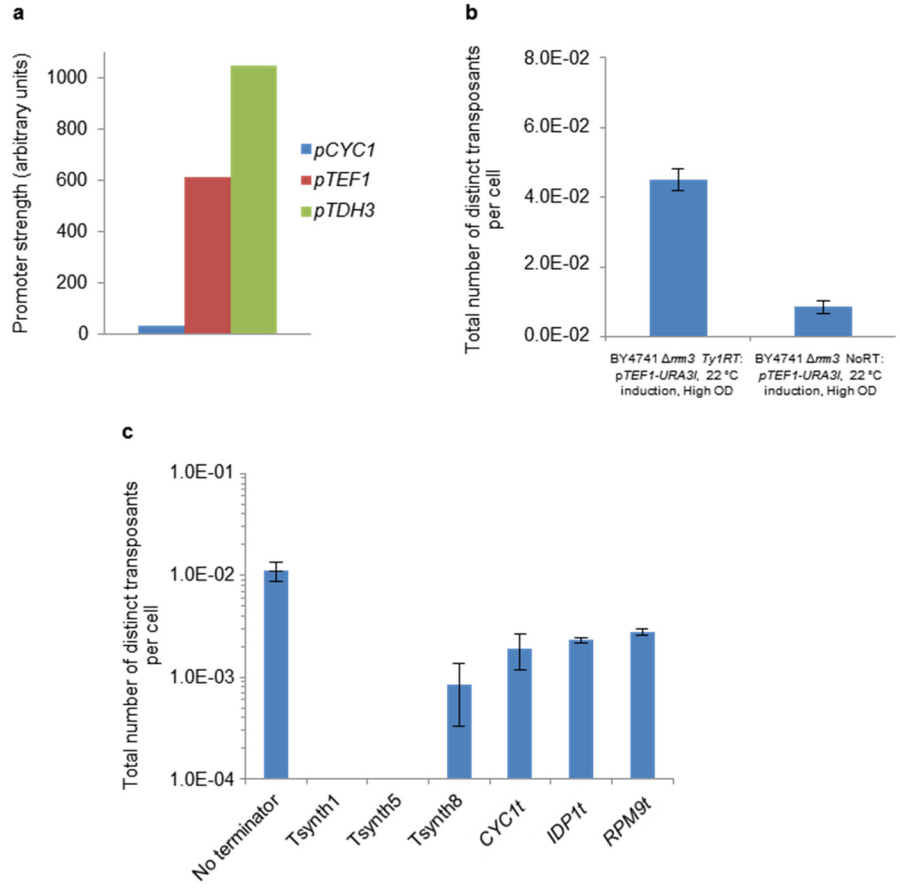
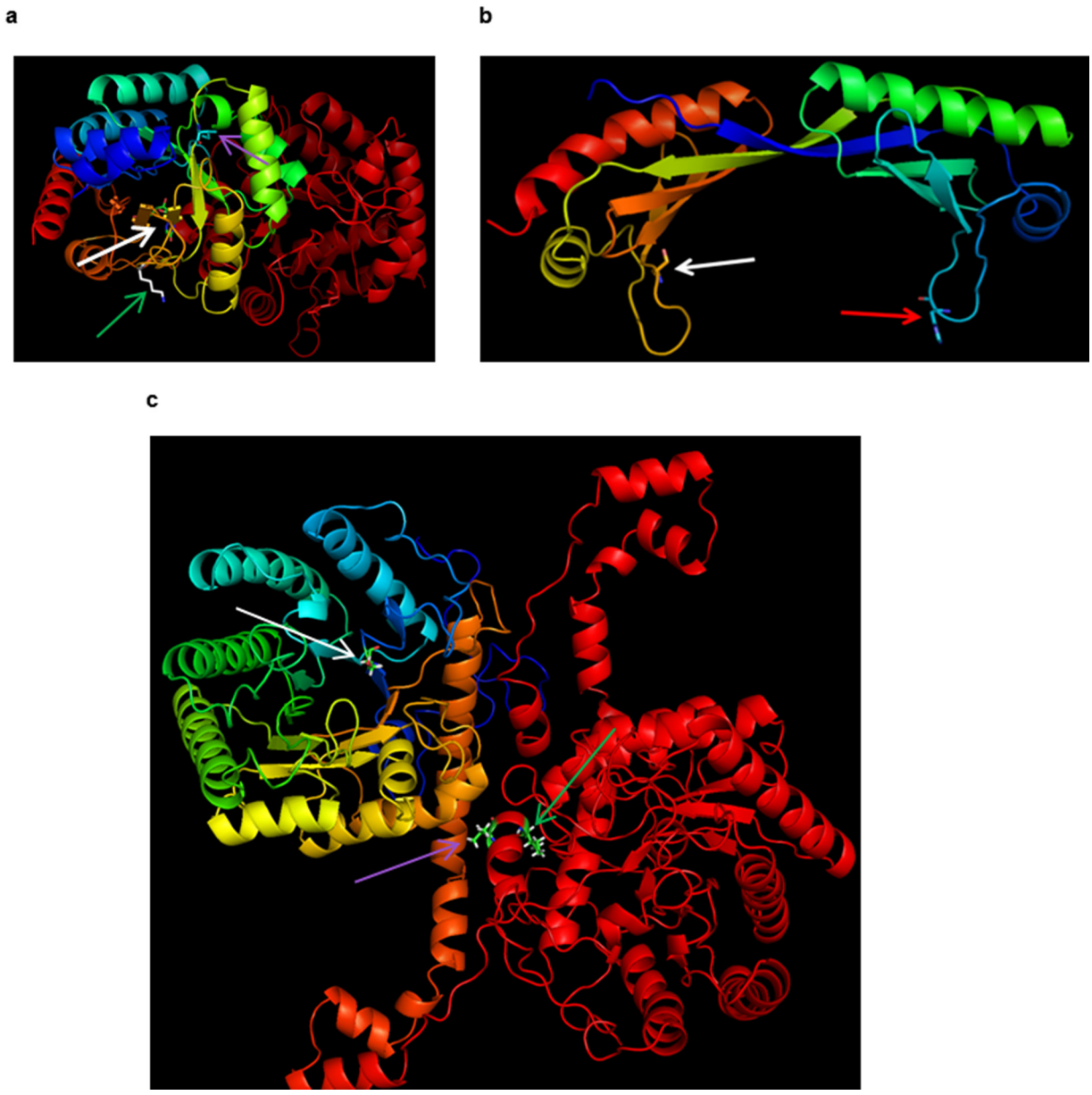
Supplementary Figures

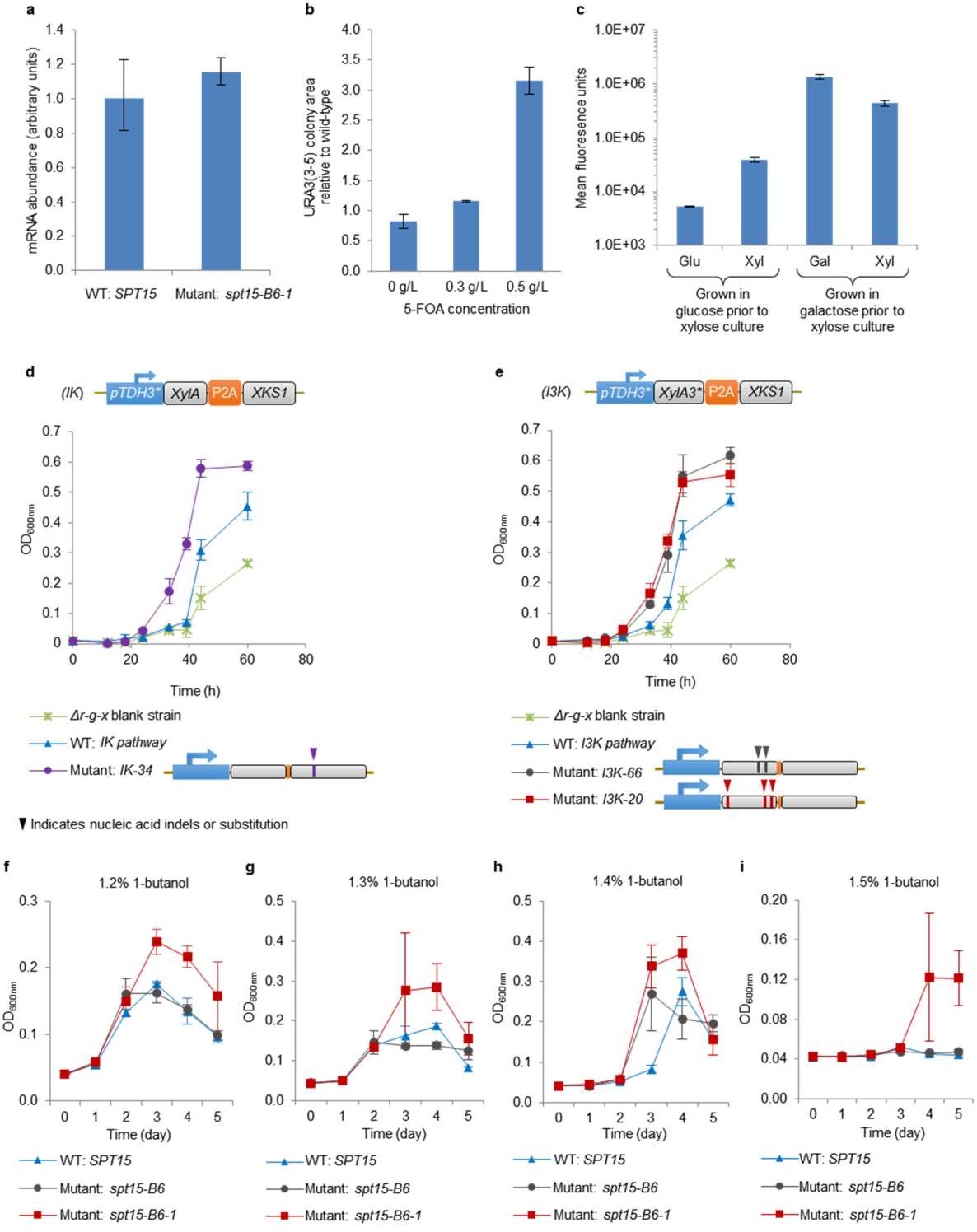


Supplementary Figure 1 | Improvement of Ty1 Transposition. **a**, Promoters used in this study. Indicated promoters were used to drive yellow fluorescent protein and fluorescence was measured using flow cytometry. **b**, Transposition of RT-containing and RT-less retroelements in a genomic context at 22ºC. **c**, The effect of terminators on transposition rate. No transpositions were observed for Tsynth1 or Tsynth5. Strains containing the appropriate retroelement were exposed to galactose at high OD for (**b**) and (**c**) for three days and then plated on uracil-deficient media to count transposants. Error bars in (**b**) and (**c**) represent the standard deviation of biological triplicates.

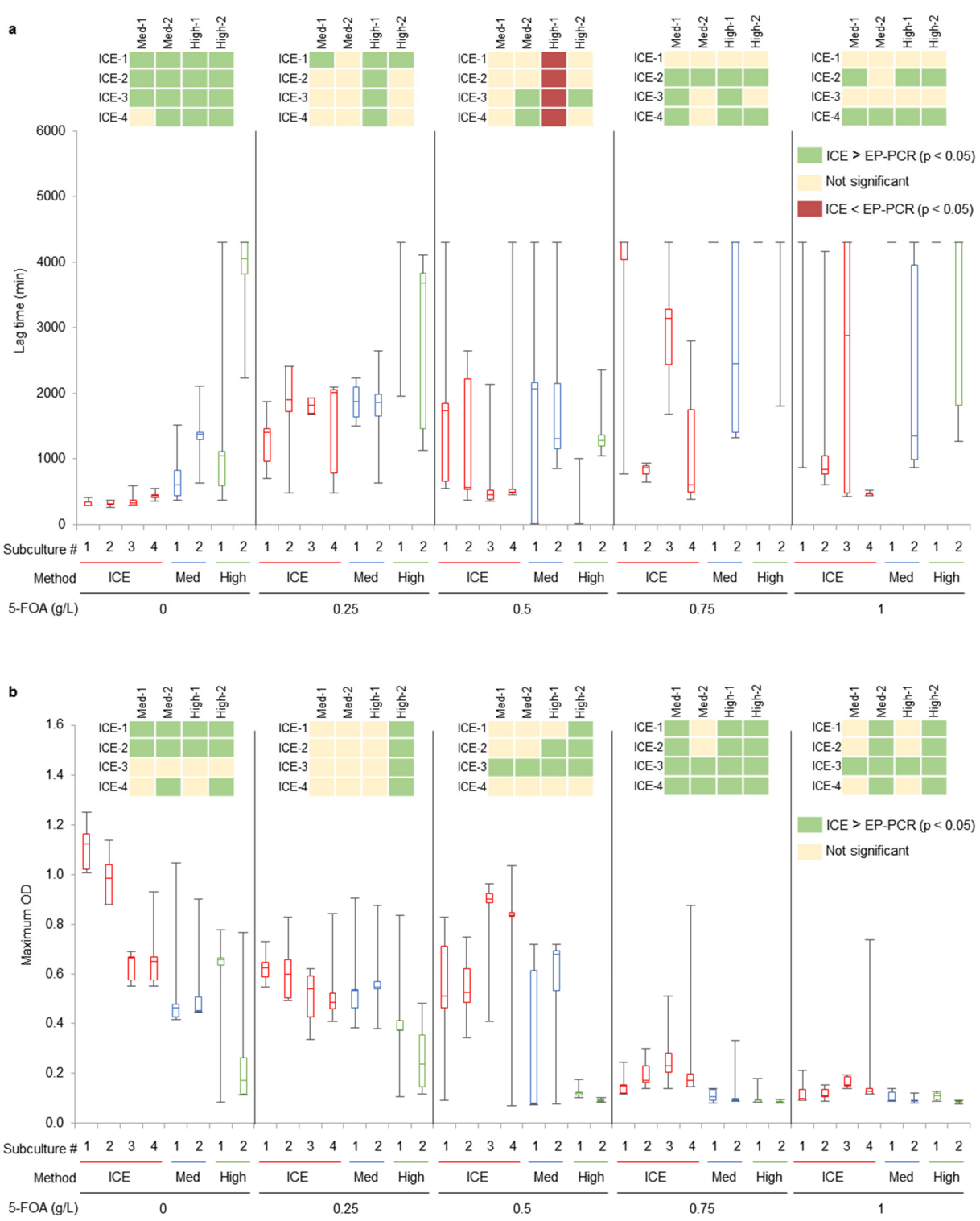


Supplementary Figure 2 | Predicted structure of ICE mutants. **a**, Predicted structure of the *ura3(3-5-2)p* homodimer. One monomer is colored red. The orotidine-5'-monophosphate ligand is shown in its catalytic site with a white arrow, the Arg^{145}Ile mutant is shown by the purple arrow, and the Arg^{186}Lys mutant is shown by the green arrow. **b**, Predicted structure of mutant *spt15-B6-1p*. The amino acid substitution Arg^{98}His indicated by the red arrow. The amino acid substitution Gly^{192}Ser which defines *spt15-B6-1* is indicated by the white arrow. **c**,

Predicted structure of the I3K-20 homodimer. One monomer is colored red. The $Ala^{48}Ser$ mutation is indicated by the white arrow, the $Ile^{433}Val$ mutation is indicated by the purple arrow, and the $Met^{435}Ile$ mutation is indicated by the green arrow.

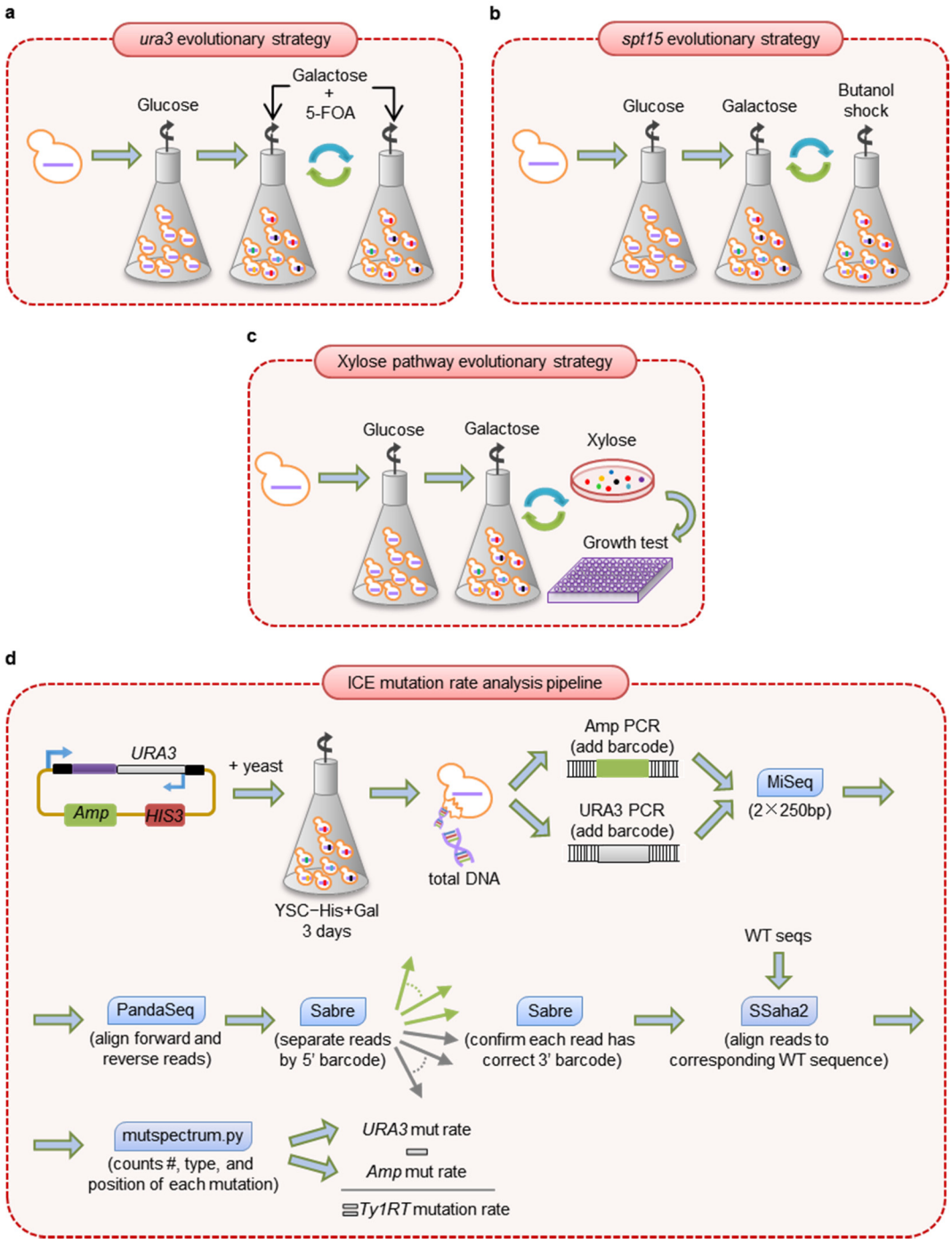

a
mRNA abundance (arbitrary units)
WT: SPT15
Mutant: spt15-B6-1
b
URA3(3-5) colony area relative to wild-type
0 g/L
0.3 g/L
0.5 g/L
5-FOA concentration
c
Mean fluoresence units
Glu
Xyl
Gal
Xyl
Grown in glucose prior to xylose culture
Grown in galactose prior to xylose culture
d
(IK)
pTDH3*
XylA
P2A
XKS1
OD600nm
Time (h)
Δr-g-x blank strain
WT: IK pathway
Mutant: IK-34
e
(I3K)
pTDH3*
XylA3*
P2A
XKS1
OD600nm
Time (h)
Δr-g-x blank strain
WT: I3K pathway
Mutant: I3K-66
Mutant: I3K-20
Indicates nucleic acid indels or substitution
f
1.2% 1-butanol
g
1.3% 1-butanol
h
1.4% 1-butanol
i
1.5% 1-butanol
OD600nm
Time (day)
WT: SPT15
Mutant: spt15-B6
Mutant: spt15-B6-1

Supplementary Figure 3 | Characterization of ICE mutants. **a**, Transcriptional rates of *SPT15* mutants. *SPT15* expression levels were measured through qRT-PCR. **b**, Growth of *URA3(3-5)*-containing strains in media lacking uracil and containing 5-FOA. Indicated strains were plated on solid media lacking uracil and containing the indicated concentration of 5-FOA. Colony area was measured using automated image analysis software. Error bars represent standard deviation observed among ~100 analyzed colonies. **c**, Activity of *pGAL1* in xylose-containing media. Strains containing a fluorescent reporter driven by *pGAL1* were cultured in the appropriate medium for several days, then re-seeded in a different carbon source overnight before measurement of fluorescence. Growth test of *xylA* (**d**) and *xylA3** (**e**) pathway mutants. Indicated strains were inoculated into 1 mL of xylose-containing minimal media at a starting optical density of 0.01 and optical density was measured over the course of 3 days. **f**, Growth test of *spt15* mutants in 1.2% 1-butanol, **g**, 1.3% 1-butanol, **h**, 1.4% 1-butanol, **i**, 1.5% 1-butanol. For (**a**), error bars represent standard deviation of technical triplicates. For (**b-i**), error bars represent the standard deviation of biological triplicates.



