

**Highly multiplexed CRISPRi repression of respiratory functions enhances mitochondrial localized
ethyl acetate biosynthesis in *Kluyveromyces marxianus***

Ann-Kathrin Löbs¹, Cory Schwartz¹, Sarah Thorwall¹, and Ian Wheeldon^{1,2 *}

1. Chemical and Environmental Engineering, University of California Riverside, Riverside CA 92521
2. Center for Industrial Biotechnology, Bourns College of Engineering, University of California
Riverside, Riverside CA 92521

*Correspondence: iwheeldon@engr.ucr.edu

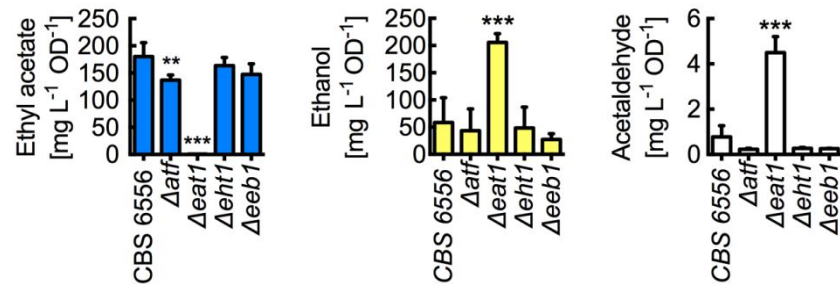


Figure S1. *EAT1* disruption eliminates ethyl acetate production and increases ethanol and acetaldehyde accumulation in *K. marxianus*. Statistical significance was determined by one-way ANOVA with pairwise comparison to the CBS6556 control (* indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; $n \geq 3$ for all samples).

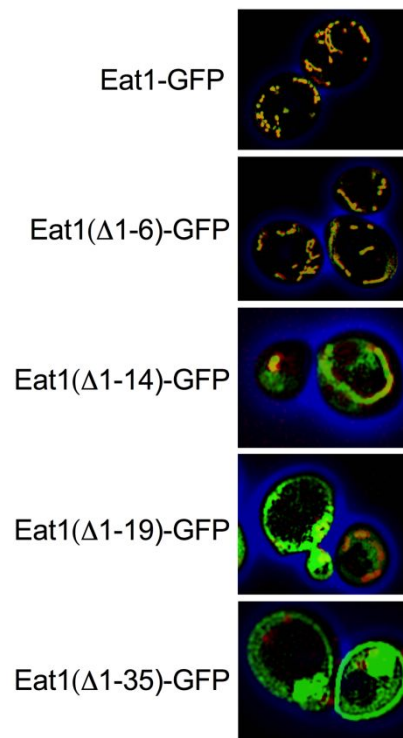


Figure S2. Intracellular localization of Eat1-GFP variants expressed in *S. cerevisiae*. Fluorescence microscopy of *S. cerevisiae* episomally expressing Eat1-GFP variants with mitochondria identified by the mitochondrial marker *OM45-DSRED*. Images were acquired using a 100x objective and merged images of green fluorescence (green), red fluorescence (red), and phase contrast (blue) are shown. Yellow color indicates colocalization of red and green fluorescence

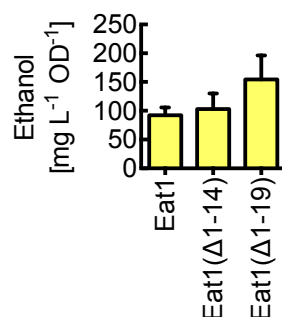


Figure S3. Ethanol production from CBS 6556 $\Delta ura3 \Delta eat1$ expressing the wildtype sequence of Eat1 and 2 N-terminal truncations. Analysis was performed after 10h of shake flask cultivation in rich media at 37°C (25mL, YPD). All bars represent arithmetic means of biological replicates and error bars represent the standard deviation.

