATTTGGAAAAAGCTGAAAAAAAAGGTTGA AACCAGTTCCCTGAAATTATTCCCCTACTTGACTAATAAGTATATAAAGACGGTAGGTATTGATT

	GTAATTCTGTAAATCTATTTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTT TAAAACACCAAGAACTTAGTTTCGAATAAACACACATAAACAAACAAA
TDH3 core promoter	CTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACTTCTTAA ATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGAATAAACAC ACATAAACAAACAAA
UAS349pr	TTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCAAAATAGGGGGCGGGTT ACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCCTGGCATCCACTAAATATA ATGGAGCCCGCTTTTTAAGCTGGCATCCAGAAAAAAAAAGAATCCCAGCACCAAAATATTGTTTT CTTCACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACT TCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGAAT AAACACACATAAACAAACAAA
UAS349-PCK1h	TTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCAAAATAGGGGGCGGGTT ACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCCT**ACGGGTGAATGGAGAT CTGG**GAGCCCGCTTTTTA**ACGGGTGAATGGAGATCTGG**AAGAATCCCAGCACCAAAATATTGT TTTCTTCACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAA CTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGA ATAAACACACATAAACAAACAAA
UAS349-FBP1h	TTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCAAAATAGGGGGCGGGTT ACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCCT**CCGGACGGATGGAATC GCCG**GAGCCCGCTTTTTA**CCGGACGGATGGAATCGCCG**AAGAATCCCAGCACCAAAATATTGT TTTCTTCACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAA CTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGA ATAAACACACATAAACAAACAAA
UAS349-Gis1	TTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCAAAATAGGGGGCGGGTT ACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCCT**TAGGGAT**ACTAAATATA ATGGAGCCCGCTTTTTAAGCT**TAGGGAT**AGAAAAAAAAAGAATCCCAGCACCAAAATATTGTTTT CTTCACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACT TCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGAAT AAACACACATAAACAAACAAA
UAS349-Gcr1D	TTAGTCAAAAAATTAGCCTTTTAATTCTGCTGTAACCCGTACATGCCCAAAATAGGGGGCGGGTT ACACAGAATATATAACATCGTAGGTGTCTGGGTGAACAGTTTATTCCTTAAAGATACTAAATATA ATGGAGCCCGCTTTTTAAGCTTAGAGATAGAAAAAAAAGAACACCCATACATCAAATATTGTTTT CTTCACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACT TCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTTAGTTTCGAAT AAACACACATAAACAAACAAA

TPI1pr	ATTTAAACTGTGAGGACCTTAATACATTCAGACACTTCTGCGGTATCACCCTACTTATTCCCTTC GAGATTATATCTAGGAACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAGACTTTTCAGCTTCC TCTATTGATGTTACACCTGGACACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGTGGCATGTG AGATTCTCCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGA CAGGTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAAC AGGGAATATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTT CTTACGTAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTTTTCTT GCTTAAATCTATAACTACAAAAAACACATACATAAACTAAAA
TPI1- FBP1h	ATTTAAACTGTGAGGACCTTAATACATTCAGACACTTCTGCGGTATCACCCTACTTATTCCCTTC GAGATTATATCTAGGAACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAGA**CCGGACGGATG** **GAATCGCCG**ATGTTACACCTGGACACCCCTTTTCTG**CCGGACGGATGGAATCGCCG**AGTGGC ATGTGAGATTCTCCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAA ACTGACAGGTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCT GTAACAGGGAATATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTT CCCTTCTTACGTAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCT TTTCTTGCTTAAATCTATAACTACAAAAAACACATACATAAACTAAAA
TPI1- Gcr1D	ATTTAAACTGTGAGGACCTTAATACATTCAGACACTTCTGCGGTATCACCCTACTTATTCCCTTC GAGATTATATCTAGGAACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAGACTTTTTACAGAAG TCTATTGATGTTACACCTGGACACCCCTTTTCTACAGAAGAGTTTTTAATCTTCAGTGGCATGTG AGATTCTCCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGA CAGGTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAAC AGGGAATATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTT CTTACGTAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTTTTCTT GCTTAAATCTATAACTACAAAAAACACATACATAAACTAAAA
TEF1pr	CACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGC GCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCCCCTCTTTCTTCCTCTA GGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTT CTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAAATTTTTTTTTTTGATT TTTTTCTCTTTCGATGACCTCCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTC AGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAA TCTAATCTAAGTTTTAATTACAAA
PGK1pr	AGAAGTACCTTCAAAGAATGGGGTCTTATCTTGTTTTGCAAGTACCACTGAGCAGGATAATAATA GAAATGATAATATACTATAGTAGAGATAACGTCGATGACTTCCCATACTGTAATTGCTTTTAGTTG TGTATTTTTAGTGTGCAAGTTTCTGTAAATCGATTAATTTTTTTTTCTTTCCTCTTTTTATTAACCTT AATTTTTATTTTAGATTCCTGACTTCAACTCAAGACGCACAGATATTATAACATCTGCATAATAGG CATTTGCAAGAATTACTCGTGAGTAAGGAAAGAGTGAGGAACTATCGCATACCTGCATTTAAAG ATGCCGATTTGGGCGCGAATCCTTTATTTTGGCTTCACCCTCATACTATTATCAGGGCCAGAAAA AGGAAGTGTTTCCCTCCTTCTTGAATTGATGTTACCCTCATAAAGCACGTGGCCTCTTATCGAGA

	AAGAAATTACCGTCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGC TCGACTTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGGTCCTAGCGACG GCTCACAGGTTTTGTAACAAGCAATCGAAGGTTCTGGAATGGCGGGAAAGGGTTTAGTACCACA TGCTATGATGCCCACTGTGATCTCCAGAGCAAAGTTCGTTCGATCGTACTGTTACTCTCTCTCTT TCAAACAGAATTGTCCGAATCGTGTGACAACAACAGCCTGTTCTCACACACTCTTTTCTTCTAAC CAAGGGGGTGGTTTAGTTTAGTAGAACCTCGTGAAACTTACATTTACATATATATAAACTTGCAT AAATTGGTCAATGCAAGAAATACATATTTGGTCTTTTCTAATTCGTAGTTTTTCAAGTTCTTAGAT GCTTTCTTTTTCTCTTTTTTACAGATCATCAAGGAAGTAATTATCTACTTTTTACAACAAATATAAA ACA
ADH2pr	TCTCTCCGGTTACAGCCTGTGTAACTGATTAATCCTGCCTTTCTAATCACCATTCTAATGTTTTAA TTAAGGGATTTTGTCTTCATTAACGGCTTTCGCTCATAAAAATGTTATGACGTTTTGCCCGCAGG CGGGAAACCATCCACTTCACGAGACTGATCTCCTCTGCCGGAACACCGGGCATCTCCAACTTAT AAGTTGGAGAAATAAGAGAATTTCAGATTGAGAGAATGAAAAAAAAAAAAAAAAAAAAGGCAGA GGAGAGCATAGAAATGGGGTTCACTTTTTGGTAAAGCTATAGCATGCCTATCACATATAAATAGA GTGCCAGTAGCGACTTTTTTCACACTCGAAATACTCTTACTACTGCTCTCTTGTTGTTTTTATCAC TTCTTGTTTCTTCTTGGTAAATAGAATATCAAGCTACAAAAAGCATACAATCAACTATCAACTATT AACTATATCGTAATACACA

References

(1) Strucko, T.; Magdenoska, O.; Mortensen, U. H. Benchmarking Two Commonly Used Saccharomyces Cerevisiae Strains for Heterologous Vanillin-β-Glucoside Production. *Metabolic Engineering Communications* **2015**, *2*, 99–108.

(2) Stovicek, V.; Borodina, I.; Forster, J. CRISPR–Cas System Enables Fast and Simple Genome Editing of Industrial Saccharomyces Cerevisiae Strains. *Metabolic Engineering Communications* **2015**, *2*, 13–22.

(3) Jessop-Fabre, M. M.; Jakočiūnas, T.; Stovicek, V.; Dai, Z.; Jensen, M. K.; Keasling, J. D.; Borodina, I. EasyClone-MarkerFree: A Vector Toolkit for Marker-Less Integration of Genes into *Saccharomyces Cerevisiae* via CRISPR-Cas9. *Biotechnol. J.* **2016**, *11* (8), 1110–1117.

(4) Soontorngun, N.; Larochelle, M.; Drouin, S.; Robert, F.; Turcotte, B. Regulation of Gluconeogenesis in Saccharomyces Cerevisiae Is Mediated by Activator and Repressor Functions of Rds2. *Mol. Cell. Biol.* **2007**, *27* (22), 7895–7905.

(5) Zhang, N.; Wu, J.; Oliver, S. G. Gis1 Is Required for Transcriptional Reprogramming of Carbon Metabolism and the Stress Response during Transition into Stationary Phase in Yeast. *Microbiology* **2009**, *155* (Pt 5), 1690–1698.