Supplementary Figure 4 | Comparison of Lag time and Max OD of ICE-derived and EP-PCR-derived mutants. a, Lag time. **b**, Max OD. Lines on boxplots represent, from top to

bottom, maximum observed value, 75th percentile, median, 25th percentile, and minimum observed value. P-values used to generate heatmaps were computed using a Mann-Whitney U-test.



Supplementary Figure 5 | Selection methods employed during evolution of genetic cargos in this study and sequencing analysis workflow. **a**) Cells with *URA3*-containing retroelements were precultured in glucose and transferred to galactose-containing media for three days to induce mutagenesis. Induced cells were exposed to increasing concentrations of 5-FOA in

uracil-deficient, galactose containing media in order to continuously generate and select for *ura3* variants maintaining uracil biosynthesis activity while reducing activity on 5-FOA. After several serial transfers, single isolates were selected. Beneficial *ura3* mutants were transferred into a fresh Ty1/yeast genomic background and the induction/selection procedures were repeated during a second round. **b**) Cells with *SPT15*-containing retroelements were precultured in glucose and transferred to galactose-containing media for three days to induce mutagenesis. Induced cells were exposed to a short "shock" of high 1-butanol concentration to deplete detrimental variants, and single isolates were selected. Beneficial *spt15* mutants were transferred into a fresh Ty1/yeast genomic background and the induction/selection procedures were repeated during a second round. **c**) Cells with xylose pathway-containing retroelements were precultured in glucose and transferred to galactose-containing media for three days to induce mutagenesis. Induced cells were plated on xylose- containing agar plates in order to continuously generate and select for pathway variants with increased activity. Single isolates were selected by choosing the largest yeast colonies and subsequently assayed for increased growth in liquid culture. **d**) Computational workflow for analysis of Ty1 mutation rates through next-generation sequencing. All computer programs are available upon request.

Supplementary Tables S1 to S14

Supplementary Table 1: Mutations identified at position 135 in d*KanMX* reversion mutants.

Codon	# recovered
TGA (stop codon in d*KanMX*)	6
TGG (Trp)	17
TGT (Cys, present in WT *KanMX*)	7
TCA (Ser)	6
AGA (Arg)	4
CGA (Arg)	4
GGA (Gly)	3
TGC (Cys)	1
TTA (Leu)	1
Total	**49**

Supplementary Table 2: Comparison of ICE to other mutagenesis techniques**.**

Method	Mutation Rate (kb^{-1})	Total mutants generated ($round^{-1}$ $cell^{-1}$)	Distinct mutants generated (per round per 10^{10} cells transformed)			Localization of mutants	Mutagenesis occurs	Continuous diversity generation?	Effort per round	Notes
			100bp	1000bp	5000bp					
ICE	0.15	$3.7*10^{-2}$ [a]	$2.7*10^{4}$ [b]	$2.7*10^{6}$ [b]	$1.6*10^{7}$ [c]	Genome-wide or along defined cargo[d]	*in vivo*	Rounds can be continuous or discrete	Cargo integrated into genome, followed by cell outgrowth.	
EP-PCR	0-16	10^{-2} [e]	$1.2*10^{7}$ [fgh]	$9.5*10^{7}$ [fg]	$9.5*10^{7}$ [fg]	Along defined cargo	*in vitro*	No	Cargo is mutated using epPCR, cloned into an expression vector, amplified in *E. coli*, and transformed into yeast. [i]	[j]
Orthogonal DNA polymerase [1]	$4*10^{-5}$	1	$3*10^{2}$ [b]	$3*10^{3}$ [b]	$1.5*10^{4}$ [b]	Along defined cargo	*in vivo*	Yes	Cargo is integrated into plasmid, followed by cell outgrowth.	[k]
Random drift [2]	$2*10^{-7}$	1	$1.5*10^{2}$ [b]	$1.5*10^{3}$ [b]	$7.3*10^{3}$ [b]	Genome-wide	*in vivo*	Yes	Cell outgrowth.	
YOGE [3]	user-defined	$2*10^{-4}$	$1.9*10^{6}$ [l]			Site-directed	*in vivo* from library generated *in vitro*	Continual recombination of existing libraries [m]	Two independent libraries generated through epPCR or oligo synthesis, cloned into *E. coli*, and then combined using YOGE through multiple yeast transformations. [in]	[o]
CRISPR-Cas9 [4]	user-defined	$7.5*10^{-5}$ [p], $2.2*10^{-5}$ [q], or 10^{-2} [r]	$7.1*10^{5}$ [lp], $2.1*10^{5}$ [lq], or $9.5*10^{7}$ [lr]			Site-directed	*in vivo* from library generated *in vitro*	Continual recombination of existing libraries [m]	Two independent libraries generated through epPCR or oligo synthesis, cloned into *E. coli*, and then combined using CRISPR/Cas9 through multiple yeast transformations. [in]	[s]
Mating/ Recombination [5]	user-defined	$6*10^{-2}$	$5.7*10^{8}$ [lt]			Site-directed	*in vivo* from library generated *in vitro*	Continual recombination of existing libraries [m]	Two independent libraries generated through epPCR or oligo synthesis, followed by cloning into *E. coli* and transformation into yeast. These two *in vivo* libraries are then combined through mating. [i]	

a) Fig. 2b.

b) This number was calculated using PEDEL[6] with a "Library size" of 10^{10} x (number of total mutants generated) and a "mean number of point mutations per sequence" of 0.15 x (cargo length). It is important to note that the number of distinct amino acid variants will be much lower than these values for random mutant libraries, ranging from 20-50%, depending on the nucleotide sequence being mutated[7]. For libraries incorporating synthetic oligonucleotides, such as the "site-directed" methods above, this efficiency can approach 100%.

c) Number of total mutants generated for a 5kb cargo has been calculated using a library size of $9.49*10^{-3}$ per cell (Supplementary Figure 2a).

d) Genome-wide mutagenesis occurs during continuous mutagenesis/selection as Ty1 integrates into alternate loci in the genome.

e) This is the maximum yeast plasmid transformation efficiency [8].

f) Consider M cells being transformed with a DNA library of size L. The probability that a particular member of L is delivered to a particular member of M is 1/L. The probability that this member of L is not delivered to this member of M is 1-(1/L). The probability that this member of L is not delivered to any member of M is $(1-(1/L))^M$. Thus, the probability that this member of L is delivered to at least one member of M is $1-(1-(1/L))^M$. Summing over all members of L, the expected value of the number of members of L which were delivered at least once to the cells in M is given by $L(1-(1-(1/L))^M)$. This calculation assumes that the DNA molecules composing L are numerous enough that successful transformation events do not significantly change the proportion of each member in L. This calculation also assumes that each member of L is at the same proportion. This is not true for error-prone-PCR-derived libraries since sequences with fewer mutations likely outnumber those with larger numbers of mutations. This bias will reduce the number of distinct mutants generated beyond the simple formula presented here.

g) For error-prone PCR, a 10^{-2} transformation efficiency implies 10^8 library-containing cells, so M for these cells is 10^8.

h) $1.2*10^7$ is the maximum number of distinct variants of a 100bp sequence which can be generated through error-prone PCR using a 16 kb^{-1} mutation rate, and so for this cell L was assumed to be $1.2*10^7$.

i) Most methods for diversity generation have been benchmarked for cell populations ~10^8 in size. Effort has been scaled accordingly to bring these methods into line with the scale of ICE.

j) For each template size we have assumed a mutation rate of 16 kb^{-1} to generate an upper bound for the number of distinct mutants attainable by error-prone PCR. Although the required library size L is different for every experiment, and has been reported exceeding 10^{12} for *E. coli* [9], we have chosen 10^9 here as this is the minimum value enabling ~95% of transformed yeast cells to contain unique library members. Percentages approaching 100% are attained with severely diminishing returns.

k) Authors note a requirement for the mutated gene to be expressed from cytoplasmic RNAP with low expression capacity.

l) Consider M cells which already contain a library of size L1 (such that the number of cells which contain each member of L1 is M/L1), and which are being transformed with a different library of size L2. The probability that a particular member of L2 is delivered to a particular member of M is 1/L2. The probability that this member of L2 is not delivered to this member of M is 1-(1/L2). The probability that this member of L2 is not delivered to any member of M containing a particular member of L1 is $(1-(1/L2))^{M/L1}$. Thus, the probability that this member of L2 is delivered to at least member of M containing a particular member of L1 is $1-(1-(1/L2))^{M/L1}$. Summing over all members of L1 and L2, the expected value of the number of unique L1-L2 combinations is $L1*L2*(1-(1-(1/L2))^{M/L1})$. This represents the best-case scenario of no biases in library representation among L1 or L2 libraries. The general form of this expression is (sum(i=1 to L1) of $L2*(1-(1-(1/L2))^{Mi})$ where Mi is # cells transformed with each member of L1. The number of distinct mutants generated will be lower if, for example, there are a large population of WT cells in L1.

m) In our opinion, the major benefit of these approaches over methods such as error-prone PCR for random mutagenesis of genes and pathways is the ability to explore combinations of two distinct libraries, so that's the mode that is highlighted here. YOGE and CRISPR-Cas9 can also be used for generation of mutations throughout the yeast genome, but as this is a different goal than that of ICE, it is not considered in detail here.

n) This is the effort required to generate one library from 10^{10} total cells. Additional effort is required to combine multiple libraries.

o) "Number of total mutants generated" is a combination of 1% electoporation survival rate and 2% recombination frequency in survivors [3]. We have chosen two libraries of size 4400, as these are the minimum values enabling ~95% of transformed yeast cells to contain unique library members. To accomplish this task using a single library, the library would need to contain $1.9*10^7$ unique members in order for $1.9*10^6$ to make it into yeast.

p) 90bp dsoligo repair template

q) 1.4kb repair template

r) Plasmid repair template, yeast plasmid transformation frequency

s) Library size for plasmid repair template assumes pre-transformation of Cas9 and repair plasmid, followed by transformation of sgRNA plasmid using the LiAc/ssDNA method, as this is the method which results in the largest theoretical library size. If instead one wishes to generate the same diversity using a single library (instead of combining multiple libraries), this library must be at least $7.2*10^6$ (90bp dsoligo repair template), $2.1*10^6$ (1.4kb repair template), or $9.6*10^8$ (plasmid repair template).

t) This has been calculated assuming L2 is # distinct variants in donor cells

Supplementary Table 3: Enzyme kinetics and sequence analysis of XylA and XylA3* pathway mutants.

Mutant	Parent	ICE performance (rounds and cycles)	Selection condition	Growth rate (h^{-1})	V_{max} (µmol min^{-1} mg $protein^{-1}$)	K_m (mM)	Mutations
***I3K*-66**	*XylA3** pathway	one round and continuous cycles	YP + 20 g L^{-1} Xylose plate	0.140	0.126 ± 0.008	90.03 ± 14.40	$Ile^{433}Val$ (in XylA3*)
***I3K*-20**	*XylA3** pathway	one round and continuous cycles	YSC −His + 20 g L^{-1} Xylose plate	0.134	0.134 ± 0.003	109.45 ± 1.33	$Ala^{48}Ser$, $Ile^{433}Val$, $Met^{435}Ile$ (in XylA3*)
***IK*-34**	*XylA* pathway	one round and continuous cycles	YSC −His + 20 g L^{-1} Xylose plate	0.136	-	-	$Glu^{164}Lys$ (in XKS1)
***XylA3** pathway Wild type*		-	-	0.106	0.118 ± 0.007	52.06 ± 16.47	-
***XylA* pathway Wild type**		-	-	0.079	-	-	-

Supplementary Table 4: Media Recipes Used in this Study.

Medium	Ingredients	Supplier
Lysogeny Broth (LB)	10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride	Teknova
LB Agar	LB, 15 g L^{-1} agar	This work
LB + ampicillin	LB + 100 μg mL^{-1} ampicillin	This work
LB + ampicillin Agar	LB + ampicillin, 15 g L^{-1} agar	This work
Yeast Extract, Peptone, Dextrose Broth (YPD)	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose.	This work
YPD + G418	YPD, 0.4g L^{-1} G418	This work
YPD Agar	YPD, 15 g L^{-1} agar	This work
YPD Agar + G418	YPD Agar, 0.4 g L^{-1} G418	This work
Yeast Extract, Peptone, Galactose Broth (YPG)	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} galactose.	This work
YPG Agar	YPG, 15 g L^{-1} agar	This work
Yeast Extract, Peptone, Xylose Broth (YPX)	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} xylose.	This work
YPX Agar	YPX, 15 g L^{-1} agar	This work
Yeast Synthetic Complete Media (YSC)	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose, 0.79 g L^{-1} CSM	This work
YSC Gal	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} galactose, 0.79 g L^{-1} CSM	This work
YSC Xyl	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} xylose, 0.79 g L^{-1} CSM	This work
YSC Agar	YSC, 15 g L^{-1} agar	This work
YSC Gal Agar	YSC Gal, 15 g L^{-1} agar	This work
YSC Xyl	YSC Xyl, 15 g L^{-1} agar	This work
YSC −His	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose, 0.77 g L^{-1} CSM −His	This work
YSC Gal −His	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} galactose, 0.77 g L^{-1} CSM −His	This work
YSC Xyl −His	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} xylose, 0.77 g L^{-1} CSM −His	This work
YSC –His Agar	YSC −His, 15 g L^{-1} agar	This work
YSC Gal –His Agar	YSC Gal −His, 15 g L^{-1} agar	This work
YSC Xyl –His Agar	YSC Xyl −His, 15 g L^{-1} agar	This work
YSC −His −Trp	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose, 0.72 g L^{-1} CSM −His −Trp	This work
YSC Gal −His −Trp	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} galactose, 0.72 g L^{-1} CSM −His −Trp	This work
YSC Xyl −His −Trp	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} xylose, 0.72 g L^{-1} CSM −His −Trp	This work
YSC −His −Trp Agar	YSC −His −Trp, 15 g L^{-1} agar	This work
YSC Gal −His −Trp Agar	YSC Gal −His −Trp, 15 g L^{-1} agar	This work
YSC Xyl −His −Trp Agar	YSC Xyl −His −Trp, 15 g L^{-1} agar	This work
YSC −Trp	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose, 0.74 g L^{-1} CSM −Trp	This work
YSC Gal −Trp	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} galactose, 0.74 g L^{-1} CSM −Trp	This work
YSC Xyl −Trp	6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} xylose, 0.74 g L^{-1} CSM −Trp	This work
YSC −Trp Agar	YSC −Trp, 15 g L^{-1} agar	This work
YSC Gal −Trp Agar	YSC Gal −Trp, 15 g L^{-1} agar	This work
YSC Xyl −Trp Agar	YSC Xyl −Trp, 15 g L^{-1} agar	This work

Agar	Agar	Teknova
Ampicillin	Ampicillin	Sigma
G418	Geneticin	Gold Biotechnology
Yeast extract	Yeast extract	Amresco
Peptone	Peptone	Amresco
Glucose	Glucose	MP Biomedicals
Galactose	Galactose	Sigma Aldrich
Xylose	Xylose	Acros
Yeast nitrogen base	(for powder): 5 g ammonium sulfate, 2 µg biotin, 400 µg calcium pantothenate, 2 µg folic acid, 2 mg inositol, 400 µg niacin, 200 µg p-aminobenzoic acid, 400 µg pyridoxine hydrochloride, 200 µg riboflavin, 400 µg thiamin hydrochloride, 500 µg boric acid, 40 µg copper sulfate, 100 µg potassium iodide, 200 µg ferric chloride, 400 µg manganese sulfate, 200 µg sodium molybdate, 400 µg zinc sulfate, 1 g monopotassium phosphate, 0.5 g magnesium sulfate, 0.1 g sodium chloride, 0.1 g calcium chloride.	Difco
CSM	(for powder): 10 mg adenine, 50 mg L-arginine, 80 mg L-aspartic acid, 20 mg L-histidine, 50 mg L-isoleucine, 100mg L-leucine, 50 mg L-lysine, 20 mg L-methionine, 50 mg L-phenylalanine, 100mg L-threonine, 50 mg L-tryptophan, 50 mg L-tyrosine, 20 mg uracil, 140 mg L-valine.	Sunrise Science Products / MP Biomedicals
CSM −His	(for powder): 10 mg adenine, 50 mg L-arginine, 80 mg L-aspartic acid, 50 mg L-isoleucine, 100mg L-leucine, 50 mg L-lysine, 20 mg L-methionine, 50 mg L-phenylalanine, 100mg L-threonine, 50 mg L-tryptophan, 50 mg L-tyrosine, 20 mg uracil, 140 mg L-valine.	Sunrise Science Products / MP Biomedicals
CSM −His −Trp	(for powder): 10 mg adenine, 50 mg L-arginine, 80 mg L-aspartic acid, 50 mg L-isoleucine, 100mg L-leucine, 50 mg L-lysine, 20 mg L-methionine, 50 mg L-phenylalanine, 100mg L-threonine, 50 mg L-tyrosine, 20 mg uracil, 140 mg L-valine.	Sunrise Science Products / MP Biomedicals
CSM −Trp	(for powder): 10 mg adenine, 50 mg L-arginine, 80 mg L-aspartic acid, 20 mg L-histidine, 50 mg L-isoleucine, 100mg L-leucine, 50 mg L-lysine, 20 mg L-methionine, 50 mg L-phenylalanine, 100mg L-threonine, 50 mg L-tyrosine, 20 mg uracil, 140 mg L-valine.	Sunrise Science Products / MP Biomedicals

Supplementary Table 5: Plasmids obtained for this study.