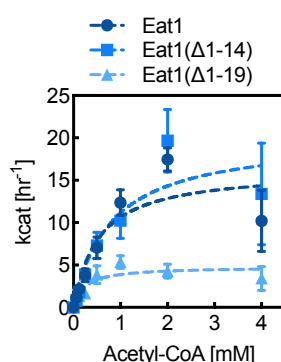


Figure S4. Kinetic analysis of Eat1 and Eat1 N-terminal truncations. His-tagged enzymes were heterologous expressed in *E. coli* and purified by nickel affinity chromatography and size exclusion chromatography as described in Methods. Kinetic parameters: Eat1, $k_{cat} = 16 \text{ hr}^{-1}$, $K_M = 0.5 \text{ mM}$; Eat1(Δ 1-14), $k_{cat} = 20 \text{ hr}^{-1}$, $K_M = 0.2$; Eat1(Δ1-19), $k_{cat} = 5 \text{ hr}^{-1}$, $K_M = 0.5 \text{ mM}$

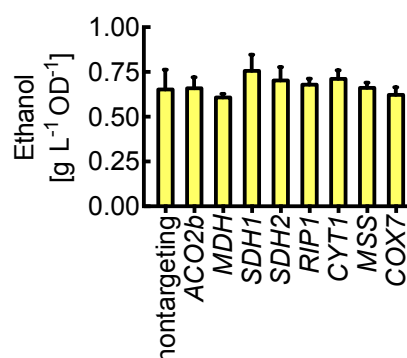


Figure S5. Ethanol production from *K. marxianus* with CRISPRi repression of selected TCA cycle and ETC genes. Cultures were grown in shake flasks to late exponential phase at 14h in selective media to maintain the plasmid (37°C, 50mL, SD-U). All bars represent arithmetic means of biological replicates and error bars represent the standard deviation.

> *K. marxianus* codon-optimized *S. pyogenes* dCas9

MDKKYSIGLAIGTNSVGWAVITDEYKVPSSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRI
CYLQEIFSNEMAKVDDSFHRLSEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLV DSTDKADLR LIYLALA
HMIKFRGHFLIEGDLNPDNSDVKLFIQLVQTYNQLFEENPINASGVDAKILSARLSKSRLENLIAQLPGEKKNGLFG
NLIALSLGLTPNFKSNFDLAEDAKLQLSKD TYDDDLNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSAS
MIKRYDEHHQDLTLLKALVRQQLPENYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKNREDLL
RKQRTFDNGSIPHQIHLGELHAILRRQEDFYPLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFE
EVVDKGASAQSFIERMTNFDKNLPNEKVLPHKSLLEYEFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNR
KVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLKKIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTY
AHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMLIHDDSLTFKEDIQKAQVSGQG
DSLHEHIANLAGSPAIIKGIQTVKVVDLVKVMGRHKPENIVEMARENQTTQKGQKNSRERMKRIEIGIKELGSQILK
EHPVENTQLQNEKLYLYLQNGRDMYVDQELDINRLSDYDVAIVPQSFLKDDSIDNKVLRSDKNRGKSDNVPSEE
VVKMKMNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRLVETRQITKHVAQILDSRMNTKYDENDKLIREV
KVITLKSCLVSDFRKDFQFYKRVREINNYHHAHDAYLNAVVG TALIKKYPKLESEFVYGDYKVYDVRKMIKSEQEI GKAT
AKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL SMPQVNIVKKTEVQTGGFSKESILPK
RNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVAKVEKGSKKLKSVKELLGITIMERSSSFENPIDFLEAKGYKEVKK
DLIILPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFY LASHYEKLKGSPEDEQKQLFVEQHKHYLDEIIE
QISEFSKRVLADANLDKVL SAYNKH RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVL DATLIHQ SIT
GLYETRIDLSQLGGD

Figure S6. *K. marxianus* codon-optimized dCas9 from *S. pyogenes*. Mutation of D10A and H840A renders Cas9 unable to cut double strand DNA (red amino acids)

Table S1. Yeast strains used in this work.

Name	Genotype	Reference
YS5	<i>S. cerevisiae</i> BY4742 MAT α <i>his3</i> Δ 1 <i>leu2</i> Δ 0 <i>lys2</i> Δ 0 <i>ura3</i> Δ 0	GE Healthcare
YS402	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i>	1
YS578	<i>S. cerevisiae</i> BY4742 OM45-DSRED	This work
YS679	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>atf</i>	1
YS963	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>iah1</i>	This work
YS964	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eht1</i>	This work
YS965	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eeb</i>	This work
YS966	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>adh9</i>	This work
YS967	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>adh8</i>	This work
YS968	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>sl1</i>	This work
YS969	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i>	This work
YS1064	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i> URA3::GPDp-EAT1-CYC1t	This work
YS1066	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i> URA3::GPDp-EAT1-GFP-Cyc1t	This work
YS1087	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i> URA3::GPDp-(Δ 1-42)EAT1-Cyc1t	This work
YS1089	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i> URA3::GPDp-(Δ 1-52)EAT1-Cyc1t	This work
YS1091	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i> URA3::GPDp-(Δ 1-42)EAT1-GFP- Cyc1t	This work
YS1093	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>eat1</i> URA3::GPDp-(Δ 1-52)EAT1-GFP-Cyc1t	This work
YS1131	<i>K. marxianus</i> CBS 6556 Δ <i>ura3</i> Δ <i>aco2b</i>	This work

Table S2. Plasmids used in this work.

Name	Sequence	Reference
pIW272	pJSKM316-GDP	Lee <i>et al.</i> ²
pIW512	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmMDH1 T1-SUP4	This work
pIW513	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmSDH1 T1-SUP4	This work
pIW514	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmSDH2 T1-SUP4	This work
pIW578	pIW272 HIS3 marker	This work
pIW601	TEF1-KmCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-PspXI-SUP4	This work
pIW602	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-PspXI4-SUP4	This work
pIW630	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-scramble DNA-SUP4	This work
pIW653	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-ACO2b T1-SUP4	This work
pIW654	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-ACO2b T2-SUP4	This work
pIW778	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmPDB1 T2-SUP4	This work
pIW779	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmPDB1 2x-SUP4	This work
pIW780	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmMDH1 T2-SUP4	This work
pIW781	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmMDH1 2x-SUP4	This work
pIW782	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmSDH1 T2-SUP4	This work
pIW783	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmSDH1 2x-SUP4	This work
pIW784	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmSDH2 T2-SUP4	This work
pIW785	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmSDH2 2x-SUP4	This work
pIW786	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmACO2b 2x-SUP4	This work
pIW821	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmMSS51 T1-SUP4	This work
pIW822	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmMSS51 T2-SUP4	This work
pIW823	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmCOX7 T1-SUP4	This work
pIW824	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmCOX7 T2-SUP4	This work
pIW825	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmRIP1 T1-SUP4	This work
pIW826	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmRIP1 T2-SUP4	This work
pIW827	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmCYT1 T1-SUP4	This work
pIW828	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmCYT1 T2-SUP4	This work
pIW829	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmMSS51 2x-SUP4	This work
pIW830	TEF1-KmdCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-KmCOX7 2x-SUP4	This work
pIW857	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-IAH1 T3	This work
pIW860	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-EHT1 T3	This work
pIW862	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-EEB1 T3	This work
pIW863	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-ADG9 T2	This work
pIW865	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-ADH8 T2	This work
pIW867	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-SLI1 T2	This work
pIW869	TEF1-KmCAS9-SV40-CYC1 KmRPR1p-Gly-tRNA-EAT1 T1	This work
pIW967	URA3 T1 HD:: GPDp-EAT1-Cyc1t	This work
pIW968	URA3 T1 HD:: GPDp-EAT1-GFP-Cyc1t	This work
pIW972	PGK1p-KmEAT1-GFP-PGK1t	This work
pIW973	PGK1p-KmEAT1(delta1-18)-GFP-PGK1t	This work
pIW977	PGK1p-KmEAT1(delta1-42)-GFP-PGK1t	This work
pIW978	PGK1p-KmEAT1(delta1-57)-GFP-PGK1t	This work
pIW998	PGK1p-KmEat1(delta1-105)-GFP-PGK1t	This work
pIW1005	URA3 T1 HD:: GPDp-EAT1(delta1-42)-CYC1t	This work
pIW1006	URA3 T1 HD:: GPDp-EAT1(delta1-57)-CUC1t	This work
pIW1007	URA3 T1 HD:: GPDp-EAT1(delta1-42)-GFP-CYC1t	This work
pIW1008	URA3 T1 HD:: GPDp-EAT1(delta1-57)-GFP-CYC1t	This work
pIW1019	pET-28b EAT1 expression HIS Tag	This work
pIW1020	pET-28b (1-42)EAT1 expression HIS Tag	This work
pIW1021	pET-28b (1-57)EAT1 expression HIS Tag	This work
pIW1023	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x RIP1 2x	This work
pIW1024	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x SDH 2	This work
pIW1025	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x ACO2b 1	This work
pIW1026	TEF1-KmdCAS9-SV40-CYC1 RIP1 2x SDH 2	This work
pIW1027	TEF1-KmdCAS9-SV40-CYC1 RIP1 2x ACO2b 1	This work
pIW1028	TEF1-KmdCAS9-SV40-CYC1 ACO2b 1 SDH 2	This work
pIW1029	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x RIP1 2x SDH 2	This work
pIW1030	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x RIP1 2x ACO2b 1	This work
pIW1031	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x RIP1 2x SDH 2 ACO2b 1	This work