Plasmid Name	Source
p413-*pTEF1*	[10]
p423-*pTDH3*	[10]
p414-*pCYC1*	[10]
p414-*pTDH3*	[10]
p424-*pTEF1*	[10]
p424-*pTDH3*	[10]
p415-*pTEF1*	[10]
p425-*pTDH3*	[10]
p416-*pCYC1*	[10]
p416-*pTEF1*	[10]
p416-*pTDH3*	[10]
p42K	[11]
pUG6	[12]
pSH47	[13]
p416-*pCYC1-YFP*	[14]
p416-*pTEF1-YFP*	[14]
p416-*pTDH3-YFP*	[14]
hCas9	[4]
p416-*pTDH3-eGFP*	[14]
p416-*pTEF1-LacZ*	[14]
p416- *pTDH3-XylA*	[15]
p416- *pTDH3-XylA3**	[15]
pUAStef-UAScit-UASclb	[16]
pmStrawberry	Clontech
p426-*pTEF1*	[10]

Supplementary Table 6: Strains obtained for this study.

Strain	Genotype	Source
***S. cerevisiae* BY4741**	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	EUROSCARF
***S. cerevisiae* CEN.PK2**	*MATa; ura3-52; trp1-289; leu2-3,112; his3Δ1; MAL2-8^{C}; SUC2*	EUROSCARF
***S. cerevisiae* S288C**	*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	ATCC
***S. cerevisiae* YSX3**	*MATα trp1-112 leu2::LEU2-PsXYL1 ura3::URA3-PsXYL2 Ty3::NEO-PsXYL3*	[17]
S. cerevisiae* BY4741 Δ*rrm3	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 rrm3::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*ice2	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 ice2::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*ckb2	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 ckb2::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*hir3	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 hir3::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*apl2	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 apl2::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*mrc1	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 mrc1::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*cac2	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 cac2::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*mre11	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 mre11::KanMX*	Yeast Haploid Knockout Collection
S. cerevisiae* BY4741 Δ*cac3	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 cac3::KanMX*	Yeast Haploid Knockout Collection
***K. lactis* CBS 2359**	*MATa*	ATCC (8585)

Supplementary Table 7: Oligonucleotides used in this study (IDT).

Primer Name	Sequence
GalCas9F	AAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAA CGTCAAGGAGAAAAAACCCCGGATATGGACAAGAAGTACTCC ATTGG
CYC1tCas9R	AGGGCGTGAATGTAAGCGTGACATAACTAATTACATGATCACA CCTTCCTCTTCTTCTTG
Cas9CYC1tF	AGGGCTGACCCCAAGAAGAAGAGGAAGGTGTGATCATGTAAT TAGTTATGTCACGCTTAC
gRNACYC1tR	AAGTGGCACCGAGTCGGTGGTGCTTTTTTTGTTTTTTATGTCTCG AGCGTCCCAAAACCT
gRNAF	TGTACGCATGTAACATTATACTGAAA
gRNAR	AAAACGACGGCCAGTGAG
PacIP416F	/5PHOS/GG-TTAATTAA-GGTACCCAATTCGCCCT
NotICYCtR	/5PHOS/GCGGCCGC-CGAGCGTCCCAAAACCT
NotIgRNAF	GG-GCGGCCGC-AGACATAAAAAACAAAAAAAGCACCA
PacIgRNAR	GG-TTAATTAA-CTTTGAAAAGATAATGTATGATTATGCTTTCA
SacIGalF	CCCCCC-GAGCTC-ACGGATTAGAAGCCGCC
SpeXbaIGalR	GG-ACTAGT-TCTAGA-GGTTTTTTCTCCTTGACGTTAAAG
GalpF1	CTCACTAAAGGGAACAAAAGCTGGAGCTCCTAGTACGGATTAG AAGCCGC
Gal1pFixR2	TATACTAGAAGTTCTCCTCGAGGCGGTAGAGGAATAAGAAGTA ATACAAACCGAAAATGT
GalpF2	CAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCAC TAAAGGGAACAAAAGCT
GalpR2	TTCCATTGTTGATAAAGGCTATAATATTAGGTATACAGAATATA CTAGAAGTTCTCCTCG
TyH3GenomeF	GCGCGGGCAAAGCCCAAAAG
TyH3GenomeR	TGCGCAAGCCCGGAATCGAA
TyH3PCRF1	TATCAACAATGGAATCCCAACAATTATCTCAACATTCACCCAA TTCTCATGGTAGCGCCT
TyH3PCRR1	GAACGGTTTCAATTGGAGAAATTGGAACAGCCTTCAAAGCTGC AATCAGGTGAATTCGTT
TyH3PCRF2	CTTCTAGTATATTCTGTATACCTAATATTATAGCCTTTATCAAC AATGGAATCCCAACAA
TyH3PCRR2	ACTTTTGGACCATCCATACCTGGTTTCAACTTAACTGGAACGGT TTCAATTGGAGAAATT
TyH3PCRF3	CTCGAGGAGAACTTCTAGTATATTCTGTATACCTAATATTATAG CC
TyH3FlankR	ACTTTTGGACCATCCATACCT
HIVnoATGF	GCTTTGAAGGCTGTTCCA
HIVRThomR	ACCGATTATTTAAAGCTGCAGCCCAAGCTTATCGATTTACAAG ATCTTTCTAATACCGGC
URA3F	TTGTAAATCGATAAGCTTGGGCTGCAGCTTTAAATAATCGGTGT TAGTTTTGCTGGCCGC
URA3AIR	GCAGAAAAGCCTCCTTTAGTCCATATTAACATACCCGCGATGA AGGTTACGATTGGTTGA

URA3R	AAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGATGTCGAA AGCTACATATAAGGAACG
URA3AIF	CTCGAATTTTTACTAACAAATGGTATTATTTATAACAGCCGCCC ATGTCTCTTTGAGCAA
AInoass2F	TCTAGAGGATCCCCGGGTACCGAGCTCGAATTTTTACTAACAA ATGGTATTATTTATAAC
AInoass3F	GTATGTTAATATGGACTAAAGGAGGCTTTTCTGCAGGTCGACT CTAGAGGATCCCCGGG
LTRF1	TGTACTAGAGGATCTATTACATTATGGGTGGTATGTTGGAATA GAAATCAACTATCATCT
LTRR1	CGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCTGAG AAATGGGTGAATGTTGA
PPTNotIF	GCGGCCGCGATCTATTACATTATGGGTGGTATGT
LTRflankR	AGGGTTTTCCCAGTCACG
HISpromF	CTTTGCCTTCGTTTATCTTGCC
HISpromR	ACCACCCATAATGTAATAGATCGCGGCCGCCTCTAGTACACTC TATATTTTTTATGCCT
P416F	GGTACCCAATTCGCCCT
P416R	GAGCTCCAGCTTTTGTTCC
Ty1RTHDF1	CCGTTCACCTTCAATCGATGCTTCTCCACCGGAAAATAATTCAT CGCACAATATTGTTCC
Ty1RTHDR1	AAAGCTGCAGCCCAAGCTTATCGATCTAATGAATCCATTTGTTA GTTAATAGTTTAAATG
Ty1RTHDF2	CGCAGATAAGTGACCAAGAGACTGAGAAAAGGATTATACACC GTTCACCTTCAATCGATG
Ty1RTHDR2	CATATTTGAGAAGATGCGGCCAGCAAAACTAACACCGATTATT TAAAGCTGCAGCCCAAG
RTmutF	TCAACAGTAAGAAAAGATCATTAGAAGA
RTmutR	GGAAGGGATGCTAAGGTAGAG
HistermR	CACCGATTATTTAAAGCTGCAGCCC
LTRTEFF2	TACCACCCATAATGTAATAGATCGCGGCCGC-ATAGCTTCAAAATGTTTCTACTCCTTTT
URA3TEFR2	GCACGTTCCTTATATGTAGCTTTCGACAT-AAACTTAGATTAGATTGCTATGCTTTCTTT
RT-URA3-BBf	ATAAGCTTGGGCTGCAGC
RT-URA3-BBr	CGATCTAATGAATCCATTTG
RT-eGFPf	CATTTAAACTATTAACTAACAAATGGATTCATTAG-TGCGTAAAGGAGAAGAACTTTTCAC
RT-eGFPr	CCGATTATTTAAAGCTGCAGCCCAAGCTTATCGAT-TTAAACTGCTGCAGCGTAG
RT-LacZf	CATTTAAACTATTAACTAACAAATGGATTCATTAG-TGACCATGATTACGGATTCAC
RT-LacZr	CCGATTATTTAAAGCTGCAGCCCAAGCTTATCGAT-TTATTTTGACACCAGACCAACTGG
RT-CAN1f	CATTTAAACTATTAACTAACAAATGGATTCATTAG-TGACAAATTCAAAAGAAGACGC
RT-CAN1r	CCGATTATTTAAAGCTGCAGCCCAAGCTTATCGAT-CTATGCTACAACATTCCAAA
Lacz-URA3-BBf	TAAATCGATAAGCTTGGGCTGC

Lacz-URA3-BBr	TTGACACCAGACCAACTGG
LacZ-eGFPf	CATTACCAGTTGGTCTGGTGTCAAAAATAA-TGCGTAAAGGAGAAGAACTTTTCAC
LTRCYCF2	CTATTCCAACATACCACCCATAATGTAATAGATCGCGGCCGC-ATTTGGCGAGCGTTGGT
URA3CYCR2	GTAGCAGCACGTTCCTTATATGTAGCTTTCGACAT-TTAGTGTGTGTATTTGTGTTTGCG
LTRGPDF2	ACCACCCATAATGTAATAGATCGCGGCCGC-AGTTTATCATTATCAATACTCGCCATTTC
URA3GPDR2	GAGTAGCAGCACGTTCCTTATATGTAGCTTTCGACAT-ATCCGTCGAAACTAAGTTCTGG
PPTNotIF	GCGGCCGC-GATCTATTACATTATGGGTGGTATGT
reGREsptR	TAACTAACAAATGGATTCATTAGATCGATAAGCTTGGGCTGCAGCTTTAAATAATCGGTG-TCACATTTTTCTAAATTCACTTAGCACAGG
reGREsptF	TGTTATAAATAATACCATTTGTTAGTAAAAATTCGGCGCGCCAAAAGCCTCCTTTAGTCCATATTAACATAC-TGGCCGATGAGGAACGTT
reGREtefF	GATGATAGTTGATTTCTATTCCAACATACCACCCATAATGTAATAGATCGCGGCCGC-ATAGCTTCAAAATGTTTCTACTCCTTTTTTACT
reGREtefR	CTAAAGGAGGCTTTTGGCGCGCCGAATTTTTACTAACAAATGGTATTATTTATAACAG-TAAACTTAGATTAGATTGCTATGCTTTCTTTC
Intron*ura3(3-5)*F	ATATGGACTAAAGGAGGCTTTTGGCGCGCCGAATTTTTACTAACAAATGGTATTATTTATAACAG-GCCTATAGGTTCCTTTGTTACTTCT
LTRTEFF2	TACCACCCATAATGTAATAGATCGCGGCCGC-ATAGCTTCAAAATGTTTCTACTCCTTTT
Ty1URA3F	CATTTAAACTATTAACTAACAAATGGATTCATTAGATCGATAAGCTTGGGCTGCAGCTTTAAATAATCGGTG-TTAGTTTTGCTGGCCGCA
Intron*ura3(3-5)*R	ATAATACCATTTGTTAGTAAAAATTCGGCGCGCCAAAAGCCTCCTTTAGTCCATATTAACATAC-CTTTTGATGTTAGCAGAATTGTCATG
(m)GAXnewintronF	CATCCCTGCGTCTACAGC
(m)GAXnewintronR	TGTAAATCTATTTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACA-CCAGAACTTAGTTTCGACGGAT
(m)GA3XnewintronXylA3for	TGGTAGCACCAGAACCTTGATAC
TART-FullMCSF	CC-TTAATTAACCGCGGGGATCCCCCGGGGCGGCCGCGATCTATTAC
TART-FullMCSR	CC-TTAATTAACCTGCAGGGAATTCCTAATGAATCCATTTGTTAGTTAATAGTTTAAATGT
NotITEFF	ATAAGAAT-GCGGCCGC-ATAGCTTCAAAATGTTTCTACTCCTT
XmaITEFR	TCCC-CCCGGG-AAACTTAGATTAGATTGCTATGCTTTCT
SacIIXylAR	C-CCGCGG-TTATTGATACATCGCGACAATAGCC
XmaIXylAF	TCCC-CCCGGG-ATGGCTAAAGAATATTTCCCTCAAA
PacIXKS1F	CC-TTAATTAA-ATGTTGTGTTCAGTAATTCAGAGAC

EcoRIXKSR	G-GAATTC-TTAGATGAGAGTCTTTTCCAGTTCG
P2AXKS1F	/5PHOS/CAAGATGGTGATGTTGAAGAAAATCCAGGACCA-TTGTGTTCAGTAATTCAGAGACAGA
P2AXylAR	/5PHOS/TTTTAATAAAGAAAAGTTGGTAGCACCAGAACC-TTGATACATCGCGACAATAGCC
XylAMCSintronF	GCGCCGAATTTTTACTAACAAATGGTATTATTTATAACAGT-CCCGGGAAACTTAGATTAGATTGC
XylAMCSintronR	GCCAAAAGCCTCCTTTAGTCCATATTAACATACTGGCTAAAGA ATATTTCCCTCAAATTC
XmaIGPDR	TCCC-CCCGGG-ATCCGTCGAAACTAAGTTCTGG
NotImegaGPDF	ATAAGAAT-GCGGCCGC-ATAGCTTCAAAATGTTTCTACTCCTT
(m)GA3XnewintronRTfor	ACCAAGAAGAACATTGCTGATGTG
(m)GA3XnewintronP2Arev	TGAATATGGAAAGAAGAATGGAGAACCGAAACAAACCTCAGG AAAACAAGAACTATACGAGGCTATTA-TCGCGATGTATCAAGGTTCTGG
XmaIXylA3F	TCCC-CCCGGG-ATGGCTAAAGAATATTTCCCTCAAA
XylAGPDnointronF	TTTGAATTTGAGGGAAATATTCTTTAGCCATATCCGTCGAAACT AAGTTCTGGTGTTTTA
XylAGPDnointronR	TAAAACACCAGAACTTAGTTTCGACGGATATGGCTAAAGAATA TTTCCCTCAAATTCAAA
GREnewF	TTTCACAGGTAGTTCTGGTCCATTGGTGAAAGTTTGCGGCTTGC AGAGCACAGAGGCCGCAGAATGT-GAACAAAAGCTGGAGCTCCTAGT
GREnewR	ACGAAATTTGCTATTTTGTTAGAGTCTTTTACACCATTTGTCTCC ACACCTCCGCTTACATCAACACCA-CTTGGCCTCCTCTAGTACACT
XbaIXKS1	CCCCCC-TCTAGA-ATGTTGTGTTCAGTAATTCAGAGA
XKS1XhoI	CCCCCC-CTCGAG-TTAGATGAGAGTCTTTTCCAGTTC
GRE3KOfor	GTAATATAAATCGTAAAGGAAAATTGGAAATTTTTTAAAGATA GCTTCAAAATGTTTCTA
GRE3KOrevnew	TTGTTCATATCGTCGTTGAGTATGGATTTTACTGGCTGGA-TTAAGCAAGGATTTTCTTAA
MRE115'F	CCCCCC-CGTACG-TCTTTCCAACAAACCAAGCG
MRE115'R	CCCCCC-GTCGAC-AGTCGAGTTTTATCGGATCTGAGC
MRE113'F	CCCCCC-ACTAGT-TTGTACTTGATCCCTATATTATATTATATCCTATTTATAACC
MRE113'R	CCCCCC-CCGCGG-AGTTCTATTGTGTGTCCAGGC
pUG6KOPCRnewF	CCAGCTGAAGCTTCGTACG
pUG6KOPCRnewR	CCGGCAGATCCGCGG
CAC35'F	CCCCCC-CGTACG-GTGGTTTGTTGTCTGTCTGG
CAC35'R	CCCCCC-GTCGAC-CTTTGAAACTAAATTTGTATATTGTTTGTCAGAA
CAC33'F	CCCCCC-ACTAGT-CCTAAACGTTCCTTGAAGCCA
CAC33'R	CCCCCC-CCGCGG-GTTCGGCTTTGGACATTTCG
BYHIR35'F	CCCCCC-CGTACG-ACTAGCAATGATTCCGTTTTACATTT
BYHIR35'R	CCCCCC-GTCGAC-AATAAGCTTTATCTAGAATCTGTGTTGAGG
BYHIR33'F	CCCCCC-ACTAGT-GATGACCATATTTTGGAAGAAGTGTG