pIW1063	TEF1-KmdCAS9-SV40-CYC1 RIP1 2x SDH 2 ACO2b 1	This work
pIW1064	TEF1-KmdCAS9-SV40-CYC1 MSS51 2x SDH 2 ACO2b 1	This work
pIW1099	TEF1-KmCAS9-SV40-CYC1 KmPRP1-Gly-tRNA-ACO2b T1-SUP4	

Table S3. Primers used in this work.

Name	Sequence (5'→3')
P1853 dCas9 1F	AAGTTTTCTAGAACTAGTGGATCCCCGGGAAAAATGGACAAGAAGTACTCTATCGGTTTGGCTATCGG
P1854 dCas9 1 R	TACCACTCTGTTGG
P1855 dCas9 2 F	TTGTGGAACGATAGCATCAACATCGTAGTCAGACAATC
P1856 dCas9 2 R	TACGATGTTGATGCTATCGTTCCACAATCCTTTTG
P1877 KmAct1Q F	ACCAAGTTGGAGTATACGCCCTAGGAAGGCAAATTAAGCCTTCG
P1878 KmAct1Q R	CCCAATGAACCCAAAGAATAACAG
P1894 KmMDH1 KD sgRNA F	GATAGCATGAGGCAAGGAGAAACC
P1895 KmMDH1 KD R	CCTCGAGGATTATCATACCAAGAAGTAGTTTTAGAGCTAGAAAATAGCAAGTTAAA
P1897 KmSDH1 KD sgRNA F	TAAACTACTTCTTGGTATGATAATCCTCGAGGTTGACACTGACGGGATTTC
P1898 KmSDH1 KD R	CCTCGAGGTTAGTATTATGTGATTGGGGTTTTAGAGCTAGAAAATAGCAAGTTAAA
P1900 KmSDH2 KD sgRNA F	CTAAAACCCCAATCACATAATACTAACCTCGAGGTTGACACTGACGGGATTTC
P1901 KmSDH2 KD R	CCTCGAGGCTTTTGAAGTCGCAAAAAAATTTTAGAGCTAGAAAATAGCAAGTTAAA
P1928 URA3 HDR Screen F	CTAAAATTTTTTTGCGACTTCAAAGACCTCGAGGTTGACACTGACGGGATTTC
P1929 URA3 HDR Screen R	TTGTATCATCACTCCCAGTCAA
AL20 pIW272 His3 F	ACTGAGTATAGTGAGTAGTAGTGTTGAT
AL21 pIW272 His3 R	CCAAAGGTGTTCTTATGTAGGACGTCAAACTGTATTATAAGTAAATGCATGTATA
AL22 His3 F	AAAAATATAGAGTGTACTAGCCTAGGAAGCGTGGTGCACCTCTCAGT
AL23 His3 R	ACTGAGAGTGCACCCAGCTCTCTAGGCTAGTACACTCTATTTTTTTATGCCT
AL45 dCas9 scrambled F	TTTACTTATAATACAGTTTTGACGTCTACATAAGAACACCTTTGGTGG
AL46 dCas9 scrambled R	TCGAATCCCGTCAGTGTCAAATTCGGATGAACGTAGGAATTTTTAGAGCTAGAAAATAGCA
AL47 URA3 T1 HD1 F	TGCTATTTCTAGCTCTAAAAATTCCTACGTTTCATCCGAATTTGACACTGACGGGATTTCGA
AL49 URA3 T2 HD1 F	CGAATTTGGAGCTCCACCGCGGTGGCGTCAACAGTACTCGAATATAATGC
AL55 MDH1 QPCR F	CGCTCGAAGGCTTTAATTTGCGCGCGGGTCTCTAGCGCACGGTGA
AL56 MDH QPCR R	CCACTTCCATCGCCAAGAAC
AL57 SDH1 QPCR F	TTTTGCTTCAAGACCTCGGC
AL58 SDH1 QPCR R	CGACAAGACCTTCGCTGATG
AL59 SDH2 QPCR F	TTTGCGCGTTTGAGTAGCAC
AL60 SDH2 QPCR R	CTTGTGTGCTTGTGTTCCAC
AL75 Aco2b 1 F	GATACCCGAGGCCTCATCTC
AL76 Aco2b 1 R	TCGAATCCCGTCAGTGTCAAATATATAGACGGAGCCAATTTTTAGAGCTAGAAAATAGCA
AL77 Aco2b 2 F	TGCTATTTCTAGCTCTAAAAATTTGGTCCGCTCTATATTTTTGACACTGACGGGATTTCGA
AL78 Aco2b 2 R	TCGAATCCCGTCAGTGTCAAACATTATCAACAGATGATATTTTTAGAGCTAGAAAATAGCA
AL116 URA3 HD1 R	TGCTATTTCTAGCTCTAAAAATATCATCTGTTGATAATGTTTTGACACTGACGGGATTTCGA
AL117 URA3 HD2 R	GTATTGATAATGATAAACTGCGGCCCATGGGCATTAAACAACCCCTCTAGGTT
AL139 KmRPR1 2nd F	AAGGGAACAAAAGCTGGTACCGCGCGTGGTCAAAGCAGCGTTTTGC
AL140 KmmsgRNA 2nd R	TTTTTTTGTTTTTATGTCTCTGCCGCGGTATACTCCAACCTTGGTCGAAAG
AL145 MDH1 2 F	ACTAAAGGGAACAAAAGCTGGTACCGCTCGAGGAGACATAAAAAACAAAAAAGCACC
AL146 MDH1 2 R	TCGAATCCCGTCAGTGTCAATAGTAGTATATGTATGTACTTTTTAGAGCTAGAAAATAGCA
AL147 SDH1 2 F	TGCTATTTCTAGCTCTAAAAAGTACATACATATACTACTATTGACACTGACGGGATTTCGA
AL148 SDH1 2 R	TCGAATCCCGTCAGTGTCAAATGCTCAGTAGATCAGTGAGTTTTAGAGCTAGAAAATAGCA
AL149 SDH2 2 F	TGCTATTTCTAGCTCTAAAACTCACTGATCTACTGAGCATTGACACTGACGGGATTTCGA
AL165 MSS51 1 F	TCGAATCCCGTCAGTGTCAACAACCTTACAATCTTTTTTTTTTAGAGCTAGAAAATAGCA
AL166 MSS51 1 R	TCGAATCCCGTCAGTGTCAAGCACATTTATCCCTATCATTTTTTAGAGCTAGAAAATAGCA
AL167 MSS51 2 F	TGCTATTTCTAGCTCTAAAAATGATAGGGATAAATGTGCTTGACACTGACGGGATTTCGA
AL168 MSS51 2 R	TCGAATCCCGTCAGTGTCAAAGTTGGAAGTATAGATACTTTTTTAGAGCTAGAAAATAGCA
AL169 COX7 1 F	TGCTATTTCTAGCTCTAAAAAGTATCTATACTTCCAACCTTGACACTGACGGGATTTCGA
AL170 COX7 1 R	TCGAATCCCGTCAGTGTCAAAAAGAGTTGTGATTAGAGAGTTTTAGAGCTAGAAAATAGCA
AL171 COX7 2 F	TGCTATTTCTAGCTCTAAAACTCTCTAATCACAACCTCTTTTTGACACTGACGGGATTTCGA
AL172 COX72 R	TCGAATCCCGTCAGTGTCAAAATAAAAAAATTTATATTTTTAGAGCTAGAAAATAGCA
AL173 RIP1 1 F	TGCTATTTCTAGCTCTAAAAATCAAAAGAAAGCATTAAATTTGACACTGACGGGATTTCGA
AL174 RIP1 1 R	TCGAATCCCGTCAGTGTCAATATTAATGCTTTCTTTTGAATTTTAGAGCTAGAAAATAGCA
AL175 RIP2 1 F	TGCTATTTCTAGCTCTAAAACTCACTGATCTACTGAGCATTGACACTGACGGGATTTCGA
AL176 RIP1 2 R	TCGAATCCCGTCAGTGTCAAAACAAAATCAAATCAGAACCGATTGACACTGACGGGATTTCGA
AL177 CYT1 1 F	TGCTATTTCTAGCTCTAAAACTCAATTTAATTTATATTCTTTGTTTTTAGAGCTAGAAAATAGCA
AL178 CYT1 1 R	TCGAATCCCGTCAGTGTCAACTTAAATTTATATTCTTTGTTTTTAGAGCTAGAAAATAGCA
AL179 CYT1 2 F	TGCTATTTCTAGCTCTAAAACTCAATTTGAGTGGTTTTTTATAGCTTTTAGAGCTAGAAAATAGCA
AL180 CYT1 2 R	TGCTATTTCTAGCTCTAAAAAGCTATAAAAAAACCACTCAATTGACACTGACGGGATTTCGA
AL181 Eht1 cPCR F	TTGGTATACCCCTTTACCGAAAAGCA
AL182 Eht1 cPCR R	TGTCAGACAATTCAAACAACCTCTCTCC
AL183 Eeb1 cPCR F	GATTAGAAGAATTGAGATTGAAGATAGTATCATGAAAAACGGG
AL185 Adh9 cPCR f	GGGAAAATAAATCCGCACATGTCTTCATATACC
AL186 Adh9 cPCR R	TCCAAGCCGCGGTGCTTCAT
AL187 Adh8 cPCR F	CTCTATGGATCCATCGGTGTTTTGG
AL188 Adh8 cPCR R	ATGAATAAATGCTGAACCTGTATTCTTGCTCTT
AL191 SLI1 cPCR F	AGTAAATGAACCATTCACGTTTATACCCCTG