BYHIR33'R	CCCCCC-CCGCGG-CAAATCTTTATCGTAATCAGATAATTTTTCCAA
CAC25'F	CCCCCC-CGTACG-AGAAAGGTCCTCAGATTGAGC
CAC25'R	CCCCCC-GTCGAC-TGTCCTGCCCCTTTGCT
CAC23'F	CCCCCC-ACTAGT-TTTTTAATATATTTAATGCGGTACATAAGAATGCC
CAC23'R	CCCCCC-CCGCGG-TCACGAGAGATGAGTCCACC
BefICE2F	ATGATTCACTGTCACTTAGTGAGC
AftACE2R	CTAAGAGTCTGTTTAGATCAACAGTCT
BefRRM1F	CATAGAACCGAGTGTAACACCA
AftRRM1R	AGGATTCTCCGAATAACCTCTAGC
APL25'F	CCCCCC-CGTACG-TATCCTGATGGAGCACTTCG
APL25'R	CCCCCC-GTCGAC-AGTTGAAACTGTTTTTTAAGTGCAGT
APL23'F	CCCCCC-ACTAGT-CTATAAACGTCCGTTGTAGTGAACT
APL23'R	CCCCCC-CCGCGG-CCTGACATCTTTGGACGTGG
IMT4p	CCG-GAGCTC-AACATCCAGT
IMT4t	GGC-GGTACC-TTATAGTCTATAGCTTAAAT
sup4p	CCG-GAGCTC-ACCATCTTGG
IMT4p	CCG-GAGCTC-AACATCCAGT
XbaITAL1for	CCG-TCTAGA-ATGTCCTCCAACTCCCTTGAAC
XhoITAL1rev	CCCG-CTCGAG-TTAGAATCTGGCTTCCAATTGTTCC
SacItefTAL1for	CCG-GAGCTC-ATAGCTTCAAAATGTTTCTACTC
SacITAL1cyc1rev	GGC-GAGCTC-CAAATTAAAGCCTTCGAGCGTCC
MCS-Fwd-SpeI	G-ACTAGT-ATGTCTAAAGGTGAAGAATTATTCACTGG
MCS-Rev-2	CCCCG-CTCGAG-TTATTTGTACAATTCATCCATACCATGGG
P2AYFPGlcF	ACAAGATGGTGATGTTGAAGAAAATCCAGGACCA-TCTAAAGGTGAAGAATTATTCACTGG
P2AstrawGlcR	TTTAATAAAGAAAAGTTGGTAGCACCAGAACC-CTTGTACAGCTCGTCCATGC
SpeImStrawberryF	CC-ACTAGT-ATGGTGAGCAAGGGCG
EcoRImStrawberryR	G-GAATTC-TTTATCGATCGATT-CTACTTGTACAGCTCGTCCAT
MCS-Fwd-SalI-2	TAACGC-GTCGAC-ATGTCTAAAGGTGAAGAATTATTCACTGG
mStraw-YFPf	GATCCCCCGGG-ATGGTGAGCAAGGGCGAG
mStraw-YFPr	ACTCGAGGAATTC-TTATTTGTACAATTCATCCATACCATGGG
His3AIgenomeflankF	ATCGATAAGCTTGGGCTGC
ARTR	TTAATTCTTAGTATTCCATGTGTCTCGT
HispromSPT15F	AAAAAATGAGCAGGCAAGATAAACGAAGGCAAAG-ATGGCCGATGAGGAACGT
HistermSPT15R	AAGCTTGGGCTGCAGCTTTAAATAATCGGTG-TCACATTTTTCTAAATTCACTTAGCACA
Spt15TEFR	AACTCCTTTAAACGTTCCTCATCGGCCAT-AAACTTAGATTAGATTGCTATGCTTTCTTT

SPT15intronR	GTTAGTAAAAATTCAAAAGCCTCCTTTAGTCCATATTAACATAC TGGCCGATGAGGAACG
SPT15intronF	AAATGGTATTATTTATAACAGTAAACTTAGATTAGATTGCTATG CTTTCTT
IntronSiteF	CGCCGAATTTTTACTAACAAATGGTATTATTTATAACAGT
IntronSiteR	CGCCAAAAGCCTCCTTTAGTCCATATTAAC
TEFmutssacF	CCC-GAGCTC-ATAGCTTCAAAATGTTTCTACTCCTT
p416ura3EcoR1rev	CCG-GAATTC-TTAGTTTTGCTGGCCGCATC
XhoISPT15R	CCCCG-CTCGAG-TCACATTTTTCTAAATTCACTTAGCACA
BefCAC2F	CGTTTCTGAGAGGTAACTGAGG
CAC2BegR	GATTCTTGCTGTGTATTTGG
CAC2EndF	TGATTTAGCATGGTCTGAGG
AftCAC2R	TTGTTGCTGTTGGTCATTGG
BefCAC3F	ACAACCACTTCACCCAAACCC
CAC3BegR	TTGGGAAGATGTAAATGAGG
CAC3EndF	AAGAAGATGGGTTAGTCAAGC
AftCAC3R	TGGAAATGTTGTAGAGTGGAGG
BefAPL2F	TTCTCAACCATCCAAGTCGG
APL2BegR	CCAACTCAACGCATAAATCC
APL2EndF	TAACGATGATGTGCTATTGG
AftAPL2R	CTTGTTGATCTTTCTTCCCACC
BefMRE11F	ATTGATGGCTGATGACGTGG
MRE11BegR	TTGGTAGAGTGACTTCTTGG
MRE11EndF	CCAACGAGCAAACCCAAACG
AftMRE11R	TGTGTTTGAGGGCTCCTTGG
BefBYHIR3F	TCTACGCGGTCCATAATCTCC
HIR3BegR	ATCTAGCGTAGGAGAAGAATTGC
HIR3EndF	ATTTGACAGCGTTTGCTTGG
AftBYHIR3R	TTGACGCAAAGGAAATGTGG
BefGRE3F	ATGGGCGCATTACTACAAGAAG
AftGRE3R	CTGTTTGACGCACTGATGGGT
BegGRE3R	GACCTTCGGAGATGGCTTTC
EndGRE3F	AACCATCCAGGCAGTACCAC
URA3RTPCRF	ATTGTTAGCGGTTTGAAGCAGGCG
URA3RTPCRR	GAGCCCTTGCATGACAATTCTGCT
Alg9F	ATCGTGAAATTGCAGGCAGCTTGG
Alg9R	CATGGCAACGGCAGAAGGCAATAA
Spt15-qPCR2f	GCAATCTAATCTAAGTTTATGGCCGATGAGGA
Spt15-qPCR2r	GTTGCTGGTTTTGTACCATCTCGATTCTGG
NGSAmp1F	ACTGAT-GCCAACTTACTTCTGACAACG
NGSAmp1R	GCTACC-CCGCCTCCATCCAGTC

NGSAmp2F	CGTACG-GCCAACTTACTTCTGACAACG
NGSAmp2R	TGACAT-CCGCCTCCATCCAGTC
NGSnointron1F	GATACA-ATCGCGGCCGCCC
NGSnointron1R	GGAACT-AGAATGGGCAGACATTACGAATG
NGSnointron2F	AGTCAA-ATCGCGGCCGCCC
NGSnointron2R	TAACCG-AGAATGGGCAGACATTACGAATG
NGSnointron3F	AGCTTT-ATCGCGGCCGCCC
NGSnointron3R	TACAAG-AGAATGGGCAGACATTACGAATG
NGSnointron4F	GGCTAC-ATCGCGGCCGCCC
NGSnointron4R	AAGCTA-AGAATGGGCAGACATTACGAATG
NGSnointron5F	ATACGA-ATCGCGGCCGCCC
NGSnointron5R	CTGATC-AGAATGGGCAGACATTACGAATG
NGSnointron6F	TTACTG-ATCGCGGCCGCCC
NGSnointron6R	AGTTCC-AGAATGGGCAGACATTACGAATG
NGSnointron7F	ACTTGA-ATCGCGGCCGCCC
NGSnointron7R	GATCTG-AGAATGGGCAGACATTACGAATG
NGSnointron8F	ACATCT-ATCGCGGCCGCCC
NGSnointron8R	AATCGT-AGAATGGGCAGACATTACGAATG
NGSnointron9F	GCCAAT-ATCGCGGCCGCCC
NGSnointron9R	CACTGT-AGAATGGGCAGACATTACGAATG
NGSnointron10F	AGAATC-ATCGCGGCCGCCC
NGSnointron10R	GCCTAA-AGAATGGGCAGACATTACGAATG
NGSnointron11F	CTGCAG-ATCGCGGCCGCCC
NGSnointron11R	ACATCG-AGAATGGGCAGACATTACGAATG
NGSnointron12F	ATCACG-ATCGCGGCCGCCC
NGSnointron12R	CACGTA-AGAATGGGCAGACATTACGAATG
NGSnointron13F	TCACAT-ATCGCGGCCGCCC
NGSnointron13R	TATAGA-AGAATGGGCAGACATTACGAATG
NGSnointron14F	TGCAAA-ATCGCGGCCGCCC
NGSnointron14R	GTGCCA-AGAATGGGCAGACATTACGAATG
NGSnointron15F	TGTTAG-ATCGCGGCCGCCC
NGSnointron15R	ATAGAA-AGAATGGGCAGACATTACGAATG
NGSnointron16F	TCGAAG-ATCGCGGCCGCCC
NGSnointron16R	GAATGA-AGAATGGGCAGACATTACGAATG
NGSnointron17F	TACAGC-ATCGCGGCCGCCC
NGSnointron17R	TCTGAG-AGAATGGGCAGACATTACGAATG
NGSnointron18F	CTATAC-ATCGCGGCCGCCC
NGSnointron18R	AGCTAG-AGAATGGGCAGACATTACGAATG
NGSnointron19F	CGGAAT-ATCGCGGCCGCCC
NGSnointron19R	GCCATG-AGAATGGGCAGACATTACGAATG

NGSnointron20F	CACGAT-ATCGCGGCCGCCC
NGSnointron20R	GCTCAT-AGAATGGGCAGACATTACGAATG
SacI-HSXf	CC-GAGCTC-GGGTTCATTACAGCAGC
PstI-HSXr	CC-CTGCAG-TATATTTACCATCAACTCCGC
EcoRITkc1F	G-GAATTC-TTTGAAAGATGATACTCTTTATTTCTAGACAG
SbfITkc1R	GG-CCTGCAGG-TATATATATATAACTGTCTAGAAATAAAGAGTATCATCTT
EcoRITkc5F	G-GAATTC-TTTGAAAGATGATACTCTTTATTTATATATATATATATATATATATA
SbfITkc5R	GG-CCTGCAGG-TATATATATATATATATATATATAAATAAAGAGTATCATCTTTCAAA
EcoRITkc8SbfIF	G-GAATTC-TTTGAAAAAATTTATTAAAAAAAAAATATATA-CCTGCAGG-CC
SbfITkc8EcoRIR	GG-CCTGCAGG-TATATATTTTTTTTTTAATAAATTTTTTCAAA-GAATTC-C
URA3AICYC1F	CCG-GAATTC-CGAGCGTCCCAAAACCTT
URA3AICYC1Rnew	CCG-CCTGCAGG-TCATGTAATTAGTTATGTCACGCTTACA
URA3AIIDP1F	CCG-GAATTC-GATGGTAATGATCCGAACTTGG
URA3AIIDP1Rnew	CCG-CCTGCAGG-TCGAATTTACGTAGCCCAATCTAC
URA3AIPRM9F	CCG-GAATTC-ATTTTCAACATCGTATTTTCCGAAGC
URA3AIPRM9Rnew	CCG-CCTGCAGG-ACAGAAGACGGGAGACACT
GREcontrol-fwd	GTCTGTTATTAATTTCACAGGTAGTTCTGGTCCATTGGTGAAAG TTTGCGGCTTGCAGAGCACAGAGGCCGCAGAATGT-GTGATGACAAAACCTCTTCCG
TART-BB-f	T-CAAACAGGAATCGAATGCAACCG
TART-BB-r	AATTGTCCTTTTAACAGCGATCGC
ip-G418f	T-GAAAGAAATGCATAAGCTTTTGC
ip-G418r	AGACTTGTTCAACAGGCC
G418-stop-f	CGAATTCACTGGCCGTCG
G418-stop-r	CAAGCTTGGCGTAATCATGG
G418-stop2-f	GACGTTGTAAAACGACGGCCAGTGAATTCGTCTGTAGCCCTCA ACGGAAA
G418-stop2-r	AGGTCCGCCGGCGTTGGACGAGCGGGTGCAACTAATTGACGGG AGT
JMW-0077	CGAATTCACTGGCCGTCG
JMW-0078	CAAGCTTGGCGTAATCATGG
JMW-0092	GACGTTGTAAAACGACGGCCAGTGAATTCGTCTGTAGCCCTCA ACGGAAA
JMW-0093	AGGTCCGCCGGCGTTGGACGAGCGGGTGCAACTAATTGACGGG AGT
JMW-0094	TGCACCCGCTCGTCCAACGCCGGCGGACCTCGGTGCAACACTA CTTCAACTTC
JMW-0095	ACAGCTATGACCATGATTACGCCAAGCTTGTTGTTCTCCTCATT CCAGGTG
JMW-0101	TCTGTAGCCCTCAACGGAAA

JMW-0102	TTGTTCTCCTCATTCCAGGTG
JMW-0163	TGACACCGATTATTTAAAGCTGCAG
JMW-0164	CTTTGCCTTCGTTTATCTTGCC
JMW-0165	TATATATATCGTATGCTGCAGCTTTAAATAATCGGTGTCAGCCT GATCTGTTTAGCTTGC
JMW-0166	TATACTAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGAC TGGATGGCGGCGTTAGTA
JA-211	AAACTTAGATTAGATTGCTATGC
JA-240	TAAAGCTGCAGCCCAA
JMW-0210	ATTCATTAGATCGATAAGCTTGG
JMW-0211	TTGCTCATTAGAAAGAAAGCAT
JMW-0225	TAGCCAGGAGGAGAGTGACGGCTCCTATGTTGTGTGG
JMW-0226	ATGACGGAAGATGCTCGGGCGGGGTATCGTATGCTTC
JMW-0227	GCGGAACCCCTATTTGTTTATT
JMW-0228	GCTGTTTCCTGTGTGAAATTGTT
JMW-0229	TTGTGAGCGGATAACAATTTCACACAGGAAACAGCTTTGCTTC TCTCTTCTTCCTGTG
JMW-0230	CACACAACATAGGAGCCGTCACTCTCCTCCTGGCTATTG
JMW-0231	AAGCATACGATACCCCGCCCGAGCATCTTCCGTCATTA
JMW-0232	GTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCCTTTTAGG CCCAGAATTAGG
JMW-0280	TTTGCTTCTCTCTTCTTCCTGTG
JMW-0281	TCCACAACCAGTTTCATTCG

Supplementary Table 8: DNA fragments generated in this study.

Fragment Name	Template	Forward Primer/Restriction Enzyme	Reverse Primer/Restriction Enzyme
CAS9	hCas9 vector	GalCas9F	CYC1tCas9R
CYC1t	p415-*pTDH3*	Cas9CYC1tF	gRNACYC1tR
***URA3*gRNA**	*URA3*gRNA	gRNAF	gRNAR
p416-*pGAL1*frag	p416-*pGAL1*	XbaI	XbaI
pGAL1	*S. cerevisiae* BY4741 genome	SacIGalF	SpeXbaIGalR
MCSBB	p416-*pGAL1-CAS9-CYC1t*-URA3gRNA	PacIP416F	NotICYC1tR
***TRP1*gRNA**	TRP1gRNA	NotIgRNAF	PacIgRNAR
***pGAL1*frag1**	*S. cerevisiae* BY4741 genome	GalpF1	Gal1pFixR2
***pGAL1*frag2**	GALfrag1	GalpF2	GalpR2
Ty1frag1	*S. cerevisiae* BY4741 genome	TyH3GenomeF	TyH3GenomeR
Ty1frag2	Ty1frag1	TyH3PCRF1	TyH3PCRR1
Ty1frag3	Ty1frag2	TyH3PCRF2	TyH3PCRR2
Ty1frag4	Ty1frag3	TyH3PCRF3	TyH3FlankR
***HIVRT*frag1**	Synthetic *HIVRT*	HIVnoATGF	HIVRThomR
***URA3AI*frag1**	*S. cerevisiae* s288c genome	URA3F	URA3AIR
***URA3*frag1**	*S. cerevisiae* s288c genome	URA3R	URA3AIF
***URA3*frag2**	URA3frag1	URA3R	AInoass2F
***URA3*frag3**	URA3frag2	URA3R	AInoass3F
LTRfrag1	Synthetic Ty1	LTRF1	LTRR1
LTRfrag2	LTRfrag1	PPTNotIF	LTRflankR
***HIS3*promfrag1**	*S. cerevisiae* BY4741 genome	HISpromF	HISpromR
p423frag1	p423-*pTDH3*	P416F	P416R
***Ty1RT*frag1**	Synthetic *Ty1RT*	Ty1RTHDF1	Ty1RTHDR1
***Ty1RT*frag2**	*Ty1RT*frag1	Ty1RTHDF2	Ty1RTHDR2
pGALmTy1frag1	pGALmTy1-*HIVRT-URA3I-pHIS3*	RTmutF	RTmutR
pGALmTy1-*Ty1RT*BB	pGALmTy1-*Ty1RT-URA3I-pHIS3*	HispromF	HistermR
***pTEF1*hom**	p416-*pTEF1*	LTRTEFF2	URA3TEFR2
***Ty1RT-URA3*-BB**	pGALmTy1-*Ty1RT-URA3I-pHIS3*	RT-URA3-BBf	RT-URA3-BBr
Cargo-*eGFP*	p416-*pTEF1-eGFP*	RT-eGFPf	RT-eGFPr
Cargo-*LacZ*	p416-*pTEF1-LacZ*	RT-LacZf	RT-LacZr
Cargo-*CAN1*	*S. cerevisiae* BY4741 genome	RT-CAN1f	RT-CAN1r

Cargo-*LacZ*-BB	pGALmTy1-*Ty1RT-LacZ-URA3I-pHIS3*	Lacz-URA3-BBf	Lacz-URA3-BBr
Cargo-*eGFP-LacZ*-ins	p416-*pTEF1-eGFP*	LacZ-eGFPf	RT-eGFPr
pGALmTy1-*HIVRT* BB	pGALmTy1-*HIVRT-URA3I-pHIS3*	HispromF	HistermR
***pCYC1*hom**	p416-*pCYC1*	LTRCYCF2	URA3CYCR2
pTDH3	p416-*pTDH3*	LTRGPDF2	URA3GPDR2
MutBB	pGALmTy1-*Ty1RT-URA3I-pHIS3*	PPTNotIF	HistermR
Smut6up	p413-*spt15-B6*	reGREsptR	reGREsptF
Smut6down	p413-*spt15-B6*	reGREtefR	reGREtefF
U3-5up	p413-*ura3(3-5)*	Intron*ura3(3-5)*F	LTRTEFF2
U3-5down	p413-*ura3(3-5)*	Ty1URA3F	Intron*ura3(3-5)*R
(m)GAXnewintron	(m)GAXnewintron(gblock)	(m)GAXnewintronF	(m)GAXnewintronR
(m)GA3Xnewintron	(m)GAX3newintron(gblock)	(m)GA3XnewintronXylA3for	(m)GAXnewintronR
P2A-RT	pGALmTy1-*Ty1RT-GAX-oldintron*	(m)GA3XnewintronRTfor	(m)GA3XnewintronP2Arev
Ty1*TAX*	pGALmTy1-*Ty1RT-TAX*	XylAMCSintronF	XylAMCSintronR
Ty1*XylAXKS*	pGALmTy1-*Ty1RT-pTEF1-XylA-XKS1*	P2AXKS1F	P2AXylAR
FullMCSFrag	pGALmTy1-*Ty1RT-URA3I-pHIS3*	TART-FullMCSF	TART-FullMCSR
pTEF1	p413-*pTEF1*	NotITEFF	XmaITEFR
XylA	p416-*pTDH3-XylA*	XmaIXylAF	SacIIXylAR
XKS1	*S. cerevisiae* BY4741 genome	PacIXKS1F	EcoRIXKSR
pTDH3*	pUAStef-UAScit-UASclb	NotImegaGPDF	XmaIGPDR
pGALmTy1-*Ty1RT-mGA3X-oldintron*frag	pGALmTy1-*Ty1RT-GA3X-oldintron*	XmaI	EcoRI
Ty1*TA3X*	pGALmTy1-*Ty1RT-TA3X*	XylAMCSintronF	XylAMCSintronR
Ty1*XylA3XKS*	pGALmTy1-*Ty1RT-pTEF1-XylA3-XKS1*	P2AXKS1F	P2AXylAR
XylA3	p416-*pTDH3-XylA3**	XmaIXylA3F	SacIIXylAR
pGALmTy1-*Ty1RT-mGAX-oldintron*frag	pGALmTy1-*Ty1RT-mGAX-oldintron*	XmaI	XbaI
pGALmTy1-*Ty1RT-mGA3X-oldintron*frag2	pGALmTy1-*Ty1RT-GA3X-oldintron*	AscI	None
XylAnointron	N/A	XylAGPDnointronF	XylAGPDnointronR
pGALmTy1-*Ty1RT-mGAX-oldintron*frag2	pGALmTy1-*Ty1RT-mGAX-oldintron*	AscI	None
pGALmTy1-*Ty1RT-SPT15I-pTEF1* cassette	pGALmTy1-*Ty1RT-SPT15I-pTEF1*	GREnewF	GREnewR

pGALmTy1-*Ty1RT-spt15-B6I-pTEF1* cassette	pGALmTy1-*Ty1RT-spt15-B6I*	GREnewF	GREnewR
pGALmTy1-*Ty1RT-URA3I-pTEF1* cassette	pGALmTy1-*Ty1RT-URA3I-pTEF1*	GREnewF	GREnewR
pGAlmTy1-*Ty1RT-ura3(3-5)I-pTEF1* cassette	pGALmTy1-*Ty1RT-ura3(3-5)I-pTEF1*	GREnewF	GREnewR
pGALmTy1-*Ty1RT-XylA3*I*-P2A-*XKS1-pTDH3 cassette**	pGALmTy1-*Ty1RT-XylA3*I*-P2A-*XKS1-pTDH3**	GREnewF	GREnewR
pGALmTy1-*Ty1RT-XylAI*-P2A-*XKS1-pTDH3 cassette**	pGALmTy1-*Ty1RT-XylAI*-P2A-*XKS1-pTDH3**	GREnewF	GREnewR
***GRE3*KO+*XKS1* cassette**	p415-*pTEF1-XKS1*	GRE3KOfor	GRE3KOrevnew
XbaI*XKS1*XhoI	*S. cerevisiae* BY4741 genome	XbaIXKS1	XKS1XhoI
pGALmTy1-*URA3I-pTEF1* cassette	pGALmTy1-*URA3I-pTEF1*	GREnewF	GREnewR
pGALmTy1-*Ty1RT-XylA3-P2A-*XKS1-pTDH3** cassette**	pGALmTy1-*Ty1RT-XylA3**-P2A-*XKS1-pTDH3**	GREnewF	GREnewR
pGALmTy1-*Ty1RT-XylA*-P2A-*XKS1-pTDH3 cassette**	pGALmTy1-*Ty1RT-XylA*-P2A-*XKS1-TDH3p**	GREnewF	GREnewR
***MRE11* cassette**	pUG6-*MRE115-KanMX-MRE113*	pUG6KOPCRnewF	pUG6KOPCRnewR
MRE115	*S. cerevisiae* BY4741 genome	MRE115'F	MRE115'R
MRE113	*S. cerevisiae* BY4741 genome	MRE113'F	MRE113'R
***CAC3* cassette**	pUG6-*CAC35-KanMX-CAC33*	pUG6KOPCRnewF	pUG6KOPCRnewR
CAC35	*S. cerevisiae* BY4741 genome	CAC35'F	CAC35'R
CAC33	*S. cerevisiae* BY4741 genome	CAC33'F	CAC33'R
***HIR3* cassette**	pUG6-*HIR35-KanMX-HIR33*	pUG6KOPCRnewF	pUG6KOPCRnewR
HIR35	*S. cerevisiae* BY4741 genome	BYHIR35'F	BYHIR35'R
HIR33	*S. cerevisiae* BY4741 genome	BYHIR33'F	BYHIR33'R
***CAC2* cassette**	pUG6-*CAC25-KanMX-CAC23*	pUG6KOPCRnewF	pUG6KOPCRnewR
CAC25	*S. cerevisiae* BY4741 genome	CAC25'F	CAC25'R

CAC23	*S. cerevisiae* BY4741 genome	CAC23'F	CAC23'R
***APL2* cassette**	pUG6-*APL25*-*KanMX*-*APL23*	pUG6KOPCRnewF	pUG6KOPCRnewR
APL25	*S. cerevisiae* BY4741 genome	APL25'F	APL25'R
APL23	*S. cerevisiae* BY4741 genome	APL23'F	APL23'R
pIMT4*-tRNA^{iMet}-*IMT4t	*pIMT4*-tRNA^{iMet}-*IMT4t* (gblock)	IMT4p	IMT4t
pSUP4*-tRNA^{iMet}-*RPR1t	*pSUP4*-tRNA^{iMet}-*RPR1t* (gblock)	sup4p	rpr1t
pSUP4*-tRNA^{iMet}-*IMT4t	*pSUP4*-tRNA^{iMet}-*IMT4t* (gblock)	sup4p	IMT4t
TAL1	*S. cerevisiae* BY4741 genome	XbaITAL1for	XhoITAL1rev
pTEF1-TAL1	p416-*pTEF1-TAL1*	SacItefTAL1for	SacITAL1cyc1rev
YFP	P416-*pTDH3-YFP*	MCS-Fwd-SpeI	MCS-Rev-2
mStraw-YFP	pGALmTy1-*Ty1RT*-*mStrawberry*-P2A-*YFP*	mStraw-YFPf	mStraw-YFPr
P2Afrag	p416-*pTDH3-mStrawberry*-*YFP*	P2AYFPGlcF	P2AstrawGlcR
df*YFP*	P416-*pTDH3-YFP*	MCS-Fwd-SalI-2	MCS-Rev-2
mStawberry	p*mStrawberry*	SpeImStrawberryF	EcoRImStrawberryR
ART BB	pGALmTy1-*Ty1RT-URA3I*-*pTEF1*	ARTR	His3AIgenomeflankF
SPT15intron	pGALmTy1-*Ty1RT*-MCS-*SPT15intronnosite-pTEF1*	IntronSiteF	IntronSiteR
Spt15intronnosite	pGALmTy1-*Ty1RT-SPT15*-*pTEF1*	SPT15intronF	SPT15intronR
pGALmTy1-*Ty1RT-SPT15*frag	pGALmTy1-*Ty1RT-SPT15*	NotI	N/A
pGALmTy1-*HIVRT-URA3I*-*pHIS3*frag	pGALmTy1-*HIVRT-URA3I*-*pHIS3*	XmaI	N/A
***SPT15-pTEF1* cassette**	p416-*pTEF1-SPT15*	LTRTEFF2	Spt15tefR
SPT15	*S. cerevisiae* BY4741 genome	HispromSPT15F	HistermSPT15R
***SPT15*clone**	*SPT15* round 1 isolate gDNA	TEFmutssacF	XhoISPT15R
spt15-B6	*SPT15* round 1 isolate gDNA	TEFmutssacF	XhoISPT15R
spt15-B6-1	*SPT15* round 2 isolate gDNA	TEFmutssacF	XhoISPT15R
URA3	*URA3* round 1 isolate gDNA	TEFmutssacF	p416ura3EcoR1rev
ura3(3-5)	*URA3* round 1 isolate gDNA	TEFmutssacF	p416ura3EcoR1rev
ura3(3-5-2)	*URA3* round 2 isolate gDNA	TEFmutssacF	p416ura3EcoR1rev
Tkc1	n/a (anneal)	EcoRITkc1F	SbfITkc1R
Tkc5	n/a (anneal)	EcoRITkc5F	SbfITkc5R

Tkc8	n/a (anneal)	EcoRITkc8SbfIF	SbfITkc8EcoRIR
CYC1t	*S. cerevisiae* BY4741 genome	URA3AICYC1F	URA3AICYC1Rnew
IDP1t	*S. cerevisiae* BY4741 genome	URA3AIIDP1F	URA3AIIDP1Rnew
PRM9t	*S. cerevisiae* BY4741 genome	URA3AIPRM9F	URA3AIPRM9Rnew
d*KanMX-pTEF1* cassette	pGALmTy1-*Ty1RT*-d*KanMX-pTEF1*	GREcontrol-fwd	GREnewR
pGALmTy1-*Ty1RT*-d*KanMX-pTEF1* cassette	pGALmTy1-*Ty1RT*-d*KanMX-pTEF1*	GREnewF	GREnewR
pGALmTy1-*Ty1RT-pTEF1* BB	pGALmTy1-*Ty1RT-URA3I-pTEF1*	TART-BB-f	TART-BB-r
KanMX	pUG6	ip-G418f	ip-G418r
KanMX-1	pGALmTy1-*Ty1RT-KanMX-pTEF1*	G418-stop-f	G418-stop-r
d*KanMX*	pGALmTy1-*Ty1RT-KanMX-1-pTEF1*	G418-stop2-f	G418-stop2-r
Linear_pUC19	pUC19	JMW-0077	JMW-0078
***KlURA3*_US-Linker**	*K. lactis* genomic DNA	JMW-0092	JMW-0093
Linker-*KlURA3*_DS	*K. lactis* genomic DNA	JMW-0094	JMW-0095
***KlURA3*_US-Linker-*KlURA3*_DS**	pUC19-Δ*KlURA3*	JMW-0101	JMW-0102
p42X-TART-*pTEF1-ScURA3AI*-NoMarker	p423-TART-*pTEF1-ScURA3AI*	JMW-0163	JMW-0164
***KanMX_Kl*Marker**	p42K	JMW-0165	JMW-0166
***KlURA3AI* Cargo**	Synthetic *KlURA3AI*	JMW-0210	JMW-0211
p42K-TART-*pTEF1*-NoCargo	p42K-TART-*pTEF1-ScURA3AI*	JA-211	JA-240
TART-*pTEF1-KlURA3AI-KanMX*	p42K-TART-*pTEF1-KlURA3AI*	JMW-0225	JMW-0226
***AmpR*-ColE1**	p42K-TART-*pTEF1-KlURA3AI*	JMW-0227	JMW-0228
***KlNDT80*_US**	*K. lactis* genomic DNA	JMW-0229	JMW-0230
***KlNDT80*_DS**	*K. lactis* genomic DNA	JMW-0231	JMW-0232
***NDT80*_US-TART-*pTEF1-KlURA3AI-KanMX-NDT80*_DS**	p42K-*NDT80*_US-TART-*pTEF1-KlURA3AI-NDT80*_DS	JMW-0280	JMW-0281

Supplementary Table 9: Plasmids generated through restriction enzyme cloning.

Plasmid Name	Backbone	Insert	Rest. Enz. 1	Rest. Enz. 2
p416-*pGAL1*	p416-*pTDH3*	*pGAL1*	SacI	XbaI
p416-*pGAL1-CAS9-CYC1t*-MCS	MCSBB	N/A	N/A	N/A
p416-*pGAL1-CAS9-CYC1t-TRP1*gRNA	p416-*pGAL1-CAS9-CYC1t*-MCS	*TRP1*gRNA	NotI	PacI
pGALmTy1-*URA3I-pTEF1*	ART BB	N/A	N/A	N/A
pGALmTy1-*Ty1RT-SPT15I-pTEF1*	*SPT15intron*	N/A	N/A	N/A
pGALmTy1-*Ty1RT*-MCS-*SPT15intronnosite-pTEF1*	*SPT15intronnosite*	N/A	N/A	N/A
p413-*pTEF1-SPT15*	p413-*pTEF1*	*SPT15*clone	SacI	XhoI
p413-*spt15-B6*	p413-*pTEF1*	*spt15-B6*	SacI	XhoI
p413-*spt15-B6-1*	p413-*pTEF1*	*spt15-B6-1*	SacI	XhoI
p413-*pTEF1-URA3*	p413-*pTEF1*	*URA3*	SacI	EcoRI
p413-*ura3(3-5)*	p413-*pTEF1*	*ura3(3-5)*	SacI	EcoRI
p413-*ura3(3-5-2)*	p413-*pTEF1*	*ura3(3-5-2)*	SacI	EcoRI
pGALmTy1-*Ty1RT-mGAX-oldintron*	pGALmTy1-*Ty1RT-TAXintron*	*pTDH3**	NotI	XmaI
pGALmTy1-*Ty1RT-TAXintron*	Ty1*TAX*	N/A	N/A	N/A
pGALmTy1-*Ty1RT-TAX*	Ty1*XylAXKS*	N/A	N/A	N/A
pGALmTy1-*Ty1RT-pTEF1-XylA-XKS1*	pGALmTy1-*Ty1RT-pTEF1-XylA*	*XKS1*	PacI	EcoRI
pGALmTy1-*Ty1RT-pTEF1-XylA*	pGALmTy1-*Ty1RT-pTEF1*	*XylA*	XmaI	SacII
pGALmTy1-*Ty1RT-pTEF1*	pGALmTy1-*Ty1RT*-FullMCS	*pTEF1*	NotI	XmaI
pGALmTy1-*Ty1RT*-FullMCS	FullMCSfrag	N/A	N/A	N/A
pGALmTy1-*Ty1RT-mGA3X-oldintron*	pGALmTy1-*Ty1RT-TA3Xintron*	*pTDH3**	NotI	XmaI
pGALmTy1-*Ty1RT-TA3Xintron*	Ty1*TA3X*	N/A	N/A	N/A
pGALmTy1-*Ty1RT-TA3X*	Ty1*XylA3XKS*	N/A	N/A	N/A
pGALmTy1-*Ty1RT-pTEF1-XylA3-XKS1*	pGALmTy1-*Ty1RT-pTEF1-XylA3*	*XKS1*	PacI	EcoRI
pGALmTy1-*Ty1RT-pTEF1-XylA3*	pGALmTy1-*Ty1RT-pTEF1*	*XylA3*	XmaI	SacII
p415-*pTEF1-XKS1*	p415-*pTEF1*	XbaI*XKS1*XhoI	XbaI	XhoI
pUG6-*MRE11*5-*KanMX-MRE11*3	pUG6-*MRE11*5-*KanMX*	*MRE11*3	SpeI	SacII
pUG6-*MRE11*5-*KanMX*	pUG6	*MRE11*5	BsiWI	SalI
pUG6-*CAC3*5-*KanMX-CAC3*3	pUG6-*CAC3*5	*CAC3*3	SpeI	SacII
pUG6-*CAC3*5	pUG6	*CAC3*5	BsiWI	SalI
pUG6-*HIR3*5-*KanMX-HIR3*3	pUG6-*HIR3*5-*KanMX*	*HIR3*3	SpeI	SacII
pUG6-*HIR3*5-*KanMX*	pUG6	*HIR3*5	BsiWI	SalI
pUG6-*CAC2*5-*KanMX-CAC2*3	pUG6-*CAC2*5-*KanMX*	*CAC2*3	SpeI	SacII
pUG6-*CAC2*5-*KanMX*	pUG6	*CAC2*5	BsiWI	SalI

pUG6-*APL25-KanMX-APL23*	pUG6-*APL25-KanMX*	*APL23*	SpeI	SacII
pUG6-*APL25-KanMX*	pUG6	*APL25*	BsiWI	SalI
p414-*pIMT4*-tRNA^{iMet}-*IMT4t*	p414-*pCYC1*	*pIMT4*-tRNA^{iMet}-*IMT4t*	KpnI	SacI
p414-*pSUP4*-tRNA^{iMet}-*RPR1t*	p414-*pCYC1*	*pSUP4*-tRNA^{iMet}-*RPR1t*	KpnI	SacI
p414-*pSUP4*-tRNA^{iMet}-*IMT4t*	p414-*pCYC1*	*pSUP4*-tRNA^{iMet}-*IMT4t*	KpnI	SacI
p424-*pIMT4*-tRNA^{iMet}-*IMT4t*	p424-*pTEF1*	*pIMT4*-tRNA^{iMet}-*IMT4t*	KpnI	SacI
p424-*pSUP4*-tRNA^{iMet}-*RPR1t*	p424-*pTEF1*	*pSUP4*-tRNA^{iMet}-*RPR1t*	KpnI	SacI
p424-*pSUP4*-tRNA^{iMet}-*IMT4t*	p424-*pTEF1*	*pSUP4*-tRNA^{iMet}-*IMT4t*	KpnI	SacI
p416-*pTEF1-TAL1*	p416-*pTEF1*	*TAL1*	XbaI	XhoI
p424-*pTEF1-TAL1-CYC1t-pIMT4*-tRNA^{iMet}-*IMT4t*	p424-*pIMT4*-tRNAiMet-*IMT4t*	*pTEF1-TAL1*	SacI	N/A
p413-*pGAL1-YFP*	p413-*pTEF1-YFP*	*pGAL1*	SacI	SpeI
p413-*pTEF1-YFP*	p413-*pTEF1*	*YFP*	SpeI	XhoI
pGALmTy1-*Ty1RT-LacZ-YFP-mStrawberry-URA3I-pHIS3*	pGALmTy1-*Ty1RT-LacZ-URA3I-pHIS3*	*mStraw-YFP*	EcoRI	N/A
pGALmTy1-*Ty1RT-mStrawberry*-P2A-*YFP*	P2Afrag	N/A	N/A	N/A
p416-*pTDH3-mStrawberry-YFP*	p416-*pTDH3-mStrawberry*	df*YFP*	SalI	xhoI
p416-*pTDH3-mStrawberry*	p416-*pTDH3*	*mstrawberry*	SpeI	EcoRI
pGALmTy1-*Ty1RT-URA3I-pTEF1-Tsynth1*	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*Tkc1*	EcoRI	SbfI
pGALmTy1-*Ty1RT-URA3I-pTEF1-Tsynth5*	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*Tkc5*	EcoRI	SbfI
pGALmTy1-*Ty1RT-URA3I-pTEF1-Tsynth8*	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*Tkc8*	EcoRI	SbfI
pGALmTy1-*Ty1RT-URA3I-pTEF1-CYC1t*	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*CYC1t*	EcoRI	SbfI
pGALmTy1-*Ty1RT-URA3I-pTEF1-IDP1t*	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*IDP1t*	EcoRI	SbfI
pGALmTy1-*Ty1RT-URA3I-pTEF1-PRM9t*	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*PRM9t*	EcoRI	SbfI
pGALmTy1-*Ty1RT-KanMX-1-pTEF1*	*KanMX-1*	n/a	n/a – blunt ligation	
pGALmTy1-*Ty1RT*-d*KanMX-pTEF1*	d*KanMX*	n/a	n/a – blunt ligation	

Supplementary Table 10: Plasmids generated through homologous recombination cloning or Gibson Assembly.