AL192 SLI1 cPCR R	TGTATGCGGACATTCCATCGAAAAAC
AL197 Aco2b qPCR F	AACATGGGTGCTGAAATCGG
AL198 Aco2b qPCR R	TCATCGGCAACCAACAAGTC
AL199 Mss51 qPCR F	AAGAGTACTGGATGGGCGAC
AL200 Mss51 qPCR R	CGTACTGGTCCTGCACAATG
AL201 Cox7 qPCR F	TGCCATCCCCACAAAGAATC
AL202 Cox7 qPCR R	AGCGAACAACCCCAAGAATG
AL203 Rip1 qPCR F	CCTCAAGCCGATTCTGACAG
AL204 Rip1 qPCR F	GAACCAACCACCGAAGTCAC
AL205 Cyt1 qPCR F	TGTTTCTCACACCAACGCTG
AL206 Cyt1 qPCR R	TCTGGCAGCTTGTTCTGTTTG
AL213 IAH1 T3	TCGAATCCCGTCAGTGTCAACCATTCTCAACGTCATATATTTTAGAGCTAGAAATAGCA
AL219 Adh9 T2	TCGAATCCCGTCAGTGTCAATTATTGAAACAGGTCCGTTTTTTTAGAGCTAGAAATAGCA
AL221 ADH8 T2	TCGAATCCCGTCAGTGTCAAAATATACCAGCAACACAAAAATTTTAGAGCTAGAAATAGCA
AL223 Sli1 T2	TCGAATCCCGTCAGTGTCAATCGAACAATAGGCTTCAACATTTTAGAGCTAGAAATAGCA
AL216 Eht1 T3	TCGAATCCCGTCAGTGTCAAGAAATTCATGTCTTTTATTTTAGAGCTAGAAATAGCA
AL218 Feb1 T3	TCGAATCCCGTCAGTGTCAAACTATCCTGATGGTGGTGAATTTTAGAGCTAGAAATAGCA
AL225 Eat1 T1	CGTCAGTGTCAACCTCGAGGGAGCCTTCAACCAACAAAAATTTTAGAGCTAGAAATAGCA
AL227 Eat1 cPCR F	GGCATATTTGCACACAATTATTAGC
AL228 Eat1 cPCR R	CCACAAGGTTAACCTTACCCAA
AL250 Feb1 cPCR T3 R	AGTGGATTGCAACTGTTTCATCAGGA
AL254 Eat1 T1 R	TGCTATTTCTAGCTCTAAAAATTTTGTGGTTGAAGGCTCCCTCGAGGTTGACACTGACG
AL270 Eat1 URA3 T1 HD F	TTCTAGAACTAGTGGATCCCCCGGGATGCTTCTCGCTTACACCGT
AL271 Eat1 URA3 T1 HD R	GACATAACTAATTACATGACTCGAGTTTAATCTCTAGCACTTTTGAGAGATTCAG
AL272 Eat1-GFP URA3 T1 HD R	CACCAGTCATGCTAGCCATCCCTCCGCCTAGGGATCCGCCTCCATCTCTAGCACTTTTGAGAGATTCAG
AL275 HDR Screen GPDp 2R	AAATGGCGAGTATTGATAATGATAAACTG
AL276 KmEat1 PGKp F	TTTCTCTTTTTACAGATCACCGCGGATGCTTCTCGCTTACACCGT
AL277 GFP PGKt R	ATCTATCGATTTCAATTCAATTCAATACTAGTTCATTTGTATAGTTCATCCATGCCAT