Plasmid Name	Fragments Used For Assembly
p416-*pGAL1-CAS9-CYC1t-URA3*gRNA	*CAS9*, *CYC1t*, *URA3*gRNA, p416-*pGAL1*frag
pGALmTy1-*HIVRT-URA3I-pHIS3*	*pGAL1*frag2, Ty1frag4, *HIVRT*frag1, *URA3AI*frag1, *URA3*frag3, LTRfrag2, *HIS3*promfrag1, and p423frag1
pGALmTy1-*Ty1RT-URA3I-pHIS*3	*Ty1RT*frag2, pGALmTy1frag1
pGALmTy1-*Ty1RT-URA3I-pTEF1*	pGALmTy1-*Ty1RT* BB, *pTEF1*hom
pGALmTy1-*Ty1RT-eGFP-URA3I-pHIS3*	*Ty1RT-URA3*-BB, Cargo-*eGFP*
pGALmTy1-*Ty1RT-CAN1-URA3I-pHIS3*	*Ty1RT-URA3*-BB, Cargo-*CAN1*
pGALmTy1-*Ty1RT-LacZ-URA3I-pHIS3*	*Ty1RT-URA3*-BB, Cargo-*LacZ*
pGALmTy1-*Ty1RT-LacZ-eGFP-URA3I-pHIS3*	Cargo-*LacZ*-BB, Cargo-*eGFP-LacZ*-ins
pGALmTy1-*Ty1RT-URA3I-pCYC1*	pGALmTy1-*Ty1RT* BB, *pCYC1*hom
pGALmTy1-*Ty1RT-URA3I-pTDH3*	pGALmTy1-*Ty1RT* BB, *pTDH3*
pGALmTy1-*Ty1RT-spt15-B6I*	MutBB, Smut6up, Smut6down
pGAlmTy1-*Ty1RT-ura3(3-5)I-pTEF1*	MutBB, U3-5up, U3-5down
pGALmTy1-*Ty1RT-XylA3*I*-P2A-*XKS1-pTDH3*	*(m)GA3X*newintron, P2A-*Ty1RT*, pGALmTy1-*Ty1RT-mGA3X-oldintronfrag*
pGALmTy1-*Ty1RT-XylAI*-P2A-*XKS1-pTDH3*	*(m)GAX*newintron, pGALmTy1-*Ty1RT-mGAX-oldintronfrag*
pGALmTy1-*Ty1RT-XylA3-P2A-*XKS1-pTDH3***	pGALmTy1-*Ty1RT-mGA3X-oldintronfrag2*, *XylAnointron*
pGALmTy1-*Ty1RT-XylA*-P2A-*XKS1-pTDH3*	pGALmTy1-*Ty1RT-mGAX-oldintronfrag2*, *XylAnointron*
pGALmTy1-*Ty1RT-SPT15-pTEF1*	pGALmTy1-*Ty1RT-SPT15*frag, *SPT15-pTEF1* cassette
pGALmTy1-*Ty1RT-SPT15*	pGALmTy1-*HIVRT-URA3I-pHIS3*frag, *SPT15*
pGALmTy1-*Ty1RT-KanMX-pTEF1*	pGALmTy1-*Ty1RT-pTEF1* BB, *KanMX*
pUC19-Δ*KlURA3*	Linear_pUC19, *KlURA3*_US-Linker, Linker-*KlURA3*_DS
p42K-TART-*pTEF1-ScURA3AI*	p42X-TART-*pTEF1-URA3AI*-NoMarker, *KanMX_Kl*Marker
p42K-TART-*pTEF1-KlURA3AI*	p42K-TART-*pTEF1*-NoCargo, *KlURA3AI* Cargo
pUC-*NDT80*_US-TART-*pTEF1-KlURA3AI-KanMX-NDT80*_DS	*AmpR*-ColE1 (pUC19-based), *KlNDT80*_US, TART-*pTEF1-KlURA3AI-KanMX*, *KlNDT80*_DS

Supplementary Table 11: Strains generated through genome editing.

Strain Name	Parent Strain	PCR Fragment
S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-SPT15I-pTEF1	Strain **6** (See below)	pGALmTy1-*Ty1RT-SPT15I-pTEF1* cassette
S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-spt15-B6I-pTEF1	Strain **6** (See below)	pGALmTy1-*Ty1RT-spt15-B6I-pTEF1* cassette
S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1	Strain **6** (See below)	pGALmTy1-*Ty1RT-URA3I-pTEF1* cassette
S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-ura3(3-5)I-pTEF1	Strain **6** (See below)	pGAlmTy1-*Ty1RT-ura3(3-5)I-pTEF1* cassette
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA3*I-P2A-XKS1-pTDH3*	Strain **14** (See below)	pGALmTy1-*Ty1RT-XylA3*I*-P2A-*XKS1-pTDH3** cassette
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylAI-P2A-XKS1-pTDH3*	Strain **14** (See below)	pGALmTy1-*Ty1RT-XylAI*-P2A-*XKS1-pTDH3** cassette
S. cerevisiae* BY4741Δ*r-g-x	*S. cerevisiae* BY4741 Δ*rrm3*	*GRE3*KO+*XKS1* cassette
S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-URA3I-pTEF1	*S. cerevisiae* BY4741 Δ*rrm3*	pGAlmTy1-*URA3I-pTEF1* cassette
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA3*-P2A-XKS1-pTDH3*	Strain **14** (See below)	pGALmTy1-*Ty1RT-XylA3**-P2A-*XKS1-pTDH3** cassette
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-xyla3xks1-66	*S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA3*-P2A-XKS1-pTDH3**	
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-xyla3xks1-20	*S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA3*-P2A-XKS1-pTDH3**	
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA-P2A-XKS1-pTDH3*	Strain **14** (See below)	pGALmTy1-*Ty1RT-XylA*-P2A-*XKS1-pTDH3** cassette
S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-xylaxks1-34	*S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA-P2A-XKS1-pTDH3**	
S. cerevisiae* BY4741 Δ*apl2* Δ*mre11	*S. cerevisiae* BY4741 Δ*apl2* Clean	*MRE11* cassette
***S. cerevisiae* BY4741 Δ*mre11* Clean**	Strain **54** (See below)	
***S. cerevisiae* BY4741 Δ*hir3* Clean**	Strain **55** (See below)	
***S. cerevisiae* BY4741 Δ*apl2* Clean**	Strain **56** (See below)	
S. cerevisiae* BY4741 Δ*mre11-2	*S. cerevisiae* BY4741	*MRE11* cassette
S. cerevisiae* BY4741 Δ*hir3-2	*S. cerevisiae* BY4741	*HIR3* cassette
S. cerevisiae* BY4741 Δ*apl2-2	*S. cerevisiae* BY4741	*APL2* cassette
S. cerevisiae* BY4741 Δ*hir3*Δ*cac3	*S. cerevisiae* BY4741 Δ*hir3* Clean	*CAC3* cassette
S. cerevisiae* BY4741 Δ*hir3*Δ*apl2	*S. cerevisiae* BY4741 Δ*apl2* Clean	*HIR3* cassette
S. cerevisiae* BY4741 Δ*hir3*Δ*mre11	*S. cerevisiae* BY4741 Δ*mre11* clean	*HIR3* cassette

S. cerevisiae* BY4741 Δ*hir3*Δ*cac2	*S. cerevisiae* BY4741 Δ*hir3* Clean	*CAC2* cassette
S. cerevisiae* BY4741 *trp1::dKanMX-pTEF1	Strain **6** (See below)	d*KanMX-pTEF1* cassette
S. cerevisiae* BY4741 *trp1::Ty1-Ty1RT-dKanMX-pTEF1	Strain **6** (See below)	pGALmTy1-*Ty1RT*-d*KanMX-pTEF1* cassette
K. lactis* CBS 2359 Δ*KlURA3	*K. lactis* CBS 2359	*KlURA3*_US-Linker-*KlURA3*_DS
K. lactis* CBS 2359 Δ*KlURA3 KlNDT80::TART-pTEF1-KlURA3AI	*K. lactis* CBS 2359 Δ*KlURA3*	*NDT80*_US-TART-*pTEF1-KlURA3AI-KanMX-NDT80*_DS

Supplementary Table 12: Strains generated through plasmid transformation.

Strain No.	Plasmid 1	Host	Growth Medium
1	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* CEN.PK2	YSC −His
2	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741	YSC −His
3	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*S. cerevisiae* BY4741	YSC −His
4	pGALmTy1-*Ty1RT-URA3I-pTEF1*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
5	None	*S. cerevisiae* BY4741 Δ*rrm3* *trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His
6	p424-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 Δ*rrm3* *trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
7	p413-*pTEF1*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
8	p413-*pTEF1-SPT15*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
9	p413-*spt15-B6*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
10	p413-*spt15-B6-1*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
11	p413-*pTEF1-URA3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
12	p413-*ura3(3-5)*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
13	p413-*ura3(3-5-2)*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
14	p424-*pIMT4*-tRNA^{iMet}-*IMT4t-TAL1*	*S. cerevisiae* BY4741Δ*r-g-x*	YSC −His −Trp
15	p424-*pIMT4*-tRNA^{iMet}-*IMT4t-TAL1*	*S. cerevisiae* BY4741Δ*r-g-x* *trp1::Ty1-Ty1RT-XylA3*-P2A-XKS1-pTDH3**	YSC −His −Trp
16	p424-*pIMT4*-tRNA^{iMet}-*IMT4t-TAL1*	*S. cerevisiae* BY4741Δ*r-g-x* *trp1::Ty1-Ty1RT-xyla3xks1-66*	YSC −His −Trp
17	p424-*pIMT4*-tRNA^{iMet}-*IMT4t-TAL1*	*S. cerevisiae* BY4741Δ*r-g-x* *trp1::Ty1-Ty1RT-xyla3xks1-20*	YSC −His −Trp
18	p424-*pIMT4*-tRNA^{iMet}-*IMT4t-TAL1*	*S. cerevisiae* BY4741Δ*r-g-x* *trp1::Ty1-Ty1RT-XylA-P2A-XKS1-pTDH3**	YSC −His −Trp
19	p424-*pIMT4*-tRNA^{iMet}-*IMT4t-TAL1*	*S. cerevisiae* BY4741Δ*r-g-x* *trp1::Ty1-Ty1RT-xylaxks1-34*	YSC −His −Trp
20	p413-*pGAL1-YFP*	*S. cerevisiae* YSX3	YSC −His
21	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
22	pGALmTy1-*Ty1RT-eGFP-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
23	pGALmTy1-*Ty1RT-CAN1-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
24	pGALmTy1-*Ty1RT-LacZ-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
25	pGALmTy1-*Ty1RT-LacZ-eGFP-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
26	pGALmTy1-*Ty1RT-LacZ-YFP-mStrawberry-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −His
27	p424-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 Δ*rrm3* *trp1::Ty1-URA3I-pTEF1*	YSC −His −Trp
28	pGALmTy1-*URA3I-pTEF1*	*S. cerevisiae* BY4741	YSC −His
29	p416-*pCYC1-YFP*	*S. cerevisiae* BY4741	YSC −Ura
30	p416-*pTEF1-YFP*	*S. cerevisiae* BY4741	YSC −Ura
31	p416-*pTDH3-YFP*	*S. cerevisiae* BY4741	YSC −Ura
32	pGALmTy1-*Ty1RT-URA3I-pCYC1*	*S. cerevisiae* BY4741	YSC −His
33	pGALmTy1-*Ty1RT-URA3I-pTDH3*	*S. cerevisiae* BY4741	YSC −His
34	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*apl2* Δ*mre11*	YSC −His
35	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*ice2*	YSC −His
36	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*ckb2*	YSC −His

37	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*hir3*	YSC −His
38	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*apl2*	YSC −His
39	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*hir3*Δ*cac3*	YSC −His
40	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*mrc1*	YSC −His
41	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*hir3*Δ*apl2*	YSC −His
42	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*hir3*Δ*mre11*	YSC −His
43	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*cac2*	YSC −His
44	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*mre11*	YSC −His
45	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*hir3*Δ*cac2*	YSC −His
46	pGALmTy1-*Ty1RT-URA3I-pHIS3*	*S. cerevisiae* BY4741 Δ*cac3*	YSC −His
47	p414-*pTDH3*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
48	p414-*pSUP4*-tRNA^{iMet}-*RPR1t*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
49	p414-*pSUP4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
50	p414-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
51	p424-*pTDH3*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
52	P424- *pSUP4*-tRNA^{iMet}-*RPR1t*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
53	P424- *pSUP4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 Δ*rrm3 trp1::Ty1-Ty1RT-URA3I-pTEF1*	YSC −His −Trp
54	pSH47	*S. cerevisiae* BY4741 Δ*mre11-2*	YSC −Ura
55	pSH47	*S. cerevisiae* BY4741 Δ*hir3-2*	YSC −Ura
56	pSH47	*S. cerevisiae* BY4741 Δ*apl2-2*	YSC −Ura
57	P424-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylA3*I-P2A-XKS1-pTDH3**	YSC −His
58	P424-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741Δ*r-g-x trp1::Ty1-Ty1RT-XylAI-P2A-XKS1-pTDH3**	YSC −His
59	p416-*pGAL1-CAS9-CYC1t-TRP1*gRNA	*S. cerevisiae* BY4741 Δ*rrm3*	YSC −Ura
60	p416-*pGAL1-CAS9-CYC1t-TRP1*gRNA	*S. cerevisiae* BY4741Δ*r-g-x*	YSC −Ura
61	pGALmTy1-*Ty1RT-URA3I-pTEF1-Tsynth1*	*S. cerevisiae* BY4741	YSC −His
62	pGALmTy1-*Ty1RT-URA3I-pTEF1-Tsynth5*	*S. cerevisiae* BY4741	YSC −His
63	pGALmTy1-*Ty1RT-URA3I-pTEF1-Tsynth8*	*S. cerevisiae* BY4741	YSC −His
64	pGALmTy1-*Ty1RT-URA3I-pTEF1-CYC1t*	*S. cerevisiae* BY4741	YSC −His
65	pGALmTy1-*Ty1RT-URA3I-pTEF1-IDP1t*	*S. cerevisiae* BY4741	YSC −His
66	pGALmTy1-*Ty1RT-URA3I-pTEF1-PRM9t*	*S. cerevisiae* BY4741	YSC −His
67	P424-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 *trp1::dKanMX-pTEF1*	YSC −His −Trp
68	P424-*pIMT4*-tRNA^{iMet}-*IMT4t*	*S. cerevisiae* BY4741 *trp1::Ty1-Ty1RT-dKanMX-pTEF1*	YSC −His −Trp
69	p416-*pGAL1-CAS9-CYC1t-TRP1*gRNA	*S. cerevisiae* BY4741	YSC −Ura
70	None	*K. lactis* CBS 2359 Δ*KlURA3 KlNDT80::TART-pTEF1-KlURA3AI*	YSC

Supplementary Table 13: Strains used in experiments described in this study.

Figure	Category	Series	Strain No.
Figure 2b: Iterative improvement of synthetic Ty1 transposition rate	BY4741 *Ty1RT*: *pHIS3-URA3I*		2
	BY4741 *Ty1RT*: *pTEF1-URA3I*		3
	BY4741 Δ*rrm3 Ty1RT*: *pTEF1-URA3I*		4
	BY4741 Δ*rrm3 Ty1RT*: *pTEF1-URA3I*		5
	BY4741 Δ*rrm3 Ty1RT*: *pTEF1-URA3I*, High OD		5
	BY4741 Δ*rrm3 Ty1RT*: *pTEF1-URA3I*, 22°C induction, High OD		5
	BY4741 Δ*rrm3 Ty1RT*: *pTEF1-URA3I*, 22°C induction, High OD, $tRNA^{iMet}$ overexpression		6
Figure 3a: Substitution of alternative promoters in retroelement	BY4741 *Ty1RT*		2
	BY4741 *Ty1RT pCYC1*		32
	BY4741 *Ty1RT pTEF1*		3
	BY4741 *Ty1RT pTDH3*		33
Figure 3b: Transposition rates for BY4741 knockout strains	Wild type		2
	Δ*apl2* Δ*mre11*		34
	Δ*ice2*		35
	Δ*ckb2*		36
	Δ*hir3*		37
	Δ*apl2*		38
	Δ*hir3*Δ*cac3*		39
	Δ*mrc1*		40
	Δ*hir3*Δ*apl2*		41
	Δ*hir3*Δ*mre11*		42
	Δ*cac2*		43
	Δ*mre11*		44
	Δ*hir3*Δ*cac2*		45
	Δ*cac3*		46
	Δ*rrm3*		21
Figure 3c: Transcript levels of engineered retroelements	Transposition Repressed	*Ty1RT* WT	3
		Ty1RT Δ*rrm3*	4
		No RT	28
	Transposition Induced	*Ty1RT* WT	3
		Ty1RT Δ*rrm3*	4
		No RT	28
Figure 3d: cDNA levels of engineered retroelements	Transposition Repressed	*Ty1RT* WT	3
		Ty1RT Δ*rrm3*	4
		No RT	28
	Transposition Induced	*Ty1RT* WT	3
		Ty1RT Δ*rrm3*	4
		No RT	28
Figure 3e: Transposition rate of strains overexpressing the	Low copy	no tRNA overexpression	47
		pSUP4-$tRNA^{iMet}$-*RPR1t*	48

initiator methionine tRNA IMT4		*pSUP4*-tRNA^{iMet}-*IMT4t*	49
		pIMT4-tRNA^{iMet}-*IMT4t*	50
	High copy	no tRNA overexpression	51
		pSUP4-tRNA^{iMet}-*RPR1t*	52
		pSUP4-tRNA^{iMet}-*IMT4t*	53
		pIMT4-tRNA^{iMet}-*IMT4t*	6
Figure 3f: tRNA^{iMet} overexpression improves cDNA synthesis	Low copy	No tRNA Glc	47
		No tRNA Gal	47
		With tRNA Glc	50
		With tRNA Gal	50
	High copy	No tRNA Glc	51
		No tRNA Gal	51
		With tRNA Glc	6
		With tRNA Gal	6
Figure 3g: Transposition rate of retroelements containing cargo of various sizes	3 Day Induction	1135 bp	21
		1890 bp	22
		2907 bp	23
		4219 bp	24
		4964 bp	25
		5768 bp	26
	5 Day Induction	1135 bp	21
		1890 bp	22
		2907 bp	23
		4219 bp	24
		4964 bp	25
		5768 bp	26
	7 Day Induction	1135 bp	21
		1890 bp	22
		2907 bp	23
		4219 bp	24
		4964 bp	25
		5768 bp	26
Figure 4a: dKanMX reversion mutation assay	d*KanMX* in genome		67
	d*KanMX* in ICE retroelement		68
Figure 4b: Mutation distribution of Ty1RT	*URA3* with RT		3
	Amp without RT		3
Figure 4c: Mutation frequencies of Ty1RT	*URA3* with RT		3
	Amp without RT		3
Figure 5: Implementation of ICE for improvement of three distinct classes of genetic cargo	a: ICE of *URA3*	WT: *URA3*	11
		Mutant: *ura3(3-5)*	12
		Mutant: *ura3(3-5-2)*	13
	b: ICE of *SPT15*	WT: *SPT15*	8
		Mutant: *spt15-B6*	9
		Mutant: *spt15-B6-1*	10
	c: ICE of xylose isomerase pathway	WT: *IK* pathway	18
		Mutant: *IK*-34	19
	d: ICE of xylose isomerase pathway	WT: *I3K* pathway	15
		Mutant: *I3K*-66	16
		Mutant: *I3K*-20	17
Figure 7: Transposition rates for alternate yeast strains	BY4741		2
	CEN.PK2		1

	K. lactis	70
Supplementary Fig. 1a: Promoters used in this study	*pCYC1*	29
	pTEF1	30
	pTDH3	31
Supplementary Fig. 1b: Transposition of RT-containing and RT-less retroelements at 22ºC	BY4741 Δ*rrm3 Ty1RT*: *TEF1p-URA3I*, 22 °C induction, High OD	6
	BY4741 Δ*rrm3* NoRT: *TEF1p-URA3I*, 22 °C induction, High OD	27
Supplementary Fig. 1c: Effect of terminators on rate of transposition	No Terminator	3
	Tsynth1	61
	Tsynth5	62
	Tsynth8	63
	CYC1t	64
	IDP1t	65
	PRM9t	66
Supplementary Fig. 3a: Transcriptional rates of *SPT15* mutants	WT: *SPT15*	8
	Mutant: *Spt15-B6-1*	10
Supplementary Fig. 3b: Growth of *ura3(3-5)*-containing strains in media lacking uracil and containing 5-FOA	0 g L^{-1}	12
	0.3 g L^{-1}	12
	0.5 g L^{-1}	12
Supplementary Fig. 3c: Activity of *Gal1p* in xylose-containing media	Glu (grown in glucose)	20
	Xyl (grown in glucose)	20
	Gal (grown in galactose)	20
	Xyl (grown in galactose)	20
Supplementary Fig. 3d: Growth test of *xylA* pathway mutants	Δ*r-g-x* blank strain	14
	WT: *IK* pathway	18
	Mutant: *IK-34*	19
Supplementary Fig. 3e: Growth test of *xylA3 pathway mutants**	Δ*r-g-x* blank strain	14
	WT: *I3K* pathway	15
	Mutant: *I3K-66*	16
	Mutant: *I3K-20*	17
Supplementary Fig. 3f-i: Growth test of *SPT15* variants	WT: *SPT15*	8
	Mutant: *SPT15-B6*	9
	Mutant: *SPT15-B6-1*	10

Supplementary Table 14: Genes Synthesized.

Name	Sequence	Source
Synthetic *HIVRT*	ATGGCTTTGAAGGCTGTTCCAATTTCTCCAATTGAAACCGT TCCAGTTAAGTTGAAACCAGGTATGGATGGTCCAAAAGTT AAGCAATGGCCTTTGACCGAAGAAAAGATTAAGGCTTTGG TTGAAATCTGCACCGAAATGGAAAAAGAAGGTAAGATCTC TAAGATCGGTCCAGAAAATCCATACAACACTCCAGTTTTCG CCATCAAGAAAAAGGATTCTACTAAGTGGAGAAAGTTGGT TGACTTCAGAGAATTGAACAAGAGAACCCAAGATTTCTGG GAAGTTCAATTGGGTATTCCACATCCAGCTGGTTTGAAGAA GAAAAAGTCTGTTACCGTTATGGATGTTGGTGATGCTTACT TTTCTGTTCCATTGGACGAAGATTTCAGAAAGTACACTGCT TTCACCATCCCATCCATTAACAACGAAACTCCAGGTATCAG ATACCAATACAACGTTTTGCCACAAGGTTGGAAAGGTTCTC CAGCTATTTTTCAATCTTCCATGACCAAGATTTTGGAACCA TTCAAGAAGCAAAACCCAGACATCGTCATCTACCAATATAT GGATGACTTGTACGTTGGTTCCGATTTGGAAATTGGTCAAC ACAGAACTAAGATCGAAGAATTGAGACAACACTTGTTGAG ATGGGGTTTGACTACTCCAGATAAGAAGCACCAAAAAGAA CCACCATTTTTGTGGATGGGTTACGAATTGCATCCTGATAA GTGGACTGTTCAACCTATAGTTTTGCCAGAAAAAGACTCTT GGACCGTTAACGACATCCAAAAATTGGTCGGTAAATTGAA CTGGGCCTCTCAAATCTATCCAGGTATTAAGGTTAGACAAT TGTGCAAGTTGTTGAGAGGTACAAAGGCTTTGACTGAAGTT ATTCCATTGACAGAAGAAGCCGAATTGGAATTGGCTGAAA ACAGAGAAATTTTGAAAGAACCAGTTCACGGTGTTTACTAC GATCCATCTAAAGATTTGATCGCCGAAATCCAAAAACAAG GTCAAGGTCAATGGACCTACCAAATCTACCAAGAACCTTTT AAGAACTTGAAAACCGGTAAGTACGCTAGAATGAGAGGTG CTCATACAAACGATGTCAAGCAATTGACTGAAGCCGTTCA AAAGATTACCACCGAATCTATAGTTATCTGGGGTAAGACTC CAAAGTTCAAGTTGCCAATTCAAAAAGAAACTTGGGAAAC CTGGTGGACTGAATATTGGCAAGCTACTTGGATTCCAGAAT GGGAATTTGTTAATACTCCACCATTGGTCAAGTTGTGGTAT CAATTGGAAAAAGAACCTATCGTTGGTGCCGAAACTTTTTA TGTTGATGGTGCTGCTAACATGGAAACTAAGTTGGGTAAA GCTGGTTACGTTACCAACAAAGGTAGACAAAAAGTTGTCC CATTGACCAACACTACCAATCAAAAGACTGAATTGCAAGC TATCTACTTGGCCTTGCAAGATTCTGGTTTGGAAGTTAACA TCGTTACCGATTCTCAATACGCCTTGGGTATTATTCAAGCT CAACCAGATAAGTCCGAATCCGAATTGGTTAATCAAATCAT CGAACAATTGATCAAGAAAGAAAAGGTTTACTTGGCTTGG GTTCCAGCTCATAAGGGTATTGGTGGTAATGAACAAGTTGA CAAGTTGGTTTCTGCCGGTATTAGAAAGATCTTGTAA	Life Technologies
Synthetic Ty1	CTCGAGGAGAACTTCTAGTATATTCTGTATACCTAATATTA TAGCCTTTATCAACAGATATCTGGTAGCGCCTGTGCTTCGG TTACTTCTAAGGAAGTCCACACAAATCAAGATCCGTTAGAC GTTTCAGCTTCCAAAACAGAAGATTGTGAGAAGGCTTCCAC TATCGATGCATGCGAATTCTTAATTAATATTACATTATGGG TGGTGGCGCGCCTGTTGGAATAGAAATCAACTATCATCTAC TAACTAGTATTTACATTACTAGTATATTATCATATACGGTG TTAGAAGATGACGCAAATGATGAGAAATAGTCATCTAAAT TAGTGGAAGCTGAAACGCAAGGATTGATAATGTAATAGGA TCAATGAATATAAACATATAAAATGATGATAATAATATTTA	Life Technologies

	TAGAATTGTGTAGAATTGCAGATTCCCTTTTATGGATTCCT AAATCCTTGAGGAGAACTTCTAGTATATTCTGTATACCTAA TATTATAGCCTTTATCAACAATGGAATCCCAACAATTATCT CAACATTCACCCATTTCTCA	
Synthetic *Ty1RT*	ATGAATAATTCATCGCACAATATTGTTCCTATCAAAACGCC AACTACTGTTTCTGAACAGAATACCGAGGAATCTATCATCG CTGATCTCCCACTCCCTGATCTACCTCCAGAATCTCCTACC GAATTTCCTGACCCATTTAAAGAACTCCCACCGATAAATTC TCGTCAAACTAATTCCAGTTTGGGTGGTATTGGTGACTCTA ATGCCTATACTACTATCAACAGTAAGAAAAGATCATTAGA AGATAATGAAACTGAAATTAAGGTATCACGAGACACATGG AATACTAAGAATATGCGTAGTTTAGAACCTCCGAGATCGA AGAAACGTATTCACCTGATTGCAGCTGTAAAAGCAGTAAA ATCAATCAAACCAATACGGACAACCTTACGATACGATGAG GCAATCACCTATAATAAAGATATTAAAGAAAAAGAAAAAT ATATCGAGGCATACCACAAAGAAGTCAATCAACTGTTGAA GATGAAAACTTGGGACACTGACGAATATTATGACAGAAAA GAAATAGACCCTAAAAGAGTAATAAACTCAATGTTTATCTT CAACAAGAAACGTGACGGTACTCATAAAGCTAGATTTGTT GCAAGAGGTGATATTCAGCATCCTGACACTTACGACTCAG GTATGCAATCCAATACCGTACATCACTATGCATTAATGACA TCCCTGTCACTTGCATTAGACAATAACTACTATATTACACA ATTAGACATATCTTCGGCATATTTGTATGCAGACATCAAAG AAGAATTATACATAAGACCTCCACCACATTTAGGAATGAA TGATAAGTTGATACGTTTGAAGAAATCACTTTATGGATTGA AACAAAGTGGAGCGAACTGGTACGAAACTATCAAATCATA CCTGATACAACAATGTGGTATGGAAGAAGTTCGTGGATGG TCATGCGTATTTAAAAACAGTCAAGTGACAATTTGTTTATT CGTAGATGATATGGTATTGTTTAGCAAAAATCTAAATTCAA ACAAAAGAATTATAGAGAAGCTTAAGATGCAATACGACAC CAAGATTATAAATCTAGGCGAAAGTGATGAGGAAATTCAA TATGACATACTTGGCTTAGAAATCAAATATCAAAGAGGTA AATACATGAAATTAGGTATGGAAAACTCATTAACTGAGAA AATACCCAAATTAAACGTACCTTTGAATCCAAAAGGAAGA AAACTTAGCGCTCCAGGTCAACCAGGTCTTTATATAGACCA GGATGAACTAGAAATAGATGAAGATGAATACAAAGAGAA GGTACATGAAATGCAAAAGTTGATTGGTCTAGCTTCATATG TTGGATATAAATTTAGATTTGACTTACTATACTACATCAAC ACACTTGCTCAACATATACTATTCCCCTCTAGGCAAGTTTT AGACATGACATATGAGTTGATACAATTCATGTGGGACACT AGAGATAAACAACTGATATGGCACAAAAACAAACCTACCG AGCCAGATAATAAACTAGTCGCAATAAGTGATGCTTCGTAT GGCAACCAACCGTATTATAAATCACAAATTGGCAACATAT ATTTACTTAATGGAAAGGTAATTGGAGGAAAGTCCACCAA GGCTTCATTAACATGTACTTCAACTACGGAAGCAGAAATAC ACGCGATAAGTGAATCTGTCCCATTATTAAATAATCTAAGT TACCTGATACAAGAACTTAACAAGAAACCAATTATTAAAG GCTTACTTACTGATAGTAGATCAACGATCAGTATAATTAAG TCTACAAATGAAGAGAAATTTAGAAACAGATTTTTTGGCAC AAAGGCAATGAGACTTAGAGATGAAGTATCAGGTAATAAT TTATACGTATACTACATCGAGACCAAGAAGAACATTGCTG ATGTGATGACAAAACCTCTTCCGATAAAAACATTTAAACTA TTAACTAACAAATGGATTCATTAG	Life Technologies
***pIMT4*-tRNA^{iMet}-*IMT4t* (gblock)**	CCGGAGCTCAACATCCAGTTCTTTCAAGTTGGCTAAGCGCC GTGGCGCAGTGGAAGCGCGCAGGGCTCATAACCCTGATGT CCTCGGATCGAAACCGAGCGGCGCTAATTTTTCATTTCTTT	IDT

	TTGCCCGCAACAATAACACAGATCGCAATCATTTAAGCTAT AGACTATAAGGTACCGCC	
***pSUP4*-tRNA^{iMet}-*IMT4t* (gblock)**	CCGGAGCTCACCATCTTGGAAGGACCGGATAATTATTTGAA ATCTCTTTTTCAATTGTATATGTGTTATGTAGTATACTCTTT CTTCAACAGCGCCGTGGCGCAGTGGAAGCGCGCAGGGCTC ATAACCCTGATGTCCTCGGATCGAAACCGAGCGGCGCTAA TTTTTCATTTCTTTTTGCCCGCAACAATAACACAGATCGCA ATCATTTAAGCTATAGACTATAAGGTACCGCC	IDT
***pSUP4*-tRNA^{iMet}-*RPR1t* (gblock)**	CCGGAGCTCACCATCTTGGAAGGACCGGATAATTATTTGAA ATCTCTTTTTCAATTGTATATGTGTTATGTAGTATACTCTTT CTTCAACAGCGCCGTGGCGCAGTGGAAGCGCGCAGGGCTC ATAACCCTGATGTCCTCGGATCGAAACCGAGCGGCGCTATC TTTCTTTTTTAATTTTCACTTATTTGCGGGTACCGCC	IDT
***URA3*gRNA (gblock)**	TGTACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAG GTTTTGGGACGCTCGAGACATAAAAAACAAAAAAAGCACC ACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC CTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACCGTGTG CATTCGTAATGGATCATTTATCTTTCACTGCGGAGAAGTTT CGAACGCCGAAACATGCGCACCAACTTTCACTTCTACAGCG TTTGACCAAAATCTTTTGAACAGAACATTGTAGGGTGTGAA AAAATGCGCACCTTTACCGCTAGCCCAAGAGGGCACTACA AAATCTAGAGTTGTACTTCAAACGTACATGTAATCACCTTG TATATACTCGAAAGAAAACATCAAGTTTCTGTATAAATATG AGTGAAAGCATAATCATACATTATCTTTTCAAAGGGTACCC AATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGC CGTCGTTTT	IDT
***TRP1*gRNA (gblock)**	AGACATAAAAAACAAAAAAAGCACCACCGACTCGGTGCCA CTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAACAGGAACTCTTGGTATTCTTGGATC ATTTATCTTTCACTGCGGAGAAGTTTCGAACGCCGAAACAT GCGCACCAACTTTCACTTCTACAGCGTTTGACCAAAATCTT TTGAACAGAACATTGTAGGGTGTGAAAAAATGCGCACCTT TACCGCTAGCCCAAGAGGGCACTACAAAATCTAGAGTTGT ACTTCAAACGTACATGTAATCACCTTGTATATACTCGAAAG AAAACATCAAGTTTCTGTATAAATATGAGTGAAAGCATAA TCATACATTATCTTTTCAAAG	IDT
***(m)GAXnewintron* (gblock)**	TATTTCCTCAAGTGACGGGATGCCGCTATTGAGTATGCACA TCCCTGCGTCTACAGCGCAAGCCAACTCATGCTCGAACGTA TGACCGGCTAGAGTAGCGTGATTAACTTCAATATTGACTTT GAAGTCTTTATCTAGATTATGAGCTTTCAAGAAACCGATTG CAGTCTCGGTGTCTACGTCGTATTGGTGTTTAGTAGGTTCC ATAGGCTTAGGTTCAATTAGGAAGGTATGTTAATATGGACT AAAGGAGGCTTTTGGCGCGCCGAATTTTTACTAACAAATGG TATTATTTATAACAGGTACCTTTAAAGCCTTTACTACGTGC ATAGTCCCTAGCCATGGTCAACATGGTTGCCATATGTTCTT TTTCTCTTTTTTGATCAGTATTAAGTAATGACATATAACCTT CTCTACCGCCCCAAAACACGTAATTTTCTGCGCCCAATTCG ATGCCTGCGTCGATTGCGTTTTTAATTTGTACAATAGCCCTC GCAACCACGTCAAAATCAGGATTAGTTGAAGCTCCGTTCAT ATACCTTTTATGACCAAATACATTAGCAGTTGACCATAATA ACTTAATCCCAGTTTCCTTCTGTTTTTCTTTCAAGTACGCGA CAACTGCCTTCAAGTTGGATTCATATTCCTCAATAGAGTTA CCTTCAGATACTAAATCCACGTCATGAAAACAATAGTAAG GAATACCTAATTTTTGCATTATCTCGAAACCAGCATCAACT TTTTGCTTTGCTATTTCAATTGCGTCTGTACCTTCGTTCCAT	IDT