AL280 delta 1-18Eat1 F	TTCTCTTTTTTACAGATCACCGCGGATGGTCAGACCATCTAACTGGTCTTT
AL281 delta 1-42Eat1 F	TTCTCTTTTTTACAGATCACCGCGGATGACCAGAAGAGCATACTCTGC
AL282 delta 1-57Eat1 F	TTCTCTTTTTTACAGATCACCGCGGATGCTGCTACTGCCAGAGCCTT
AL283 delta 1-105Eat1 F	TTCTCTTTTTTACAGATCACCGCGGATGCCAATCAAGGAACTGTGATATGG
AL285 delta 1-42Eat1F Km int URA3	TTCTAGAACTAGTGGATCCCCCGGGATGACCAGAAGAGCATACTCTGC
AL286 delta 1-57Eat1F Km int URA3	TTCTAGAACTAGTGGATCCCCCGGGATGCTGCTACTGCCAGAGCCTT
AL287 Ecoli Eat1 F	GTTTAACTTTAAGAAGGAGATATACCATGCTTCTCGCTTACACCGT
AL288 Ecoli Eat1 R	AGTGGTGGTGGTGGTGGTGGTCTCGAGATCTCTAGCACTTTTGAGAGATTCAG
AL289 Ecoli Eat1(1-42) F	GTTTAACTTTAAGAAGGAGATATACCATGACCAGAAGAGCATACTCTGC
AL290 Ecoli Eat1(1-57) F	TTCTAGAACTAGTGGATCCCCCGGGATGACCAGAAGAGCATACTCTGC
AL291 qPCR GFP F	TCGGGATCTGTACGACGATG
AL292 qPCR GFP R	TTACCCCTCTCCACTGACAG
Cr_1539	GTGGTGCTTTTTTTGTTTTTATGTCTctgCCGCTATACTCCAACCTGGTCGAAAGATG
Cr_1540	CAATCAAGCTCGGATTACGGTGTTCACTCCAGACATAAAAAACAAAAAAGCACCACC
Cr_1541	GGAGTGAACACCGTAATCCGAGCTTGATTGTATACTCCAACCTGGTCGAAAGATG
Cr_1542	CATCTTTTCGACCAAGTTGGAGTATACCGCAGACATAAAAAACAAAAAAGCACCACC
Cr_1547	CACTAAAGGGAACAAAAGCTGgtaCCGCAGACATAAAAAACAAAAAAGCACCACC
Cr_1548	CGCTCGAAGGCTTTAATTTGCcttCCTAGTATACTCCAACCTGGTCGAAAGATG
Cr_1549	CTTTTCGACCAAGTTGGAGTATAcgcCCTAGAGACATAAAAAACAAAAAAGCACCACC
Cr_1551	GTCCTGCTGTACTCACGGTCTGTAATCCAAGACATAAAAAACAAAAAAGCACCACC
Cr_1552	TGGATTACAGACCGTGAGTAACAGCAGGACTATACTCCAACCTGGTCGAAAGATG
Cr_1598	GACGCTCGAAGGCTTTAATTTGCcttCCTAGGTATACTCCAACCTGGTCGAAAGATG
Cr_1599	CTTTTCGACCAAGTTGGAGTATAcgcCCTAGAGACATAAAAAACAAAAAAGCACCACC
Cr_1645	GCCAGCAAACTAAAAAACTGTATTATAAGTAAATGCAGGCTTTAATTTGCCTTCTTAGG
Cr_1646	ATTGAAGCTCTAATTTGTGAGTTAGTATACATGCACCTCACTAAAGGGAACAAAAGC