	GGGAAACTTTTAGTCCCTCCTCCAAATTGATCTGCTCCTTC AGCACACAAGGTGTGCCACCAAGCCATGGCAAATCTTAAC CAGTCTTTCATTTTTTTACCCATGACCTCTTTTTCGGCATCA TAGTAATGAAAGGCCAATGGGTTCTTACTATCTTTACCCTC GAATTTTATCTTTTGAATTTGAGGGAAATATTCTTTAGCCAT cccgggatccgtcgaaactaagttctggCTTCTCAACTTTCAACTCTTTCGC CAACGGAACTGGAGTCGTTAAGC	
***(m)GA3Xnewintron* (gblock)**	TGGTAGCACCAGAACCTTGATACATCGCGATAATAGCCTCG TATAGTTCTTGTTTTCCTGAGGTTTGTTTCGGTTCTCCATTC TTCTTTCCATATTCATAGACTTGCTCCAATGTCAGTTTCCCA TCTTCAAAATCTTTTCCTATTCCAGAATCGAATGAAGCATA TCTTTCCTTCTTCATTTTTGTGTATGGAGACTCCTGTAACAA TTTAGCTGCATTCTCCAATGCACGAGCCATGGCATCCATAC CAGACACATGAGCGATAATAATATCTTCTAAGTCAGTCGA ATTACGTCTTGTCTTAGCGTCGAAGTTTGTTCCACCGGTTAC AAAACCGCCACCCCTTATAATTTCCATCCAAGCCTGTACCA ATTCGTACTGGTCGATAGGAAACTGGTCAGTGTCCCAGCCA TTTTGATAGTCACCCCTATTGGCATCTATGCTACCTAACATC CCTGCGTCTACAGCGCAAGCCAACTCATGCTCGAACGTATG ACCGGCTAGAGTAGCGTGATTAACTTCAATATTGACTTTGA AGTCTTTATCTAGATTATGAGCTTTCAAGAAACCGATTGCA GTCTCAGTGTCTACGTCGTATTGGTGTTTAGTAGGTTCCAT AGGCTTAGGTTCAATAAGGAAGGTATGTTAATATGGACTA AAGGAGGCTTTTGGCGCGCCGAATTTTTACTAACAAATGGT ATTATTTATAACAGGTACCTTTAAAGCCTTTACTACGTGCA TAGTCCCTAGCCATGGTCAACATGGTTGCCATATGTTCTTTT TCTCTTTTTTGATCAGTATTAAGTAATGACATATAACCTTCT CTACCGCCCCAAAACACGTAATTTTCTGCGCCCAATTCGAT GCCTGTGTCGATTGCGTTTTTAATTTGTACAATAGCCCTCGC AACCACATCAAAATCAGGATTAGTCGAAGCTCCGTTCATAT ACCTTTTATGACCAAATACATTAGCAGATGACCATAATAAC TTAATCCCAGTTTCCTTCTGTTTATCTTTCAAGTACGCGACA ACTGCCTTCAAGTTGGATTCATATTCCCCAATAGAGTTACC TTCAGATACTAAATCCACGTCATGAAAACAATAGTAAGGA ATACCTAATTTTTGCATTATCTCGAAACCAGCATCAACTTTT TGCTTTGCTATTTCAATTGCGTCTGTACCTTCGTTCCATGGG AAACTTTTAGTCCCTCCTCCAAATTGATCTGCTCCTTCAGCA CACAAGGTGTGCCACCAAGCCATGGCAAATCTTAACCAGT CTTTCATTTTTTTACCCATGACCTCTTTTTCGGCATCATAGT AATGAAAGGCCAATGGGTTCTTGCTATCTTTACCATCGAAT TTTATCTTTTGAATTTGAGGGAAATATTCTTTAGCCATcccgg gatccgtcgaaactaagttctgg	IDT
***KlURA3AI* (gBlock)**	ATTCATTAGATCGATAAGCTTGGGCTGCAGCTTTAAATAAT CGGTGTTAATGGGGAGCGCTGATTCTCTTTTGGTACGCTTC CCATCCAGCATTTCTGTATCTTTCACCTTCAACCTTAGGATC TCTACCCTTGGCGAAAAGTCCTCTGCCAACAATGATGATAT CTGATCCACCACTTACAACTTCGTCGACGGTTCTGTACTGC TGACCCAATGCATCGCCTTTGTCGTCTAAACCTACACCTGG GGTCATGATTAGCCAATCAAACCCTTCTTCTCTGGTATGTT AATATGGACTAAAGGAGGCTTTTCTGCAGGTCGACTCTAGA GGATCCCCGGGTACCGAGCTCGAATTTTTACTAACAAATGG TATTATTTATAACAGCCTCCCATATCGTTCTGAGCAATGAA CCCAATAACGAAATCTTTATCACTCTTTGCAATATCAACGG TACCCTTAGTATATTCACCGTGTGCTAGAGAACCCTTGGAA GACAATTCAGCAAGCATCAATAATCCCCTTGGTTCTTTGGT GACCTCTTGCGCACCTTGTTTCAAGCCAGCAACAATACCAG	IDT

CACCAGTAACCCCGTGGGCGTTGGTGATATCAGACCATTCT
GCGATACGGTAAACGCCCGATGTATATTGTAATTTGACTGT
GTTACCGATATCGGCGAATTTTCTGTCCTCAAATATCAAGA
ACTTGTATTTCTCTGCCAATGCTTTCAATGGAACGACAGTA
CCCTCATAACTGAAATCATCCAAGATATCAACGTGTGTTTT
CAAAAGGCAAATGTATGGACCCAACGTTTCAACAAGTTTC
AATAGCTCATCAGTCGAACGAACGTCAAGAGAAGCACACA
AATTGGTCTTCTTTTCATCCATTAAACGTAAAAGTTTCGAT
GCAACCGGACTTGCATGAGTCTCAGCTCTACTGGTATATGA
TTTTGTGGACATAAACTTAGATTAGATTGCTATGCTTTCTTT
CTAATGAGCAA

Supplementary Notes

Supplementary Note 1: Analysis of *in vivo* mutation rate through next-generation sequencing

In order to gain a detailed picture of the mutational rate and spectrum enabled by Ty1, yeast cells containing *URA3*-containing, Ty1 retrotransposons were induced in galactose for three days, after which intron-less *URA3* amplicons were generated via PCR of total DNA. As a negative control encompassing background genetic drift, PCR error rate, and sequencing error, a region of the Ampicillin resistance gene (*Amp*) of the same length (which is not reverse transcribed) was also amplified (See Methods). These amplicons were then sequenced (Supplementary Fig. 5d). Analysis of identified mutants showed a uniform error distribution across each amplicon, with *URA3* consistently showing a higher mutation rate (0.28 kb^{-1}) than *Amp* (0.13 kb^{-1}) (Fig. 4b). This 0.15 kb^{-1} increase in error rate above the combined effects of drift, PCR error, and sequencing error was thus due to Ty1 and was also reflected in an increased frequency of observing a given number of mutations per 200bp read in *URA3* versus *Amp* (Fig. 4c). Finally, Ty1 exhibited a mutational spectrum commensurate with other commonly-used error-prone polymerases and displayed an error rate which is useful for directed evolution of genes and pathways (Table 1). Collectively, this analysis indicated that the Ty1 retrotransposon is a useful vehicle for introducing mutations to defined genes and pathways *in vivo*.

Supplementary Note 2: Analysis of *in vivo* mutation rate using dKanMX reversion assay

In order to directly compare the rates of Ty1-induced mutations and background genetic drift, the d*KanMX* marker was constructed for use in a mutation reversion assay, containing two point mutations that together prevent functional activity. The first is T405A, which introduces an artificial stop codon at the 135th residue, in the middle of the proposed active site[18].

Interestingly, this mutation alone does not inactivate *KanMX*, and expression of this variant confers G418 resistance. The second mutation, T530A, is a missense variant resulting in Trp177Leu. Together, these mutations inactivate *KanMX*, and do not allow growth in the presence of G418.

To separate the effect of background genetic drift absent the optimized Ty1 retroelement, d*KanMX* was either integrated directly into the genome (g-dKanMX) or incorporated into a Ty1 mutagenesis cassette, which was also integrated into the genome (ICE-dKanMX). Both strains were grown to stationary phase and exposed to galactose, then plated on media containing G418. The rate of reversion mutations could then be measured by counting G418-resistant colonies. Genomic DNA from 39 colonies of both strains was extracted, a PCR designed to amplify position 135 of d*KanMX* was performed, and amplicons were sequenced via Sanger sequencing. In 34 of 39 resistant colonies sequenced, mutations at position 135 reverted the artificial stop codon to one of several non-stop codons, thus activating the gene and conferring a G418-resistant phenotype. We hypothesize that the 5 remaining colonies displaying a G418-resistant phenotype in the absence of a mutation at residue 135 may be due to a mutation elsewhere in *KanMX* (e.g. position 177), or a mutation elsewhere in the yeast genome (e.g. a mutation generating an amber suppressor tRNA). To this end, we repeated this experiment with an additional 10 colonies, this time amplifying and sequencing both mutated loci (position 135 and 177). Of these 10, 9 have mutations at position 135 that were previously observed. However, no other mutations in *dKanMX* were observed, including at position 177, in any of the 10 sequenced revertants. As stated above, the generation of an amber suppressor tRNA may be the cause of the remaining resistant isolate. We therefore conservatively conclude that at least 88% (43/49) of G418-resistant colonies appeared as a result of direct Ty1-mediated mutagenesis to d*KanMX*.

This conservative rate of reversion mutants present in ICE-d*KanMX* above that of g-d*KanMX* (Fig. 4a) was then compared to the expected rate of mutation based on sequencing experiments described previously. With this established rate of 0.15 point mutations per bp, each transposition of the 717 bp d*KanMX* gene will result in an average of 0.10755 mutations. Given an approximate transposition rate of 10^8 per liter of culture (based on the length of the cargo cassette), 1.0 mL of culture plated on G418-containing media is expected to contain 10,755 mutations in d*KanMX*. Neglecting indel mutations, there are 8 mutations out of the 2151 possible that will eliminate the stop codon from d*KanMX*; assuming the mutations are distributed randomly and uniformly throughout the gene, this results in approximately 40 reversion mutations per 1.0 mL of culture that will be able to grow on the G418 plate. This matches remarkably closely to the rate of colony formation observed (0.88*58 colonies per mL of culture= 51 per mL of culture), confirming the utility of this assay and the rate of mutation in ICE cargo.

Supplementary Note 3: Characterization of ura3 variants

The first-round mutant *ura3(3-5)* contained a single coding mutation ($Arg^{145}Ile$) that resides on an outer loop of the URA3p $(\beta/\alpha)_8$ barrel and which is distal to both the homodimer interface and catalytic site (Supplementary Fig. 2a). After isolation and sequencing of *ura3* mutants enriched by the first round of screening, the capacities of each *ura3* variant to convert 5-FOA to 5-fluorouracil, while maintaining activity in the uracil biosynthesis pathway, were performed by integrating each variant into a low-copy vector and transformed this expression cassette into BY4741 Δ*rrm3*. Cells containing either a mutant *ura3* or wild-type *URA3* were then plated on solid media lacking uracil and containing 5-FOA, and relative growth rate was quickly determined by comparing colony size. Strains expressing *ura3(3-5)* enabled over 3-fold increases

to colony area relative to those expressing the wild-type gene, indicating a decreased propensity of these mutants to convert 5-FOA into 5-fluorouracil while retaining their function in the uracil biosynthesis pathway (Supplementary Fig. 3b). Importantly, cells containing this mutant did not exhibit decreased growth rate relative to those containing wild-type *URA3* in uracil-deficient media *without* 5-FOA, indicating that their increased specificity came with no observable fitness tradeoff under these conditions.

ura3(3-5-2), the best-performing mutant from the second round, contained a second coding mutation ($Arg^{186}Lys$) that was likewise distal to the active site and the homodimer interface (Supplementary Fig. 1a). *ura3(3-5-2)* was also cloned into a low-copy vector and transformed into BY4741. Cells containing either *ura3(3-5)*, *ura3(3-5-2),* or wild-type *URA3* were then subjected to growth assays in liquid media lacking uracil but containing from 0 g L^{-1} 5-FOA to 1 g L^{-1} 5-FOA (which is the concentration generally used for efficient counter-selection of the *URA3* marker[19]). Maximum exponential growth rates at each condition were measured in order to compare the relative specificity of each enzyme. In all conditions, strains containing *ura3(3-5-2)* grew the fastest followed by *ura3(3-5)* and finally *URA3*. In particular, cells containing *ura3(3-5)* and *ura3(3-5-2)* attained IC_{50} values for 5-FOA 1.6- and 2.5-fold higher than strains expressing wild-type *URA3*, respectively (Fig. 4a). Again, neither mutant strain exhibited decreased growth rate relative to those containing wild-type *URA3* in uracil-deficient media without 5-FOA, confirming this increased specificity did not practically affect native activity.

Supplementary Note 4: Characterization of spt15 variants

The best-performing mutant isolated from the first round, *spt15-B6*, contains a single coding mutation ($Arg^{98}His$) near the DNA-binding domain of this protein (Supplementary Fig.

2b), suggesting a putative mode of action. *Spt15-B6-1,* the best-performing mutant from the second round of ICE contained a second coding mutation ($Gly^{192}Ser$) along with two indels in the TEF1 promoter (Fig. 4b). This coding sequence mutation, like that of *spt15-B6*, resides in the DNA-binding domain (Supplementary Fig. 2b).

After isolation and sequencing of *spt15* mutants enriched by our initial screening, viability analyses of *spt15* mutants were performed by integrating each *spt15* mutant into a low-copy vector and transforming this expression cassette into wild-type BY4741. These strains, along with controls expressing either wild-type *SPT15* or an empty vector, were grown to stationary phase and then subjected to a killing concentration of 1-butanol (3.5% by volume). After 0, 1, 2, and 3.5 hours, a small volume of each culture was plated to determine the number of viable cells remaining (see Methods). This analysis indicated that cells containing *spt15-B6* exhibited a 1.7-fold higher viability in lethal 1-butanol concentrations compared to wild-type, while cells containing *spt15-B6-1* exhibited up to 1.95-fold higher viability relative to wild-type under the same conditions (Fig. 4b).

These mutants were then further tested for any potential growth improvements in butanol-containing media. The same strains, along with controls expressing either wild-type *SPT15* or an empty vector, were grown to stationary phase and then resuspended at a low OD in media containing between 1.2% and 1.5% butanol. These were then grown in anaerobic sealed culture tubes. *Spt15-B6-1* conferred 32% and 44% increased growth over wild-type at 1.3% and 1.4% 1-butanol, respectively ($p<0.05$). As *spt15-B6-1* also contained mutations to *pTEF1*, a qRT-PCR experiment was carried out to investigate potential changes to transcription levels in order to provide insights into the observed phenotype (see "Expression Analysis" above). However, expression measurements indicated no difference between the rate of transcription

enabled by *pTEF1* and the promoter contained in *spt15-B6-1* (Supplementary Fig. 3a) under these test-tube, exponential growth conditions.

Supplementary Note 5: Development of an optimal *in vivo* mutagenesis host for screening xylose pathway variants

We performed several modifications to our optimized Ty1-containing strain in order to enable selection for growth on xylose. In particular, *GRE3*, which encodes an aldose reductase, was knocked out in order to reduce competitive xylose utilization and allow any potential improvements in xylose isomerase activity to confer a greater phenotypic advantage, thus increasing the sensitivity of our growth-based screen[20]. An additional copy of *XKS1* was also integrated into the genome to boost downstream metabolic flux. Finally, since overexpression of transaldolase (*TAL1*) has been shown to improve xylose consumption rate[21], the *TAL1* gene was expressed under the control of *pTEF1* and cloned into the $tRNA^{iMet}$ overexpression vector. The resulting plasmid was introduced into BY4741 Δ*rrm3* containing the *GRE3* knockout and *XKS1* overexpression, resulting in BY4741 Δ*r-g-x*. The two-gene xylose catabolic pathways consisting of xylose isomerase and xylulokinase were driven by a strong hybrid *pTDH3* promoter (UAS_{TEF}-UAS_{CIT}-UAS_{CLB}-P_{GPD})[22] and pathway genes were joined using ribosome-cleavable 2A sites[23]. These pathways were then inserted into the synthetic retroelement and integrated into the genome of BY4741 Δ*r-g-x*, collectively forming the parent strains for ICE of each xylose pathway.

Supplementary Note 6: Characterization of xylose pathway variants

The *I3K-66* and *I3K-20* strains contain mutants with one ($Ile^{433}Val$) and three ($Ala^{48}Ser$, $Ile^{433}Val$, $Met^{435}Ile$) amino acid substitutions, respectively. *I3K-66* also contains one silent mutation in *xyla3** (A1029G). Interestingly, A48S lies inside the $(\beta/\alpha)_8$ barrel of *xylA*, which

houses the dual Mg catalytic core and thus potentially influences the active site of this enzyme. Ile^{433}Val and Met^{435}Ile both lie proximal to the homodimer interface and potentially influence the stability of the XylA3*p catalytic tetramer[24] (Supplementary Fig. 2c). Enzymatic assays of the isolated mutants indicated increased V_{max} values (0.126 ± 0.008 and 0.134 ± 0.003 μmol min^{-1} mg protein^{-1} for *I3K*-66 and *I3K*-20, respectively) compared to wild-type (0.118 ± 0.007 μmol min^{-1} mg protein^{-1}) (Supplementary Table 3). In comparing these values to prior work, it is important to note that *XKS1* was not overexpressed during the characterization of xylose consumption kinetics in[15], whereas it was in this work. However, in both works, Vmax and Km are computed using whole cell extracts, so the entire pathway will contribute to these values. Since isomerization is reversible, high xylulose concentrations due to the absence of sufficient *XKS1* may have increased the apparent Km in the Lee, *et. al.* study. However, because we overexpressed *XKS1* in this study, xylulose is readily removed as it is formed and thus does not compete with xylose for active sites in *XylA*, thus lowering the apparent Km.

After screening, the growth rates of strains containing xylose pathway variants were characterized through growth in 1mL cultures containing 20 g L^{-1} xylose (Supplementary Fig. 3d and 3e). It was observed that mutant *IK-34* displayed a 1.7-fold increase in growth rate over the control as well as a significantly shorter lag phase. For the *I3K* multi-gene cassette, *I3K-66* and *I3K-20* conferred roughly 1.3-fold improvements to growth rate (Supplementary Fig. 3d and 3e, Supplementary Table 3) concomitantly with a decrease in lag time. In order to characterize the growth curves of each mutant in more detail, optical density was measured over short (15 minute) intervals through the use of a Bioscreen C. After scaledown from 1 mL to 200 μL in this setting, mutants *IK-34, I3K-66*, and *I3K-20* displayed 20.5%, 14.0%, and 16.2% improvements to growth rate in xylose-containing media over their respective wild-type,

respectively (Fig. 4c). Mirroring results at the 1mL scale, mutant *IK-34* exhibited an 18 hour shorter lag, while mutants *I3K-66* and *I3K*-20 exhibited a 6 hour shorter lag phase. These analyses produced similar results and indicated that variants of strains containing *IK* and *I3K* exhibited significantly increased growth rates and significantly reduced lag times compared with that of their parent strains.

These mutants were isolated at the following frequency at the end of the selection experiment: IK-34: 2/52. I3K-66: 1/66, I3K-20: 1/26. To assess and calculate actual enrichment values, we first calculated the ratio of these clones we would have expected based on no selective pressure. To generate these variants, we induced mutagenesis in 4.6kb pathway for 3 days. Based on Supplementary Figure 2a, this yields ~3*10^-3 transposants per cell. Since we induced mutagenesis in 50mL of OD=1 culture, the total number of transposants generated is 50*10^7*1*3*10^-3=1.5*10^6. Given that IK-34 and I3K-66 have one mutation each while 13K-20 has three, we can compute the expected copy number of each 1-mutant and 3-mutant variants in our generated library using PEDEL[6]. This analysis shows that each 1-mutant variant is at a copy number of 37.6, while each 3-mutant variant is unique. This indicates that the expected probability of finding IK-34 and I3K-66 is 37.6/1.5*10^6=2.5*10^-5, and of finding I3K-20 is 6.7*10^-7 if no selection was occurring. Given the observed recovery frequencies of these variants, we concluded that IK-34 is enriched 1500-fold, I3K-66 610-fold, and I3K-20 57000-fold over the initial library.

We were initially surprised that none of these variants contained mutations in all of the promoter and coding sequences. As one potential explanation, we posit that in multigene pathways, it is only really necessary to improve the rate-limiting step in order for pathway performance to be increased, which may only map to one enzyme or promoter region. For long

evolutionary time courses, multiple genes and regulatory elements will each eventually undergo mutations as they become the rate-limiting step in further improvements.

Supplementary References

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