Table S4. sgRNA sequences

Name	20 Sequence and PAM (5'-> 3')
ATF1 KO	ATATAGTCTTCGGCAACACCGGG
EAT1 KO	GAGCCTTCAACCAACAAAAAGGG
IAH1 KO	CCATTTCTCAACGTCATATAGGG
EHT1 KO	AGAAATTCAATGTCTTTTATGGG
EEB1 KO	ACTATCCTGATGGTGGTGAAGGG
SLI1 KO	TCGAACAATAGGCTTCAACAGGG
ADH8 KO	ATATACCAGCAACACAAAAAGGG
ADH9 KO	TTATTGAAACAGGTCCGTTTGGG
ACO2b KO	CTTGTAAGTTAATGAAGGAGTGGG
URA3 for HR	TTGCCGAGTTATCGTCCAAGGGG
Scrambled sgRNA	ATCCGGATGAACGTAGGAAT (no PAM)
ACO2b sgRNA 1	AATATATAGACGGAGCCAATCGG (TATA, antisense)
ACO2b sgRNA 2	AACATTATCAACAGATGATATGG (TSS, antisense)
MDH1 sgRNA 1	ATTATCATACCAAGAAGTAGAGG (TATA, antisense)
MDH1 sgRNA 2	TAGTAGTATATGTATGTACTTGG (TSS, antisense)
SDH1 sgRNA 1	TTAGTATTATGTGATTGGGGGGG (TATA, antisense)
SDH1 sgRNA 2	ATGCTCAGTAGATCAGTGAGAGG (TSS, sense)
SDH2 sgRNA 1	TCTTTGAAGTCGCAAAAAAATGG (TATA, antisense)
SDH2 sgRNA 2	CAACTTACAAATCTTTTTTTTGG (TSS, sense)
RIP1 sgRNA 1	TATTAATGCTTTCTTTTGAATGG (TSS, antisense)
RIP1 sgRNA 2	TCGGTTCTGATTTGATTTTGTGG (TATA, sense)
CYT1 sgRNA 1	CTTAAATTTATATTCTTTGTTGG (TSS, antisense)
CYT1 sgRNA 2	TTGAGTGGTTTTTTTATAGCAGG (TATA, sense)
MSS51 sgRNA 1	GCACATTTATCCCTATCATTTGG (TSS, antisense)
MSS51 sgRNA 2	AGTTGGAAGTATAGATACTTTGG (TATA, sense)
COX7 sgRNA 1	AAAGAGTTGTGATTAGAGAGAGG (TSS, sense)
COX7 sgRNA 2	ATAATAAAAAAATTTATATGG (TATA, sense)

References

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2. Lee, K.S. et al. Characterization of *Saccharomyces cerevisiae* promoters for heterologous gene expression in *Kluyveromyces marxianus*. *Applied microbiology and biotechnology* **97**, 2029-2041 (2013).